Cambridge Crops

July 07, 2021

Paulette Gaynor, PhD
GRAS Notification Program
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5001 Campus Drive
College Park, MD 20740

GRAS NOTIFICATION FOR SILK PROTEIN DERIVED FROM *BOMBYX MORI* COCOONS.

Dear Dr. Gaynor,

On behalf of Cambridge Crops, Inc., I hereby submit a notification of the generally recognized as safe (GRAS) determination for silk protein as derived from *Bombyx mori* cocoons.

Cambridge Crops previously submitted a notification of its GRAS determination for the same substance (filed as GRN 000930 by FDA on July 10, 2020). Following discussions with FDA, the notifier submitted an amendment on November 18, 2020 to address FDA's concerns and requests for clarification. Without calling safety into question, the FDA review team discussed the importance of the "general recognition" aspect at the time of submittal and requested refinement of exposure estimates during a teleconference on February 16, 2021. Following these discussions with FDA, and at FDA's recommendation, the notifier requested that FDA cease to evaluate GRN 000930 (April 5, 2021; FDA cease-to-evaluate letter issued May 25, 2021). FDA stated that they do not have safety concerns regarding the silk fibroin described in GRN 930. Communications are attached hereto.

This new GRAS notice contains the following substantive differences from the previous GRN 930:

- Refinement of exposure estimates based on specific addition levels of the GRAS substance per category of food and the removal of USDA-regulated foods (Part 3.3);
- Reference to published, peer-reviewed report of toxicology and allergenicity studies (Part 6.3, Yigit et al. 2021[1]);
- "Non-Novelty Determinations Regarding Mori Silk™" by Food Directorate, Health Products and Food Branch, Health Canada (June 21, 2021) (Appendix K);
- Clarification of relevance of cited toxicology studies to Cambridge Crops' exact notified substance (Part 6.3);
- Addition of support vector machine (SVM) bioinformatics searches to investigation of allergenic potential (Part 6.4);
- Refinement of *in vitro* pepsin digestion studies (Part 6.2.1);
- Clarification of manufacturing process including GRAS salts as process aids (Part 2.4);
- Refinement of heavy metals specifications (Part 2.5); and
- Citation of standard analytical methods for specification parameters and batch analysis results (Part 2.5).

[1] Yigit, Sezin, et al. "Toxicological assessment and food allergy of silk fibroin derived from Bombyx mori cocoons." *Food and Chemical Toxicology* 151 (2021): 112117.

If you have any questions or require any additional information, please do not hesitate to contact me.

Sincerely,

[redacted]

Laith M. Abu-Taleb, Esq.,
General Counsel and Vice President of Corporate Affairs
Cambridge Crops, Inc. d/b/a Mori

CC: Adam M. Behrens, Ph.D., adam@mori.com, Cambridge Crops, Inc. d/b/a Mori
Joseph V. Rodricks, Ph.D., DABT, jrodricks@ramboll.com, Ramboll

Attachment: Email communications about the notifier's cease-to-evaluate request for GRN 000930 (March 22 and April 5, 2021).

Enclosures: Electronic copies of all submission documents.

mori

Laith Abu-Taleb <laith@mori.com>

Cease to Evaluate Request

Eischeid, Anne <Anne.Eischeid@fda.hhs.gov> Fri, May 28, 2021 at 12:01 PM
To: Laith Abu-Taleb <laith@mori.com>
Cc: Joseph V Rodricks <JRodricks@ramboll.com>, "Pelonis, Evangelia C." <pelonis@khlaw.com>, Gavin P Thompson <gthompson@ramboll.com>, Cassie Huang <CHUANG@ramboll.com>, Adam Behrens <adam@mori.com>

Hi Laith,

I hope you are all well. I am attaching the CTE letter for GRN 930. Your email is timely, as it was just completed this week.

Thank you for your patience and please let me know if you have any questions.

Have a nice weekend,

Anne

From: Laith Abu-Taleb <laith@mori.com>
Sent: Wednesday, May 26, 2021 1:00 PM
To: Eischeid, Anne <Anne.Eischeid@fda.hhs.gov>
Cc: Joseph V Rodricks <JRodricks@ramboll.com>; Pelonis, Evangelia C. <pelonis@khlaw.com>; Gavin P Thompson <gthompson@ramboll.com>; Cassie Huang <CHUANG@ramboll.com>; Adam Behrens <adam@mori.com>
Subject: Re: [EXTERNAL] Cease to Evaluate Request

Dear Dr. Eischeid,

Thank you very much for your email and your confirmation that the letter will reflect that FDA does not have safety concerns regarding the silk fibroin described in GRN930.

We would love to resubmit as soon as we can. Do you recommend our resubmittal prior to the issuance of a Cease to Evaluate letter from FDA? If not, do you have an estimated time of completion of that letter?

Thanks again. I hope you and the team are doing well.

Best regards,

Laith

On Mon, Apr 5, 2021 at 11:12 AM Eischeid, Anne <Anne.Eischeid@fda.hhs.gov> wrote:

Dear Laith,

I hope you are doing well too. Congratulations on the acceptance of your paper!

I have confirmed that we will not be able to honor your request to review the cease to evaluate letter, as we do not do that in the GRAS program. As we have discussed in our calls, it will reflect that FDA does not have safety concerns regarding the silk fibroin described in GRN 930.

I apologize for the delay in my response.

Please let me know if you have any further questions,

Anne

From: Laith Abu-Taleb <laith@mori.com>
Sent: Monday, March 22, 2021 7:33 PM
To: Eischeid, Anne <Anne.Eischeid@fda.hhs.gov>
Cc: Joseph V Rodricks <JRodricks@ramboll.com>; Pelonis, Evangelia C. <pelonis@khlaw.com>; Gavin P Thompson <gthompson@ramboll.com>; Cassie Huang <CHUANG@ramboll.com>; Adam Behrens <adam@mori.com>
Subject: [EXTERNAL] Cease to Evaluate Request

Dear Dr. Eischeid,

We hope this message finds you doing well.

This email follows up on the discussion we had with you and your team on February 16. Based on this discussion we have decided to request that FDA cease to evaluate GRAS Notice 930, which was filed on July 10, 2020. We are making the request based on our understanding that FDA, after reviewing the information in the GRAS Notice, agrees there is no safety concern with silk fibroin.

That said, we understand that the FDA would prefer that we submit a new and revised GRAS Notice for silk fibroin after certain safety information is published in a peer reviewed scientific journal. To that end, we thank you for your discussion with us on types of peer-reviewed journals that are acceptable.

We are happy to report that the Journal of Food and Chemical Toxicology accepted our article "Toxicological assessment and food allergy of silk fibroin derived from Bombyx mori cocoons" for publication on March 8. (*Yigit, S.* et al,. Toxicological assessment and food allergy of silk fibroin derived from Bombyx mori cocoons. *Food and Chemical Toxicology*, 112117. https://doi.org/10.1016/j.fct.2021.112117, 2021).

Thus, we will be submitting a new and updated GRAS Notice for silk fibroin soon with reference to this published article. We understand that the publication, in addition to our reported scientific talks given to the food-safety community, will fully address the general recognition and public availability concerns expressed by the Agency. On February 16, 2021, we also discussed a request by FDA to update the exposure estimate to make it more precise regarding which product categories were associated with the highest use level. This updated exposure estimate will also be included in the new GRAS Notice. Finally, the new GRAS Notice will also include the additional information that has already been submitted to FDA in the amendment dated November 18, 2020. This information was submitted in response to questions from FDA raised in calls dated September 15, 2020 and October 29, 2020.

It is our understanding that the cease to evaluate letter will note that FDA does not find any specific safety issues with silk fibroin for its intended use but has two remaining concerns with GRN 930: (1) certain safety information was not yet publicly available to achieve general recognition of safety at the time of submittal and (2) the exposure estimate needs to be updated to address which product categories are intended to be used at the highest levels of use. If at all possible, we would appreciate an opportunity to see a copy of the cease to evaluate letter before it is issued.

Once again, we sincerely thank you for your time. Looking forward to speaking with you again shortly.

Best regards,
Laith

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formerly Cambridge Crops

GRN 930 CTE letter_FINAL_transmittal.pdf
237K

Prepared for
Cambridge Crops

Prepared by
Ramboll US Consulting, Inc.

Date
June 2021

GENERALLY RECOGNIZED AS SAFE (GRAS) DETERMINATION FOR THE USE OF CAMBRIDGE CROPS MORI SILK AS A COATING FOR FOODS

RAMBOLL

Cambridge Crops

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APPENDICES

PART 1. SIGNED STATEMENTS AND CERTIFICATION

1.1 Name and Address of the Notifier

Adam Behrens, Ph.D. CEO adam@mori.com	Laith Abu-Taleb, Esq. General Counsel & Vice President of Corporate Affairs laith@mori.com
Cambridge Crops, Inc. d/b/a Mori 440 Rutherford Avenue Boston, MA 02129	

1.2 Name of Notified Substance

The subject of this generally-recognized-as-safe (GRAS) notice (GRN) is silk protein, primarily consisting of silk fibroin, as extracted from *Bombyx mori* cocoons. It is isolated from additional proteins within the *Bombyx mori* cocoons through a relatively simple process. It is manufactured as per current Good Manufacturing Processes (cGMP) and is made soluble via the addition (and later, the removal) of commonly found salts. The silk protein shall be marketed under the trade name Mori Silk.

1.3 Background

Silk has been approved by regulatory bodies and used in various industries including healthcare and cosmetics. Silk fibroin was first utilized as a suture in the 1800s, and nonabsorbable silk surgical sutures designed to be used *in vivo* are included as a "General and Plastic Surgery Device" in 21 CFR §878.5030 since 1993. Further, silk fibroin was approved by United States Food and Drug Administration (FDA) as a surgical scaffold to be used *in vivo* in a 510(k) premarket submission by SERI Surgical (Allergan) in 2013 (K123128).

Other countries have also approved the use of silk fibroin *in vivo* by their regulating authorities. In South Korea, for example, Tympasil® has been approved as a silk fibroin patch for ear drum perforations. In China, silk fibroin has been approved for use as a clinical wound dressing (Sidaiyi®).

In Canada, *Bombyx mori* silkworms and *Bombyx mori* powder, both containing silk fibroin, are listed as Non-Novel Foods by Health Canada. They may both be consumed across the country and can be purchased at retailers all over Canada. Health Canada recently (June 21, 2021) confirmed the non-novel food status of fibroin protein isolate from silkworm (*Bombyx mori*) cocoons; *Non-Novelty Determinations Regarding Mori Silk™ (Case # 2021-025133)* (Appendix K).

The Cosmetic Ingredient Review ("CIR") panel published a "Safety Assessment of Silk Proteins as Used in Cosmetics" (CIR 2016, Johnson et al. 2020) that reviewed the use of ten variations of silk ingredients as conditioning and bulking agents in cosmetic products. The

CIR noted the various uses of silk proteins in cosmetics such as powders and hairsprays, as well as non-cosmetics such as surgical sutures.

Generally, silk fibroin is a well-characterized protein approved for uses in ancillary industries by regulating bodies. Furthermore, silkworms and silkworm-derived food products are consumed by people in regions of the world both in and outside of North America.

Cambridge Crops previously submitted a GRN for Mori Silk to FDA in 2020, filed as GRN 000930 on July 7, 2020. Cambridge Crops requested that FDA cease to evaluate GRN 000930 on March 22, 2021. Following FDA CFSAN's consultations, Cambridge Crops now submits a revised GRN that includes additional information along with a peer-reviewed publication of the toxicological studies described herein (Yigit et al., 2021 - Appendix J1).

1.4 Intended Conditions of Use of the Notified Substance

Mori Silk primarily consists of silk fibroin isolated from *Bombyx mori* cocoons and applied to various foods including fruits, vegetables, fish, cheeses, and select processed foods, i.e., hard candy, candy bars (including chocolate bars), nuts, cookies, cereal or meal bars, chewing gum results in a thin edible coating to preserve food in accordance with allowed mechanisms described in 21 CFR 170.3(o). It will be used at levels consistent with cGMP. Mori Silk is intended to extend the shelf life of foods by forming a protective barrier on the outside of the food. It will provide a thin, edible, and tasteless barrier to protect against degradation, moisture loss, and oxidation. Mori Silk is effective in extending the shelf life of a variety of foods, from whole- and cut-produce to fish, and select processed foods. There are three main ways that Mori Silk may be added to a food, further described in **Part 3**: (1) Wetting the food in a container containing Mori Silk solution, (2) mixing a Mori Silk solution in with food, or (3) spraying food with Mori Silk solution. The estimated 90th percentile daily intake of Cambridge Crops Mori Silk on foods consumption is 2.9 milligrams (mg) Mori Silk / kilograms (kg) body weight / day (**Table 1**). The estimated daily intake by age group is reported in **Table 6** and **Table 7** in **Part 3.3**.

Table 1: Estimated Daily Intake[1] of Mori Silk as a food coating by the U.S. population

mg Mori Silk / kg body weight / day		mg Mori Silk / day	
Mean	90th Percentile	Mean	90th Percentile
1.3	2.9	82	170
Notes: General population presented is the U.S. population ages 2 years and older.			

[1] As calculated over every individual with reported body weight surveyed in the United States Department of Health and Human Services' 2013-2014 and 2015-2016 National Health and Nutrition Examination Surveys (NHANES). Overall, the 2013-2014 and 2015-2016 NHANES had a sample size of 16,085 people from all ages, including infants, children, teenagers, and adults with a reported body weight.

1.5 Statutory Basis for GRAS Determination

The use of Cambridge Crops Mori Silk as an ingredient in food at the levels described herein has been determined to be safe and GRAS based on data generally available in the public domain, using scientific procedures[2], in accordance with the Federal Food, Drug and Cosmetic Act (FFDCA), Section 201(s) and Section 170.30 of Part 21 of the Code of Federal Regulations (21 CFR §170.30).

Ramboll, on behalf of Cambridge Crops, organized a panel of experts qualified by training and experience to evaluate the safety of food and food ingredients. The GRAS status of uses of Mori Silk also were confirmed by consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of ingredients as a component of food (Appendix A). This panel of experts ("GRAS Panel") evaluated the Mori Silk food ingredient, the intended conditions of use as presented in GRN 000930, which had intakes approximately 1-2x greater than the intakes presented herein, and the safety of the proposed intake of Mori Silk based on generally available and accepted information.

The GRAS Panel is composed of the following experts:

- Joseph F. Borzelleca, PhD
  - Professor Emeritus of Pharmacology and Toxicology; Virginia Commonwealth University School of Medicine
- John Erdman, PhD
  - Professor Emeritus of Food Science and Human Nutrition; and Professor of Nutrition at the University of Illinois at Urbana-Champaign
- Richard Goodman, PhD
  - Research Professor; Food Science and Technology Department at the University of Nebraska, Lincoln; member of the Food Allergy Research and Resource Program (FARRP)
- Stephen Taylor, PhD
  - Professor Emeritus; Food Science and Technology Department at the University of Nebraska, Lincoln; founding director of the Food Allergy Research and Resource Program (FARRP)
- Duncan Turnbull, DPhil, DABT, Technical Advisor to GRAS Panel
  - Toxicologist and Senior Science Advisor (retired) at Ramboll Environment & Health

These experts, independently and collectively, critically evaluated the safety assessment as presented in GRN 000930 including the supporting data[3]. The safety dossier incorporated publicly available information regarding the safety of Mori Silk including published reports of

[2] 21 CFR §170.3 Definitions. (h) Scientific procedures include the application of scientific data (including, as appropriate, data from human, animal, analytical, or other scientific studies), information, and methods, whether published or unpublished, as well as the application of scientific principles, appropriate to establish the safety of a substance under the conditions of its intended use.

[3] Since the Expert Panel's review of the GRN 000930 dossier, the following items have been added to this dossier: (1) daily intakes (EDIs) and cumulative EDIs have been reduced and are currently based on a reduced set of foods for intended uses and refinements of the intake calculations; (2) SVM allergenicity tests, which were performed by Dr. Goodman at the request of FDA; and (3) the published version of the peer-reviewed article (Yigit et al., 2021) of which Dr. Goodman was a co-author.

toxicological studies, unpublished[4] supporting data from the Notifier, and estimates of the potential human exposure to Mori Silk resulting from its intended use as an ingredient in coating for foods. Cambridge Crops and the GRAS Panel concluded that the proposed uses of Mori Silk described herein are safe and GRAS based on scientific procedures. The GRAS Expert Panel Consensus Statement is provided in Appendix A. Curricula Vitae of the Panel members and advisor are provided in Appendix A1.

1.6 Exemption from Premarket Approval Requirements of the FFDCA

Cambridge Crops has determined that the proposed food ingredient uses of Mori Silk in food, wherein Mori Silk is manufactured as described herein in accordance with Good Manufacturing Practice (GMP) and meets the specifications described herein, are exempt from the premarket approval requirements of the FFDCA because Cambridge Crops determined such uses to be safe and GRAS. This determination was made in compliance with the Substances Generally Recognized as Safe regulation [21 CFR § 170.30, as published in the Federal Register, Vol. 81, No. 159, FR 54960, August 17, 2016] and meets the requirements of the final regulation (21 CFR §170.35).

Cambridge Crops concluded that the uses of Mori Silk described herein are safe and GRAS based on scientific procedures at the proposed levels of inclusion in food, and thus, these uses of Mori Silk are excluded from the definition of a food additive, are not subject to the premarket approval requirements of Section 201(s) of the FFDCA and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

1.7 Availability of Data and Information to FDA

Should FDA ask to see the data and information that are the basis for the conclusion of GRAS status of the food ingredient uses of Cambridge Crops Mori Silk as described herein, Cambridge Crops

i. agrees to make the data and information available to FDA; and

ii. agrees to the following procedures: upon FDA's request, Cambridge Crops will allow FDA to review and copy the data and information as provided at 21 CFR §170.225(c)(7).

1.8 Freedom of Information Act (FOIA)

None of the data and information in this GRN is exempt from disclosure under the Freedom of Information Act, 5 U.S. Code 552.

1.9 Certification

To the best of the knowledge of Cambridge Crops, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to Cambridge Crops and pertinent to the evaluation of the safety and GRAS status of the proposed food ingredient uses of Cambridge Crops Mori Silk described herein.

[4] Currently published as Yigit et al., 2021 and considered pivotal safety data in this GRN.

1.10 Name, Position, and Signature of Certifier

Based on an evaluation of relevant data as described herein, the notifier has determined that Cambridge Crops Mori Silk is safe for its intended uses and GRAS under the terms of 21 CFR §170.30. We also have concluded that other "experts qualified by scientific training and experience to evaluate the safety of food and food ingredients" would agree with this determination.

[redacted]

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Telephone: +1 (617) 520-4016
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[redacted]

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Telephone: +1 (617) 520-4012
Email: laith@mori.com

PART 2. IDENTITY, METHOD OF MANUFACTURE, AND SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

2.1 Identity

Silk fibroin is a naturally occurring protein derived from the silk fibers of the domesticated silkworm (*Bombyx mori*). Fibroin consists of three components (Kaplan and McGrath 2012, p106; Vapari and Kaplan 2007; Mondal et al. 2007):

1. ~325-390 kilodaltons (kDa) heavy chain.
2. ~25-26[5] kDa light chain.
3. ~25-30 kDa P25 glycoprotein.

The three proteins coexist in fibroin with a molar ratio between fibroin's heavy chain, light chain, and P25 of 6:6:1 (Inoue et al. 2000). Silk fibroin is dominated by the heavy chain, which has an approximate 15 times higher molecular weight than the light chain. Because of that, silk fibroin's identity is dominated by the heavy chain's characteristics. Taken together, the proteins are known as "silk fibroin." Fibroin has a Chemical Abstracts Service Registry Number (CASRN) of 9007-76-5. Silk fibroin's amino acid composition consists primarily of glycine (approximately 42-46%), alanine (approximately 29-31%), and serine (approximately 9-12%) as shown in **Table 2** and Table B1 in **Appendix B**. Independently, fibroin's light chain is comprised of alanine (approximately 17%), aspartic acid/asparagine (approximately 15%), glycine (approximately 10%), glutamic acid/glutamine (approximately 8%), and valine (approximately 7%) (Sashina et al. 2006). P25, a smaller protein of approximately 220 amino acids, is composed of 20 amino acids with leucine at the highest proportion (10%), followed by alanine (7%), aspartic acid, isoleucine, asparagine, arginine, and serine (6% each) with other amino acids in proportions ≤5% (**Appendix E**). Because of the relative molecular weights, the light chain and P25 do not impact the amino acid concentration of silk fibroin to a significant degree. The light chain and P25 compose relatively little of the overall fibroin content.

Generally, fibroin consists of repeating segments of amino acid sequences. The heavy chain comprises twelve crystalline domains interspersed with non-repetitive primary sequences known as "linkers". The heavy chain is primarily made of glycine (G)-X repeats, with X being alanine (A), serine (S), or tyrosine (Y) (Zhou et al. 2001). Each domain consists of sub-domain hexapeptides made up of these glycine-X repeats including: GAGAGS, GAGAGY, GAGAGA or GAGYGA. These sub-domains end with tetrapeptides such as GAAS or GAGS (Zhou et al. 2001; Zhou et al. 2000; Gage and Manning 1980). The less crystalline forming linker regions are between 42-44 amino acid residues with identical 25 amino acid residues composed of charged amino acids (Zhou et al. 2001; Vepari and Kaplan 2007).

Occurrence of fibroin in silkworm cocoon

The silkworm cocoon naturally contains fibroin protein that is intermingled with sericin in the cocoon fibers. Silk fibers are composed of two proteins (**Figure 2**): fibroin fibers (center) are held together by sericin proteins like a glue or coat (Kaplan and McGrath 2012, Barbosa and

[5] Different publications report different weights for each of these components due to different methodological approaches.

Martins 2017). Fibroin and sericin are different in amino acid profile and solubility. Fibroin mostly consists of four amino acids in the following approximate proportions: 42-46% glycine, 29-31% alanine, 9-12% serine, and 4-5% tyrosine (Mondal et al. 2007, Wray et al. 2011, Kaplan and McGrath 2012). This amino acid profile is distinct from sericin, which is composed of approximately 10-20% glycine, 6% alanine, 30% serine, and 15% aspartic acid/asparagine (Wray et al. 2011, Kaplan and McGrath 2012) (**Appendix B**). Due to the distinct amino acid profiles and the solubility profile of fibroin, fibroin and sericin can be separated during a process known as "degumming." The sericin components represents 20-30% of the silk cocoon by mass. The degumming process has been reported to alter the molecular weight of silk fibroin and lead to a smear of protein below 390 kDa (Yamada et al. 2001; Jiang et al. 2006). This aligns with the range reported by other publications, where the smear starts at approximately 375-446 kDa (Kaplan and McGrath 2012, Vepari and Kaplan 2007, Mondal et al. 2007). Due to the distinct amino acid profiles and the hydrophobic nature of fibroin, fibroin and sericin can separated during the degumming process, and the removal of sericin during the degumming process can be verified.

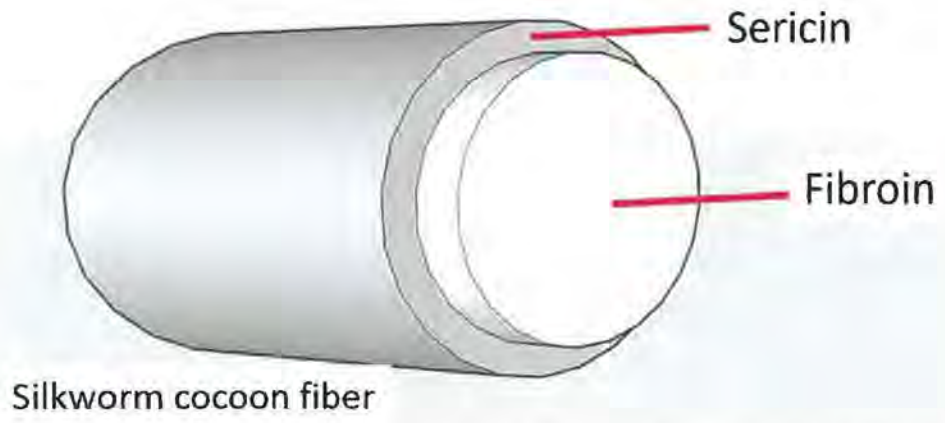

Figure 1: Cross section of a representative silk cocoon fiber. Source: Cambridge Crops

2.2 Physical and Chemical Properties

The physical and chemical properties of fibroin as described in the scientific literature are listed in **Table 2**). The abbreviated amino acid composition as described in the literature is in **Table 2**, with details in **Appendix B**. The molecular weight of silk fibroin ranges between approximately 375 kDa and 446 kDa, as described in **Part 2.1**. As silk fibroin is a large nonuniform molecule with varying structure, different publications report varying values for solubility, pH, and molecular weight due to different methodological approaches. Fibroin is insoluble without further processing, and solubility varies in literature based on methodology. Fibroin is commercially available as a powder or in solution.

2.3 Characterization of Production Organism

Cambridge Crops' Mori Silk is derived from the cocoons of the domesticated silkworm *Bombyx mori*. Silkworms were domesticated in China thousands of years ago primarily for the use of producing silk for textile as well as the edible pupae (Durst et al. 2010).

B. mori silkworms feed solely on the leaves of the mulberry plant and exist only as domesticated – wild *B. mori* silkworms are either rare or nonexistent. *Bombyx mori* silkworms are within the *Bombyx* genus, the *Bombycidae* family, of the order of *Lepidoptera* of the *Insect* class.

The silkworm's silk glands are divided into three functionally different regions. Fibroin is synthesized in the posterior region (Kaplan and McGrath 2012) where it moves by peristalsis to the middle region where it is stored as a viscous aqueous solution until needed for spinning. Sericin is produced in the middle region of the gland. When the silkworms secrete the liquid silk during the spinning, it passes through the anterior gland and expelled out through the spinneret opening (Mondal et al. 2007). As such – the silkworm itself that is eaten in various countries in and outside of North America contains fibroin at all times.

2.4 Manufacturing Process

Isolation of Mori Silk begins with cocoons from *Bombyx mori* silkworms. Silkworms are first removed from the cocoons. The silkworm-free cocoons then undergo a "degumming" step to separate the fibroin from the sericin "glue", which is glue-like due to hydrogen bonds between serines. As the sericin is more readily soluble in aqueous conditions than fibroin, it is easily removed by boiling in alkaline solutions (Kaplan and McGrath 2012), as highlighted in Step 1 in **Figure 2**. After a period of neutral-to-high pH boiling, the insoluble fibroin is removed and thoroughly rinsed (Step 2a in **Figure 2**). A salt solution is added to the rinsed fibroin and agitated to solubilize the fibroin (Step 2b in **Figure 2**). The salt, which may be calcium chloride or another similar GRAS salt, is then removed from the solution by any traditional method (e.g., dialysis) (Step 3 in **Figure 2**), thus leaving Mori Silk in solution (Product D in **Figure 2**). No antibiotics are used in the production process. Only food-grade substances and processing aids are used in the manufacture of Mori Silk.

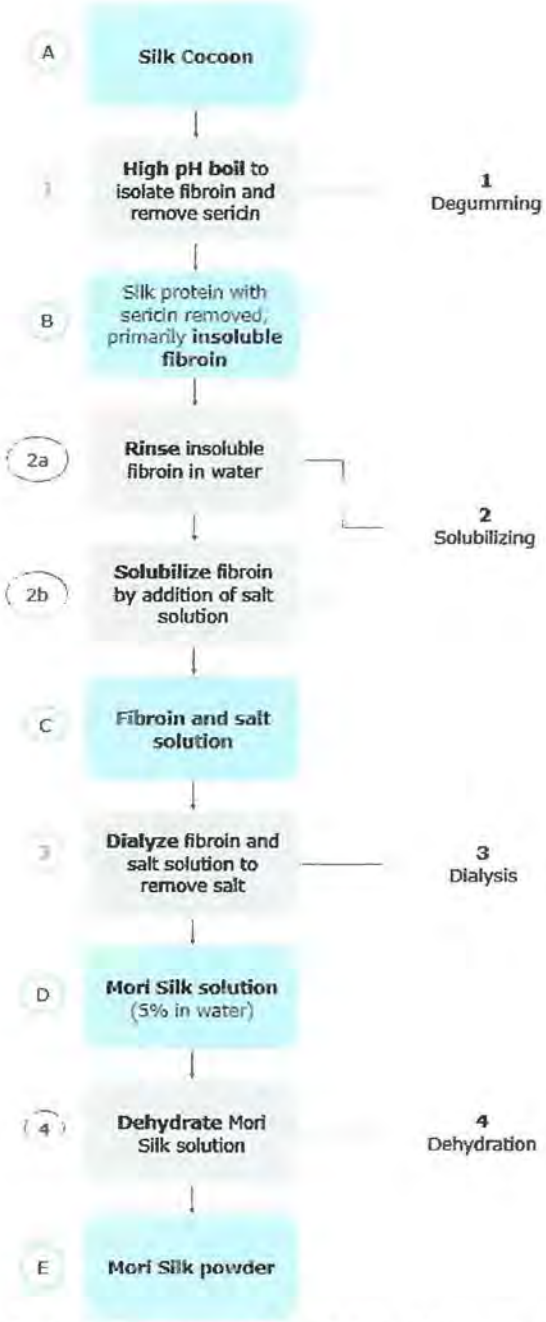

Figure 2: Manufacturing process of Cambridge Crops Mori Silk

Product D (Mori Silk solution) may be applied to foods to extend the underlying food's shelf-life. Further, Product D may be dehydrated until only powder remains (Product E, "Mori Silk powder"). This powder may then be reconstituted in water prior to application on a food.

2.5 Product Characteristics and Specifications

The identity of Mori Silk is confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid analysis (**Appendix B** contains additional details on amino acid analysis) and compared to literature values. The amino acid analysis confirms that Mori Silk is made up of almost entirely the fibroin heavy chain, as the light chain has different proportions of amino acid concentrations (**Appendix B**). The molecular weight range of Mori Silk (smear of fragments of molecular weights below 460 kDa, pictured in **Appendix I**) aligns with the molecular weight range determined from literature (**Table 2**), and the amino acid profile of Mori Silk corresponded with that reported in literature (**Table 2**). Mori Silk is comprised of silk protein, primarily fibroin heavy chain with minimal presence of fibroin light chain and P25.

Table 2: Chemical and Physical Properties of Silk Fibroin

Property	Silk Fibroin Based on Published Values	Mori Silk Manufactured by Cambridge Crops
Chemical		
Molecular weight	Range 375-446 kDa[a] Smear of molecular weights below 390 kDa[b]	Smear of molecular weights at approximately 460 kDa[c]
Amino acid profile[d,e]		
Glycine	42-46%	43-46%
Alanine	29-31%	30-32%
Serine	9-12%	7-10%
Tyrosine	4-5%	4-6%
Valine	1-3%	2-3%
Physical		
Appearance (Form)	Colorless to yellow aqueous solution White or off-white powder	Colorless to yellow aqueous solution White or off-white powder
Solubility	Variable[f]	150-250 mg/mL
pH	≥4.5[g], 6.8[h]	Approximately 7

Abbreviations:
kDa = kiloDalton
[a]Kaplan and McGrath 2012, p106; Vepari and Kaplan 2007; Mondal et al. 2007
[b]Yamada et al. 2001
[c]SDS-PAGE yields a smear of molecular weights with an upper bound at or around 460 kDa.
[d]Fibroin heavy chain is presented as it makes of the majority of fibroin; based on eight publications, see Appendix B for details
[e]Cambridge Crops amino acid analysis, see Appendix B for full results.
[f]Fibroin is a large molecule and solubility varies based on method of isolation and processing and resultant structure
[g,h]Advanced BioMatrix, Chen et al. 2008; pH varies depending on structure of fibroin and the part of *Bombyx mori* from which the fibroin was extracted.

Mori Silk will be applied to foods in solution form or as a reconstituted powder—both of which will have identical specifications. Between solution (Part D in **Figure 2**) and powder (Part E in **Figure 2**) is a dehydration process for shipping and storage purposes. Both forms will contain instructions on dilution to particular concentrations. The products will meet specifications for identity and purity including specifications for microbiological contaminants and heavy metals (**Table 3a**). All analytical methods were validated for respective analytes for protein, ash, fat, carbohydrate, and elemental analyses using internationally recognized standard methods and all AOAC, EPA, BAM and USDA/FSIS methods are appropriate for silk fibroin test matrix.

Mori Silk will be added to the foods either by mixing it in with the food, spraying it onto the food, or placing the food in a solution containing Mori Silk.

The salt solution used to solubilize fibroin may contain common GRAS salts such as calcium chloride or other GRAS chaotropic salts.

Table 3a: Specifications of Mori Silk*

Parameter	Product Specification	
	Specification	**Method**
Protein	3.5 - 6.0%wt (35 - 60 mg/g)	AOAC 992.15
Ash	<0.1%wt	AOAC 920.153
Fat	<0.1%wt	AOAC 922.06
Carbohydrates	<0.1%wt	Calculation
Arsenic	<1 mg/L	EPA 200.8
Lead	<1 mg/L	EPA 200.8
Escherichia coli	<10 cfu/g	AOAC 991.14
Listeria monocytogenes	Negative/25g	USDA/FSIS MLG 8.05
Salmonella	Negative/25g	AOAC 2009.03

* Mori Silk solution or Mori Silk powder recocalnstituted at 5% weight/volume in water (50 mg Mori Silk in 1 mL potable water, mixed or stirred to solution).

Abbreviations: AOAC = Association of Analytical Chemists; cfu = colony-forming units; g = gram(s); ICP-OES = inductively coupled plasma optical emission spectrometry; mg = milligram(s); ppmw = parts per million by weight; wt = weight

The product will meet specifications for identity and purity including specifications for microbiological contaminants and heavy metals.

In addition to the parameters in the specification, Cambridge Crops also routinely monitors the ingredient for the following parameters on a frequent basis as part of its food safety plan. These parameters were selected to monitor for product quality and are not part of the specification. As part of Cambridge Crops' food safety plans under Good Manufacturing Practices, the following parameters are expected to not be present within Mori Silk. Under

Cambridge Crops' food safety and Hazard Analysis and Critical Control Points (HACCP) plans, preventative controls are applied to control for such parameters.

Table 3b: Additional Parameters Measured for Mori Silk*

	Unit	Method
Magnesium	mg/L	AOAC 985.01
Mercury	mg/kg	EPA 7471B
Bacillus cereus	mpn/g	AOAC 980.31/ISO 7932
Cronobacter	None	PCR
Enterobacteriaceae	cfu/g	AOAC 2003.01
Staphylococcus aureus	cfu/g	AOAC 2003.07
Mold	cfu/g	BAM Ch. 18
Yeast	cfu/g	BAM Ch. 18

* Mori Silk solution or Mori Silk powder reconstituted at 5% weight/volume in water (50 mg Mori Silk in 1 mL potable water, mixed or stirred to solution).

Abbreviations: AOAC = Association of Analytical Chemists; cfu = colony-forming units; g = gram(s); kg = kilogram(s); mpn = most probable number; PCR = polymerase chain reaction.

2.6 Batch Data

Three independently manufactured, nonconsecutive batches of approximately 5% Mori Silk in water were analyzed for protein, carbohydrates, fat, and contaminants including metals and microbes to demonstrate that Mori Silk consistently met specifications set for identity and purity (**Table 4**; **Appendix C1 and C2**). All three batches met the established specifications demonstrating that Mori Silk complies with appropriate specifications for food-grade materials and that a consistent product may be produced. Specifications are set and can be seen in Table 3**a**. All analytical methods were validated for respective analytes for protein, ash, fat, carbohydrate, and elemental analyses using internationally recognized standard methods and all AOAC, EPA, BAM and USDA/FSIS methods are appropriate for silk fibroin test matrix. Corresponding certificates of analysis for Mori Silk are in **Appendix C2**. Cambridge Crops demonstrated that protein was the main constituent (98.6%) of Mori Silk in one batch of Mori Silk powder, spray-dried from a 5% in water Mori Silk solution (**Appendix C3**).

Mori Silk is also periodically monitored for additional parameters. (**Table 3b**). The batch analysis also includes analysis of these parameters in **Table 5**.

Table 4. Analysis of Production Batches of Mori Silk Solution*

Parameter	Specification	Unit	Batches[1]			Method
			Batch 342	Batch 337	Batch 335	
			10/24/2020	10/15/2020	10/05/2020	
Specification Parameters						
Protein	3.5 - 6.0 (35 - 60)	%wt (mg/g)	4.15	3.69	5.44	AOAC 992.15
Fat	<0.1	%wt	0.02	0.06	0.05	AOAC 922.06
Carbohydrates	<0.1	%wt	0.1	<0.1	<0.1	Calculation
Ash	<0.1	%wt	0.04	0.04	0.04	AOAC 920.153
Arsenic	<1	mg/L	<0.5 mg/L	<0.5 mg/L	<0.5 mg/L	EPA 200.8
Lead	<1	mg/L	<0.5 mg/L	<0.5 mg/L	<0.5 mg/L	EPA 200.8
Escherichia coli	<10	cfu/g	<10	<10	<10	AOAC 991.14
Listeria monocytogenes	Negative/25g	none	Negative/10g	Negative/10g	Negative/10g	USDA/FSIS MLG 8.05
Salmonella	Negative/25g	none	Negative/10g	Negative/10g	Negative/10g	AOAC 2009.03
Other Parameters						
Calcium	250 mg/L	mg/L	210 mg/L	211 mg/L	216 mg/L	AOAC 985.01
Magnesium	N/A	mg/L	3.75	1.74	2.54	AOAC 985.01
Mercury	N/A	mg/kg	<0.050	<0.050	<0.050	EPA 7471B
Bacillus cereus	N/A	mpn/g	<3	<3	<3	AOAC 980.31/ISO 7932
Cronobacter	N/A	none	Negative/10g	Negative/10g	Negative/10g	PCR
Enterobacteriaceae	N/A	cfu/g	<10	<10	<10	AOAC 2003.01
Staphylococcus aureus	N/A	cfu/g	<10	<10	<10	AOAC 2003.07
Mold	N/A	cfu/g	<10	<10	<10	BAM Ch. 18
Yeast	N/A	cfu/g	<10	<10	<10	BAM Ch. 18

Notes: Protein was provided on a weight basis in mg protein/g sample.
* Mori Silk solution before dehydration
[1] Values provided with "<" are the limits of detection for those analytes.

Abbreviations: AOAC = Association of Analytical Chemists; BAM = Bacteriological Analytical Manual; cfu = colony-forming units; g = gram(s); ICP-OES = inductively coupled plasma optical emission spectrometry; LOD = limit of detection; mg = milligram(s); mL = milliliter(s); N/A = not applicable (no specification); PCR = polymerase chain reaction; ppmw = parts per million by weight; USDA/FSIS MLG = United States Department of Agriculture Food Safety and Inspection Service Microbiology Laboratory Guidebook

2.7 Stability

Mori Silk powder was determined to be stable for approximately 317 days (accelerated conditions in a stability study lasting 28 days and extrapolated using Arrhenius equation). Containers carrying Mori Silk powder will be labeled with a stability time of 300 days from time of manufacturing. The stability study was conducted by preparing Mori Silk in three different food-grade packaging materials: (1) Compostable Plant Fiber container (World Centric, SKU CU-SC-U2); (2) Metallized Mylar container (QQStudio, ASIN B071VDZXWX); and (3) Compostable PLA Souffle container (World Centric, SKU CP-CS-2S). Each of the samples were stored at 60°C and 30% relative humidity for the 28 days. At the start of the study and once every seven days, a bicinchoninic acid protein assay (BCA assay) was conducted with part of the sample to quantify the total protein in each sample after reconstitution in water. The standard BCA assay has a margin of error of ±10 mg/ml. The total protein remained substantially similar to baseline throughout the 28-day stability study. Protocol and detailed results are provided in **Appendix D**.

2.8 Allergenic Potential

There are no published instances of allergenic episodes from the consumption of silk fibroin.

Potential allergens within the silkworm itself were researched, as silk fibroin is a substance produced by the silkworm. Details of this bioinformatics investigation are in **Part 6.4**. Furthermore, samples of the *Bombyx mori* pupae and cocoon along with Cambridge Crops in-process fibroin and Mori Silk were analyzed for potential allergenicity by way of mass spectrometry (**Part 6.4**). Results were published in *Food and Chemical Toxicology* (Yigit et al. 2021).

2.9 Intended Technical Effect

Cambridge Crops' Mori Silk is intended to be used as a coating on various foods including fruits, vegetables, fish, cheeses, and select processed foods, i.e., hard candy, candy bars, (including chocolate bars), nuts, cookies, cereal or meal bars, and chewing gum to preserve food in accordance with allowed mechanisms described in 21 CFR 170.3(o) including as a surface finishing agent, a substance used to increase palatability, preserve gloss, and inhibit discoloration of foods, including glazes, polishes, waxes, and protective coatings (21 CFR 170.3(o)(30)).

Cambridge Crops' Mori Silk will be used at levels consistent with current Good Manufacturing Practice. [6]

[6] Good manufacturing practice (21 CFR §182.1) includes the following restrictions: (1) The quantity of a substance added to food does not exceed the amount reasonably required to accomplish its intended physical, nutritional, or other technical effect in food. (2) The quantity of a substance that becomes a component of food as a result of its use in the manufacturing, processing, or packaging of food, and which is not intended to accomplish any physical or other technical effect in the food itself, shall be reduced to the extent reasonably possible. (3) The substance is of appropriate food grade and is prepared and handled as a food ingredient.

PART 3. DIETARY EXPOSURE

3.1 Background Intake Level

Foods consisting of or containing silkworm, pupae, or cocoons are available for purchase as a specialty food in the United States. However, the background intake level of silkworm, silkworm pupae or silkworm cocoons in the United States is unknown.

Jones (1931), working with the United States Department of Agriculture (USDA), established factors for converting the percentage of nitrogen in foods and feeds into percentages of proteins and established that the silk protein, fibroin, as a source of dietary protein, contains 15.9% of nitrogen. Silk is listed along with a multitude of other common dietary proteins, ranging from almonds to lentils to milk to shrimp.

Outside of the U.S., fibroin-containing *Bombyx mori* silkworms and silkworm-derived products, including fibroin powder (Rajakumar et al., 2014), are eaten frequently and by people of all age groups. Within North America, *Bombyx mori* silkworms and *Bombyx mori* powder are listed in Health Canada's List of Non-Novel Determinations for Food and Food Ingredients[7].

3.2 Uses in Food

Uses of silkworm, larvae or cocoons either as food itself or within food are common in countries including Canada, China and Japan (see **Part 3.1** Background Intake Level and **Part 5.1** Naturally Occurring Silk Fibroin). Silkworms and silkworm-derived food products exist and are sold in the United States; however, consumption is likely well below rates of consumption in other parts of the world. In fact, *Bombyx mori* silkworms have been consumed as far back as 5,000 years ago in China (Durst et al., 2010), with populations in China, Thailand, India, and other countries treating the silkworms as part of their common diet (Yang 1999 via Yang et al., 2009, Chen et al. 2009, Feng et al 2018). There are no known widely consumed food uses in the United States.

Cambridge Crops intends to use Mori Silk to extend the shelf life of foods. Specifically, it intends to use Mori Silk with fruits, vegetables, cheese, fish, and select processed foods, i.e., hard candy, candy bars (including chocolate bars), nuts, cookies, cereal or meal bars, and chewing gum for ingestion by the general population in accordance with 21 CFR 182.1(b).[8] Those food categories represent approximately 390 grams of food per day at the 90th percentile of intake among the U.S. population 2 years and older. The concentration of Mori Silk on foods is dependent on the food category itself. As regulated by 21 CFR 182.1(b), Cambridge Crops intends to only use an amount "reasonably required to accomplish its intended physical, nutritional, or other technical effect in food."

Mori Silk will be applied to foods in three ways, depending on the food product:

1. Wetting the food in a container with Mori Silk solution;
2. Mixing Mori Silk in with the food; or
3. Spraying the food with a Mori Silk solution.

[7] Government of Canada List of non-novel determinations for food and food ingredients. Accessed April 20, 2021. https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/requesting-novelty-determination/list-non-novel-determinations.html

[8] Mori Silk is not intended to be used in USDA regulated products at this time and is not intended to be used in infant formula.

The difference between applications will depend on the underlying food processing lines and methods. Mori Silk is designed to be effective for use with existing processor lines or equipment with minimal changes from farmers, growers, shippers, or packers.

The estimated daily intake is 2.9 mg Mori Silk per kg body weight per day for the 90th percentile consumer ages 2 years and older (details are presented in **Part 3.3**).

3.3 Estimated Intakes of Mori Silk from the Proposed Uses

Estimates of potential intakes of ingredient resulting from these intended uses were calculated using food consumption data reported in the United States Department of Health and Human Service's 2013-2014 and 2015-2016 National Health and Nutrition Examination Surveys (NHANES).

Mori Silk is intended to be used on a variety of foods, including fruits, vegetables, fish, cheese, and select processed foods. In order to estimate potential intake of the Mori Silk, Cambridge Crops analyzed data from the 2013-2014 and 2015-2016 NHANES which had a total sample size of 16,085 people with reported body weights. The 2013-2016 NHANES data are the most recent datasets that have been published. Each food was analyzed to determine consumption of foods belonging to the following food categories: fish, fruits, vegetables, cheese, and select processed foods. Further details of NHANES calculations are provided in **Appendix H**. Cambridge Crops anticipates some food items in each of the categories to be coated with Mori Silk.

Each of the foods coated may require a different amount of Mori Silk for the coating to be effective. **Table 5** provides coating concentrations by food category. Cambridge Crops will follow good manufacturing practice (21 CFR §182.1(b)) and only use the amount necessary. Further, there is an economic reason to only use what is necessary.

In addition, Cambridge Crops assumed the very unlikely scenario where *each and every* piece of food in the five food categories eaten by the individuals surveyed by NHANES is coated with Mori Silk.

Further, the consumption amounts below do not take into account any sort of rinsing that may occur at any stage in the supply chain, whether on the processing line, in a commercial kitchen, or at home. For some foods, Mori Silk may be readily rinsed or washed off.

These conservative assumptions were used to estimate the daily intake of Mori Silk on foods by age group and food category (**Table 8** and **Table 9**). **Part 6** further describes the safety of such amounts as eaten by the consumer.

Table 5: Estimated Mori Silk Coating Concentrations by Food Category	
Food Category	**Mori Silk concentration (mg/kg food)**
Fish	415
Fruits	385
Vegetables	315
Cheese	750
Select processed foods	980
Notes: * The select processed foods are hard candy, candy bars (including chocolate bars), nuts, cookies, cereal or meal bars, and chewing gum. Additional details are provided in Appendix H.	

Table 6. Estimated Daily Intake of Mori Silk by Kilograms-Body Weight as a Food Coating by Age Group

Population	*n*	g **food** consumed / kg bodyweight / day		mg **Mori Silk** / kg bodyweight / day	
		Mean	90th percentile	**Mean**	**90th percentile**
Infants (0-11 months)	487	1.39	3.75	0.60	2.00
Toddlers (12-35 months)	820	8.45	17.73	4.22	8.27
Children (3-11 years)	2983	5.20	11.80	2.64	5.73
Teenagers (12-19 years)	2464	2.01	4.71	1.03	2.38
Adults (age 18-79 years)	9967	2.43	5.43	1.12	2.38
General population (2 years and older)	15862	2.82	6.36	1.34	2.92
Abbreviations: bw = body weight, g = gram(s), kg = kilogram(s), mg = milligram(s)					

Table 7. Estimated Daily Intake of Mori Silk as a Food Coating by Age Group

Population	*n*	g **food** consumed / day		mg **Mori Silk** / day	
		Mean	90th percentile	**Mean**	**90th percentile**
Infants (0-11 months)	488	12.81	47.51	5.59	18.26
Toddlers (12-35 months)	828	105.59	228.87	53.15	106.29
Children (3-11 years)	3002	133.02	286.28	67.53	139.26
Teenagers (12-19 years)	2491	122.21	284.04	62.84	141.71
Adults (age 18-79 years)	10041	188.02	412.63	86.47	182.40
General population (2 years and older)	15986	175.10	388.93	81.89	173.52

Abbreviations: g = gram(s), mg = milligram(s)

Table 8. Estimated Daily Intake of Mori Silk by Kilograms-Body Weight as a Food Coating by Age Group and Food Category

Population	***n***	**Food category**	**g food consumed / kg bodyweight / day**		**mg Mori Silk consumed / kg bodyweight / day**	
			Mean	**90th percentile**	**Mean**	**90th percentile**
Infants (0-11 months)	487	Fish & Shellfish	0.01	5.19	0.02	0.00
		Fruits	0.73	0.00	1.24	3.95
		Vegetables	0.45	11.51	0.59	2.25
		Cheese	0.07	5.88	0.26	0.67
		Select Processed Foods	0.13	2.23	0.48	1.56
		Total	1.39	3.75	0.60	2.00
Toddlers (12-35 months)	820	Fish & Shellfish	0.14	0.00	0.07	0.00
		Fruits	4.40	11.51	1.91	4.72

Table 8. Estimated Daily Intake of Mori Silk by Kilograms-Body Weight as a Food Coating by Age Group and Food Category

Population	*n*	Food category	g food consumed / kg bodyweight / day		mg Mori Silk consumed / kg bodyweight / day	
			Mean	90th percentile	Mean	90th percentile
		Vegetables	1.78	5.88	0.62	2.05
		Cheese	0.75	2.23	0.64	1.85
		Select Processed Foods	1.37	3.75	1.54	3.92
		Total	8.45	17.73	4.22	8.27
Children (3-11 years)	2983	Fish & Shellfish	0.17	0.00	0.08	0.00
		Fruits	2.37	6.98	1.02	2.84
		Vegetables	1.28	3.85	0.45	1.32
		Cheese	0.43	1.25	0.36	1.02
		Select Processed Foods	0.95	2.48	1.07	2.74
		Total	5.20	11.80	2.64	5.73
Teenagers (12-19 years)	2464	Fish & Shellfish	0.10	0.00	0.05	0.00
		Fruits	0.67	2.21	0.31	1.00
		Vegetables	0.66	1.94	0.25	0.73
		Cheese	0.20	0.55	0.19	0.48
		Select Processed Foods	0.39	1.08	0.46	1.19
		Total	2.01	4.71	1.03	2.38
Adults (age 18-79 years)	9967	Fish & Shellfish	0.19	0.68	0.09	0.31
		Fruits	0.61	1.99	0.26	0.85
		Vegetables	1.13	2.95	0.40	0.99
		Cheese	0.18	0.50	0.16	0.41
		Select Processed Foods	0.32	0.86	0.35	0.93
		Total	2.43	5.43	1.12	2.38
All (2 years and older)	15862	Fish & Shellfish	0.18	0.55	0.08	0.26
		Fruits	0.89	2.67	0.38	1.12
		Vegetables	1.12	2.99	0.40	1.01
		Cheese	0.22	0.58	0.19	0.49
		Select Processed Foods	0.42	1.12	0.47	1.22
		Total	2.82	6.36	1.34	2.92

Abbreviations: bw = body weight, g = gram(s), kg = kilogram(s), mg = milligram(s)
Notes: The select processed foods are hard candy, candy bars (including chocolate bars), nuts, cookies, cereal or meal bars, and chewing gum.

Table 9. Estimated Daily Intake of Mori Silk by as a Food Coating by Age Group and Food Category

Population	*n*	Food category	g food consumed / day		mg Mori Silk consumed / day	
			Mean	90th percentile	Mean	90th percentile
Infants (0-11 months)	488	Fish & Shellfish	0.12	0.00	0.22	0.00
		Fruits	6.72	17.87	11.14	32.73
		Vegetables	4.13	8.21	5.45	18.97
		Cheese	0.65	0.00	2.42	6.06
		Select Processed Foods	1.19	0.00	4.43	14.68
		Total	12.81	47.51	5.59	18.26
Toddlers (12-35 months)	828	Fish & Shellfish	1.75	0.00	0.83	0.00
		Fruits	54.90	145.19	23.84	58.23
		Vegetables	21.77	69.51	7.64	23.53
		Cheese	9.59	29.04	8.10	23.66
		Select Processed Foods	17.59	50.16	20.22	51.52
		Total	105.59	228.87	53.15	106.29
Children (3-11 years)	3002	Fish & Shellfish	4.47	0.00	2.11	0.00
		Fruits	58.78	165.28	25.32	70.01
		Vegetables	34.27	99.19	12.15	34.21
		Cheese	11.01	31.42	9.40	25.14
		Select Processed Foods	24.48	60.88	27.71	66.69
		Total	133.02	286.28	67.53	139.26
Teenagers (12-19 years)	2491	Fish & Shellfish	6.00	0.00	3.06	0.00
		Fruits	39.90	132.03	18.52	63.88
		Vegetables	40.41	124.94	15.53	45.17
		Cheese	12.72	35.15	11.80	31.27
		Select Processed Foods	23.18	61.01	27.95	72.35
		Total	122.21	284.04	62.84	141.71
Adults (age 18-79 years)	10041	Fish & Shellfish	14.91	50.99	7.06	23.55
		Fruits	46.78	158.03	20.07	68.26
		Vegetables	87.55	227.00	31.01	76.86
		Cheese	14.39	41.98	12.43	31.87
		Select Processed Foods	24.38	67.50	27.13	72.81
		Total	188.02	412.63	86.47	182.40

Table 9. Estimated Daily Intake of Mori Silk by as a Food Coating by Age Group and Food Category

Population	*n*	Food category	g food consumed / day		mg Mori Silk consumed / day	
			Mean	90th percentile	Mean	90th percentile
All (2 years and older)	15986	Fish & Shellfish	12.57	39.88	5.97	19.13
		Fruits	48.47	159.25	20.93	68.49
		Vegetables	76.15	205.32	27.07	69.78
		Cheese	13.61	38.85	11.79	31.50
		Select Processed Foods	24.30	66.46	27.20	71.41
		Total	175.10	388.93	81.89	173.52

Abbreviations: g = gram(s), mg = milligram(s)
Notes: The select processed foods are hard candy, candy bars (including chocolate bars), nuts, cookies, cereal or meal bars, and chewing gum. Consumption of 0.00 indicates that the individuals surveyed in that age group did not consume that food category during the NHANES survey.

Assuming the 90th percentile food consumption rate, an average adult in the United States weighing 83.4 kg[9] would consume 182 mg of Mori Silk each day[10]. An adult consuming the mean amount of these foods per day would consume 86 mg. Adults have the highest overall intake of Mori Silk per day and the next highest overall intake group is children ages 3-11 years old, consuming 140 mg Mori Silk per day at the 90th percentile, and 68 mg per day at the mean.

[9] Average body weight based on participants in NHANES 2013-2016

[10] At the 90th percentile, the consumption of Mori Silk amounts to 0.36% (2.9 mg/kg bw/day) of the total daily protein consumption (0.8 g/kg-bw/day) recommended by National Academy of Medicine (NAM, formerly the Institute of Medicine (IOM)). The mean consumption of Mori Silk amounts to 0.16% (1.3 mg/kg bw/day) of the NAM value.

PART 4. SELF-LIMITING LEVELS OF USE

The intended uses of Cambridge Crops' Mori Silk are not self-limiting.

PART 5. EXPERIENCE BASED ON COMMON USE IN FOOD

The statutory basis for Cambridge Crops' conclusion of the GRAS status of Mori Silk is based on scientific procedures appropriate to establish the safety of the ingredient based on generally available data under the conditions of its intended uses in food. The following is presented for background information purposes only.

5.1 Naturally Occurring Silk Fibroin: Silkworms are eaten in many parts of the world

The *Bombyx mori* silkworm may be the oldest domesticated insect in the world – domesticated for both the silk textile as well as the edible pupae as far back as 5,000 years ago in China (Durst et al. 2010). The *B. mori* silkworm is reported to be a "well-studied insect and people from many countries have eaten silkworm, larvae, pupae, and adults for centuries." (Mitsuhashi, 1997). The larvae, pupae, and adults (as well as the silkworm cocoon pelade, the innermost layer of silkworm cocoon, commonly eaten as well) all contain fibroin. *B. mori* silkworms are known for having high protein content and are relatively easy to breed. Yang et al (2009) proposed silkworms as a suitable source of protein for humans on the International Space Station. The silkworm silk fiber contains more than 98% protein (Feng, 2004 via Yang et al., 2009) and fibroin itself contains 18 kinds of amino acids (Yang et al., 2009).

In fact, *Bombyx mori* silkworms and *Bombyx mori* powder (both containing fibroin) are listed in Health Canada's List of Non-Novel Determinations for Food and Food Ingredients.[11] And because of silk fibroin's specific amino acid sequence, it is commonly used as a marker for the standardization of processed mature silkworm powder that is used as a food and food ingredient. (Lee et al., 2018). Health Canada recently (June 21, 2021) confirmed the non-novel food status of fibroin protein isolate from silkworm (Bombyx mori) cocoons; Non-Novelty Determinations Regarding Mori Silk™ (Case # 2021-025133) (Appendix K).

Outside of North America, fibroin-containing *Bombyx mori* silkworms and silkworm-derived products, including fibroin powder (Rajakumar et al., 2014), are eaten frequently and by people of all age groups. In a United Nations Food and Agricultural Organization Workshop, scholars from around the world emphasized the positive effects of consuming *Bombyx mori* silkworms (Durst et al., 2010). In Europe, *Bombyx mori* consumption may be found in countries such as Belgium, France and Italy (Superior Health Council of Belgium, 2014; Melgar-Lalanne et al., 2019).

Further, silkworms, silkworm pupae and silkworm cocoons have been eaten for years in Asian countries including China, Japan, Thailand and India (e.g., Yi et al. 2010, Rajakuma et al. 2014, Buhroo et al. 2018). *Bombyx mori* silkworms are consumed during all stages of the silkworm's life cycle: in particular, the silkworms themselves are a popular food among people in Shandong province, China; people in the northeast region of China commonly consume silkworm pupae; and people in Henan province and Southern China commonly consume silkworm moths (Yang et al., 1999 via Yang et al., 2009) – all of which contain fibroin. The cocoon pelade, the inner and unreelable layer of cocoon, is also consumed in Japan where it is hydrolyzed from the waste of silk fibers via enzymatic treatment and further chemically refined to become a final food product intended to be consumed with milk or coffee as a nutritional supplement (Rajakumar et al., 2014).

[11] Government of Canada List of non-novel determinations for food and food ingredients. Accessed April 20, 2021. https://www.canada.ca/en/health-canada/services/food-nutrition/genetically-modified-foods-other-novel-foods/requesting-novelty-determination/list-non-novel-determinations.html

Silkworm and pupae are also eaten in other forms. For example, silkworm and silkworm pupae are commonly added to food in powder form as a protein source in Hong Kong, China, Korea and Japan (Savithri and Sujathamma 2016). The Henan Province in China is cited as an area where the larvae and adults of *Bombyx mori* and tussah silkworms (*Antheraea pernyi*) are common foods (Feng et al., 2018). Larvae and adults are also popular cuisines in other regions of China, Japan, and east Asian countries (Yang 1999 *in Chinese* via Yang et al. 2009, Chen et al. 2009, Feng et al 2018). Another species of silkworm is domesticated in India for textiles and serves as a food (Durst et al. 2010). In Thailand, cooked silkworm pupae are a popular snack in rural villages and urban areas like Bangkok (Durst et al. 2010). Silkworms are served boiled in a sweet-sour sauce in Japan as well as in Vietnam, roasted silkworm pupae are sold by street vendors in China, and another boiled and seasoned preparation of silkworm is common in Korea (Savithri and Sujathamma 2016). Fibroin protein from silkworms, silkworm larvae, and silkworm cocoons has been consumed by large populations for many years.

PART 6. NARRATIVE

6.1 Introduction

Silkworms, pupae, and cocoons have been eaten in many countries with diverse populations for centuries. Though there is no known large-scale consumption noted in the United States, the safety of consuming silkworm and silkworm-derived products is supported by scientific procedures, and a documented history of safe consumption in other parts of the world. In addition, silk fibroin products have been approved by FDA and other regulating bodies around the world for non-food use in humans.

Mori Silk is directly derived from silkworm cocoons. Such cocoons mainly consist of two proteins, sericin and fibroin. Sericin is removed, leaving fibroin for use as a food coating. **Part 2** provides details on the identity and manufacturing process of Cambridge Crops Mori Silk as well as information on analytical testing to demonstrate the absence or acceptable levels of manufacturing residuals including chemical byproducts, processing aids, solvents, bacteria, bacterial proteins, and endotoxins. In this part (**Part 6**), the safety data for Cambridge Crops' Mori Silk and supporting studies are presented and discussed. Cambridge Crops developed a toxicology testing program with a repeated dose 28-day oral toxicity study (**Part 6.3.2.1**) and other supporting studies for the Mori Silk ingredient based on a pre-notification consultation meeting to GRN 000930 with FDA (September 10, 2019) and subsequent communications. In addition to the results of Cambridge Crops studies, studies of other *Bombyx mori*-derived substances are also presented and discussed.[12]

6.2 Absorption, distribution, metabolism, and excretion of silk fibroin

Silk fibroin is a protein present within the *Bombyx mori* silkworm cocoon as a double-stranded fiber coated with proteins called sericin (Wray et al. 2011). The silk fiber mass is comprised of 70-80% silk fibroin, 20-30% sericin, 0.4-0.8% wax matter, 1.2-1.6% carbohydrates, 0.7% inorganic matter, and 0.2% as pigment (Mondal et al., 2007). The core of the silk fiber, silk fibroin, is comprised predominately of a crystalline portion that contains repeating amino acids, e.g., glycine, alanine, and serine, that form the antiparallel ß-sheet providing the silk fiber its stability and mechanical properties (Cao and Wang 2009). The amino acid molar composition of fibroin consists of 46.4 mol% of glycine, 31.6 mol% of alanine, 9.48 mol% of serine, and 4.98 mol% of tyrosine, plus residual amounts of other amino acids (Mondal et al. 2007).

In general, digestion of dietary proteins begins in the stomach and continues into the lumen of the duodenum (Lentner et al. 1981). Within the digestive tract of humans, proteins are broken down via hydrolysis primarily into free amino acids. Approximately 10% of the proteins in the gastrointestinal tract do not undergo hydrolysis and are excreted in the feces. (Lentner et al. 1981). Silk fibroin heavy chain, most similar to the composition of Mori Silk, is composed predominately of glycine and alanine and, in smaller proportions, tyrosine and serine and trace amounts of other amino acids (Mondal et al. 2007). Glycine is a non-essential amino acid capable of being endogenously generated from serine with very low acute oral toxicity (European Chemicals Agency (ECHA) 2020a). Following absorption, glycine is transported into the liver and distributed throughout the body where it is involved

[12] For the literature searches, the following search terms (using Medical Subject Headings (MeSH), if available) were utilized in the National Library of Medicine's PubMed database: silk, fibroin, protein, safety toxicology, bombyx, silkworm, mutagenicity, genotoxicity, oral, ingestion.

in the biosynthesis of DNA, phospholipids, heme, and collagen (ECHA 2020a). Glycine undergoes metabolism primarily via the glycine cleavage system leading to the formation of ammonia and carbon dioxide. Glycine can also be converted to serine and then metabolized to pyruvate or oxidized to oxalate in the liver via hepatic lactate dehydrogenase (ECHA 2020a).

The amino acid alanine, also a primary component of silk fibroin, is a prominent amino acid within the human body that is utilized in the synthesis of proteins (ECHA 2020b). The liver accumulates plasma alanine and metabolizes alanine to pyruvate, which is oxidized or utilized to form glucose by gluconeogenesis (ECHA 2020b). L-alanine is absorbed in the gastrointestinal tract and taken up via active transporters into the mucosal cells, where it may undergo distribution, utilization, or be further broken down by the mucosal cells and excreted. Alanine and its metabolites are water soluble and are filtered and eliminated in the urine by the kidneys. (ECHA 2020b).

L-tyrosine and L-serine, the other major amino acid components of silk fibroin, are highly concentrated in cell membranes and are found in high concentrations in the muscle tissue. Serine undergoes non-oxidative deaminated to pyruvate via L-serine dehydratase. Influenced by the catalytic enzyme, serine hydroxymethyltransferase, serine maintains equilibrium with glycine and has a similar metabolic pathway (Lentner et al. 1981). Finally, L-tyrosine is metabolized in humans by the enzyme tyrosine aminotransferase to yield 4-hydroxyphenylpyruvate, which is further metabolized to acetoacetate and fumarate (Lentner et al. 1981). Serine and tyrosine, like other amino acids, are rapidly metabolized and broken down within the liver and other cells via conversion into urea and other metabolites. Amino acids and their metabolites are filtered by the kidneys, where they are actively reabsorbed, or filtered into the urine for excretion (ECHA 2020c, ECHA 2020d).

6.2.1 Mori Silk Demonstrated Digestible in *in vitro* Digestibility Study in Simulated Human Gastric Fluid

Cambridge Crops conducted *in vitro* digestibility studies in simulated human gastric fluid using Mori Silk. The digestibility of Mori Silk was tested both at 10 units pepsin as well as 1 unit pepsin per microgram (µg) Mori Silk, per FDA's recommendation. Pepsin was diluted in simulated gastric fluid with a pH of 2.0. The pepsin solution was tested for proteolytic activity by digestion of Mori Silk within 24 hours of each assay day. A limit of detection study was performed prior to digestion to ensure that 10% residual protein was detectable using SDS-PAGE and Coomassie blue staining. 350 µg of Mori Silk with and without pepsin were measured at different time points from 2 minutes to 60 minutes.

The results show that Mori Silk was rapidly digested in pepsin at pH 2.0 at both tested ratios (10:1 and 1:1 pepsin:µg Mori Silk). The SDS-PAGE Coomassie blue gel staining method demonstrated that over 90% of the protein was digested in less than two minutes. The full study is provided in **Appendix I**, and results from both the 10:1 ratio and the 1:1 ratio studies are also described in Yigit et al. (2021).

6.3 Safety of Mori Silk Demonstrated in Toxicological Studies

Published studies confirm that Cambridge Crops Mori Silk is safe for its intended uses (Yigit et al. 2021). Safety data on Mori Silk was also presented at the Food Allergy and Anaphylaxis Meeting (FAAM) during the European Academy of Allergy & Clinical Immunology (EAACI) Annual Meeting (October 16, 2020); as well as at the International Association for Food

Protection Annual Meeting (October 28, 2020) (Appendix J). A discussion of key studies is presented in this **Part 6.3** and in **Table 10** "Summary of Toxicological Studies Regarding Mori Silk". Studies of related *Bombyx mori*-derived substances (i.e. silkworm powder) are also included to support the safety of consumption of silk fibroin (**Table 11**). Studies of silk fibroin substances that have been significantly altered after isolation from *Bombyx mori* or their cocoons were not included as such substances may be different from silk fibroin (Coelho et al. 2020, Naserzadeh et al. 2018).

The test substance used in Cambridge Crops' toxicology studies was Mori Silk solution at approximately 5% in water. The Mori Silk solution was then further diluted or concentrated to create the concentrations necessary for the studies.

6.3.1 Mutagenicity and Genotoxicity Studies

6.3.1.1 Mori Silk is not mutagenic or genotoxic

Mutagenicity

To evaluate the potential mutagenicity of Cambridge Crops Mori Silk, an Ames test was conducted by Product Safety Labs (PSL, 2394 US Highway 130, Dayton NJ, 08810) using Salmonella Typhimurium strains TA1535, TA1537, TA98, TA100 and *E. coli* strain WP2 uvrA (Yigit et al. 2021, **Appendix G1**) following OECD Guideline 471 and complying with Good Laboratory Practice (GLP). Based on this Ames test, Mori Silk did not show evidence of bacterial mutagenicity.

Mori Silk was tested at levels of 31.6, 100, 316, 1000, 3160, 10,000, 31,600, and 100,000 ug/plate using the plate incorporation method in both the absence and presence of metabolic activation (chemically-induced rat liver S9 mix), with a confirmatory assay using the same dose levels in a pre-incubation assay (30 minutes at 37°C), with and without metabolic activation. The mean revertant colony counts for each strain treated with the vehicle were close to or within the expected range. The vehicle control was sterile water, the same substance with which the Mori Silk test substance was diluted. The positive control substances yielded the expected substantial increases in revertant colony counts in both the absence and presence of S9 in each phase of the test, confirming the sensitivity of the test and the activity of the S9 mix. The positive control substances used in the test were the following: sodium azide (15 ug/mL in sterile water) for Salmonella Typhimurium TA100, TA1535, ICR 191 acridine (10 ug/mL in sterile water) for Salmonella Typhimurium TA1537, daunomycin (60 ug/mL in sterile water) with Salmonella Typhimurium TA98, methyl methanesulfonate (25 uL/mL in sterile water) with *E. coli* WP2 uvrA, and 2-aminoanthracene (100 ug/mL in DMSO) for all strains tested. Precipitation and toxicity were not present in any of the strains; contamination that did not impact evaluation of mutagenicity, was noted in individual plates for three strains tested (*E. coli* WP2 uvrA, Salmonella Typhimurium TA1535 and TA1537). No concentration-related or substantial test substance related increases in the number of revertant colonies were reported with any of the *Salmonella* or *E. coli* strains tested in both the absence and presence of metabolic activation using either the plate incorporation or the pre-incubation method. The study met the requirements of appropriate Good Laboratory Practice Standards with the exception that positive control substances and verification of concentration of positive control substances in their carriers were not determined analytically; however, the purity of the materials used were certified by a

reputable supplier and all preparations were thoroughly documented. Under the conditions of the study, Cambridge Crops Mori Silk was not mutagenic.

Genotoxicity

To evaluate the potential genotoxicity of Cambridge Crops Mori Silk, (i.e., damage to the chromosomes or mitotic spindle apparatus of erythroblasts), an *in vivo* mouse erythrocyte micronucleus test was performed by Product Safety Labs following OECD Guideline 474 and complying with GLP (Yigit et al. 2021, **Appendix G2**). The test substance was administered by gavage to 5 male and 5 female Institute of Cancer Research (ICR) mice at 1000 mg/kg·bw/day in a volume of 20 mL/kg for two days. The limit dose of 1000 mg/kg·bw/day was selected because it was the maximum achievable dose level of the test substance formulation in this test system. Distilled water served as the vehicle control and cyclophosphamide was the positive control. There were no mortalities and no clinical findings were reported in any animal over the course of the study. A minimum target of 4,000 polychromatic erythrocytes per animal was scored for incidence of micronucleated immature erythrocytes.

The test substance did not induce a statistically significant increase in frequency of micronucleated reticulocytes in male or female mice. The frequency of reticulocyte and micronucleated normochromatic erythrocytes did not differ significantly after treatment with the test substance. The negative control and positive control results were valid, and the study met the requirements of appropriate Good Laboratory Practice Standards. Under the conditions of the study, Cambridge Crops Mori Silk was not genotoxic with respect to micronucleus induction.

6.3.1.2 Other studies evaluating genotoxicity of *Bombyx mori*-derived substances (not Mori Silk)

No mutagenicity or genotoxicity noted with silkworm extract powder (Heo et al. 2013)

The test substance used by Heo et al. (2013) was silkworm extract powder from *Bombyx mori* larvae frozen in liquid nitrogen and lyophilized. The lyophilized larvae were extracted with ethanol and lyophilized again to produce the silkworm extract powder. The final test substance powder likely contained fibroin along with other components of the silkworm. Heo et al. (2013) performed a bacterial reverse mutation assay, chromosomal aberration assay, and a mouse bone marrow micronucleus assay.

Silkworm extract powder was tested for mutagenicity in a bacterial reverse mutation test (Ames test) with Salmonella Typhimurium TA100, TA1535, TA98, TA1537, and *E. coli* WP2 *uvr*A. The bacterial strains were treated with 10, 50, 150, 500, 1500, or 5000 mg/plate silkworm extract powder with or without S9 metabolic activation. There were no increases of the means of revertant per plate for all doses of silkworm extract powder in all test strains both in the presence and absence of metabolic activation. Precipitation was seen in the 1500 and 5000 ug/plate. Positive control plates had results validating the test system. Under the conditions of the study, silkworm extract powder showed no evidence for mutagenicity.

In a chromosomal aberration assay, Chinese hamster lung cells were incubated with silkworm extract powder at concentrations of 0, 150, 275, 300, 550, 600, 900, 700, or 1100 ug/mL for 6 hours with an 18-hour recovery period or 0, 150, 300, 600, or 700 ug/mL for 24

hours with no recovery period. One hundred metaphases per culture (200 metaphases per dose) were evaluated for chromosome aberrations. The number of aberrant metaphases in all of the treatment series in the presence and absence of the metabolic activation system were not significantly increased. The positive controls had results verifying the validity of the test system. Silkworm extract powder showed no evidence for generating chromosomal aberrations under the conditions of this study with Chinese hamster lung cells.

In a mouse bone marrow micronucleus assay, silkworm extract powder was orally administered to 6 male ICR mice per group at doses of 0, 1250, 2500, or 5000 mg/kg for two consecutive days. Cyclophosphamide monohydrate was used as a positive control and was administered intraperitoneally once on day two. No mortality or abnormalities were reported. The frequency of micronucleated bone marrow polychromatic erythrocytes was not increased at any of the silkworm extract powder dose levels tested. No significant differences in polychromatic erythrocyte/red blood cell (PCE:RBC) ratio were reported at any of the doses of silkworm extract powder tested. In the positive control, the PCE:RBC ratio was decreased significantly. Silkworm extract powder did not show evidence for genotoxicity with respect to micronucleated erythrocytes under the conditions of the study.

Based on the results of the bacterial reverse mutation test, the chromosomal aberration assay, and the mouse bone marrow micronucleus assay, silkworm extract powder had little to no mutagenic or genotoxic potential under the conditions of the studies.

6.3.2 Oral Toxicity: Silk Fibroin Does Not Induce Toxic Effects After Repeated Ingestion by Rats

6.3.2.1 Mori Silk was not toxic in a subchronic 28-day oral toxicity test with rats

14-day range-finding study

Cambridge Crops commissioned a 14-day repeated dose range-finding study with Product Safety Labs following OECD Guideline 407. In this GLP study, Sprague-Dawley rats (5 per sex per group) were administered 0, 125, 250, or 500 mg/kg·bw/day Cambridge Crops' Mori Silk in distilled water via oral gavage (Yigit et al. 2021, PSL 2020a, **Appendix G3**). The highest dose tested was 500 mg/kg·bw/day limited by the solubility of Mori Silk for a single gavage. The test substance had no reported effect on mortality, body weight, body weight gain, food consumption, food efficiency, or clinical observations at any dose tested. No histopathological abnormalities nor macroscopic findings at necropsy were reported at any dose tested.

28-day repeated dose oral toxicity study

Cambridge Crops commissioned a 28-day repeated dose oral toxicity study with Product Safety Labs. The repeated dose oral toxicity of Cambridge Crops Mori Silk was evaluated in a 28-day study conducted with Sprague-Dawley rats consistent with OECD Guidelines for Testing of Chemicals Test No. 407[13], US EPA Health Effects Test Guidelines: OPPTS 870.3050[14], and US FDA Redbook[15] (Yigit et al. 2021, PSL 2020b, **Appendix G4**). Male and female rats (10 per sex per dose group) were administered Mori Silk (Batch 215) gavage

[13] OECD Guidelines for testing of chemicals, Section 4, Test No. 407: Health Effects, Repeated Dose 28-Day Oral Toxicity Study in Rodents (adopted 1995; updated October 2008).

[14] US EPA Health Effects Test Guidelines: OPPTS 870.3050 Repeated Dose 28-day Oral Toxicity Study in Rodents (2000)

[15] US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, Revised 2007 IV.C.4.A. Subchronic Toxicity Studies with Rodents (2003).

doses of 0 (distilled water), 125, 250, or 500 mg/kg·bw/day. Individual doses were calculated based on the most recent body weights and were adjusted each week to maintain the targeted dose level for each rat. The average concentration of the highest dose tested was 44.8 mg/mL, 89.6% of the target 50 mg/mL. The rats were randomly distributed, stratified by body weight among the four dosing groups on the day of study start. Mori Silk doses were administered daily for 28 consecutive days.

Animals were observed twice daily for viability. Cage-side observations were performed daily. Individual body weights and food consumption were measured one day prior to study start and weekly thereafter. Food efficiency was also reported. The animals were weighed prior to sacrifice. Clinical pathology was performed for clinical chemistry, hematology, and coagulation on blood collected at necropsy. All animals in the study were subjected to a gross necropsy, which included examination of the external surface of the body, all orifices, musculoskeletal system, and the cranial, thoracic, abdominal, and pelvic cavities with their associated organs and tissues including adrenals, brain, epididymides, kidneys, liver, heart, spleen, thymus, and reproductive organs. All gross lesions were recorded. Histological examination was performed on the preserved organs and tissues of the animals from both the control and high dose groups.

No significant changes were reported in clinical observations, food efficiency, clinical chemistry, hematology, or coagulation at any of the doses tested. All animals survived to the end of study and no test substance-related gross or microscopic findings were reported. No adverse effects were reported. The reported No-Observed-Adverse-Effect-Level (NOAEL) was the highest dose tested, 500 mg/kg·bw/day[16]. The highest dose was limited by solubility of Mori Silk in water for a single gavage per day (Yigit et al. 2021).

6.3.2.2 Toxicological studies evaluating *Bombyx mori*-derived test substances (not Mori Silk)

No evidence for acute or subchronic toxicity was observed in a study of silkworm extract powder (Heo et al 2013)

In the following acute and subchronic oral toxicity GLP studies consistent with OECD Guidelines, the test substance, silkworm extract powder, came from *Bombyx mori* larvae frozen in liquid nitrogen and lyophilized (Heo et al. 2013). The lyophilized larvae were extracted with ethanol and lyophilized again to produce the silkworm extract powder.

A single oral dose of silkworm extract powder was administered to five male and five female Sprague-Dawley rats at doses of 0, 1250, 2500, or 5000 mg/kg. Clinical signs of toxicity and mortality were recorded continuously for the first hour, and once per hour for the next 5 hours, then once daily for 14 days. No deaths were reported at any of the administered doses. Soft stool was reported in 3 of 5 males at 2500 mg/kg, all 5 males at 5000 mg/kg and 2 of 5 females at 5000 mg/kg on the second day. No treatment-related changes were reported in body weight and no gross findings were recorded at necropsy. The study authors concluded that the approximate lethal dose of the silkworm extract powder was greater than 5000 mg/kg.

[16] While the study called for 500 mg/kg·bw/day, PSL achieved an average of 89.6% of the targeted high dose, leaving the high dose as measured during Concentration Verification Analysis to be 448 mg/kg bw/day. Further details provided in the 28-day study report in Appendix G4.

Silkworm extract powder was administered by gavage at doses of 0, 500, 1000, or 2000 mg/kg·bw/day to groups of ten male and ten female Sprague-Dawley rats for 90 consecutive days in a subchronic toxicity study. Additional groups of five male and five female rats were orally administered 0 or 2000 mg/kg·bw/day for 90 days followed by a 28-day recovery period. The doses for the subchronic study were determined in a 4-week dose range-finding study in which doses of 0, 250, 500, 1000, and 2000 mg/kg·bw/day were administered. The authors reported the absence of toxic effects at any dose and the highest dose tested, 2000 mg/kg·bw/day, was selected as the highest dose for the subchronic study. No significant changes related to silkworm extract powder exposure were reported in body weights, food and water consumption, ophthalmological findings, hematological values, or histopathological findings. Results of the urinalysis indicated a significant increase in ketone bodies in males dosed with 2000 mg of silkworm extract powder/kg·bw/day. The study authors did not elaborate further on this observation. Specific gravity was significantly elevated among males in all doses of the test substance, and pH was significantly increased among females dosed with 500 and 2000 mg/kg·bw/day of the test substance compared to controls. There were significant increases in absolute adrenal and kidney weights in males at 500 and 200 mg/kg·bw/day, and a significant increase in relative liver weights in males at 2000 mg/kg·bw/day. The absolute weight of liver, kidney, lung, brain, and fasting weight were significantly higher in both males and females in the recovery group for the highest dose tested. However, changes in absolute and relative organ weight were not considered treatment related by the study authors. Under the conditions of the study, no significant toxic effects of silkworm extract powder were reported in rats at doses up to 2000 mg/kg·bw/day of silkworm extract powder.

No evidence for toxicity was observed in a study of silkworm powder administered to rats (Rattana et al 2017)

An acute oral toxicity test evaluated the toxicity of silkworm powder. Three varieties of *Bombyx mori* silkworms in Thailand were freeze-dried at the larval stage and ground into a fine powder. The silkworm powder contained fibroin along with other components of the silkworm. The silkworm powder from three varieties of *Bombyx mori* were administered by gavage to three groups of male Albino Wistar rats, 6 rats/group at single doses of 0, 1000, 1500 and 2000 mg/kg body weight for a total of ten groups. Behavioral changes, general toxicity, mortality, body weight, and clinical signs were reported each day for 14 days. No treatment-related mortalities, signs of toxicity or changes in body weights of control or treated rats were reported.

In a sub-acute toxicity test, 60 male albino Wistar rats were divided into 10 groups, 6 rats per group, and treated orally with 2000 mg silkworm powder/kg body weight every two days) from one of the three varieties of *Bombyx mori* for 6 weeks. Body weights and blood glucose levels were measured weekly for six weeks. Hematological values, lipid profiles, and blood chemistry parameters were analyzed at the end of the experiment. The body weights among control and silkworm powder treated rats were not significantly different. Blood glucose levels and blood chemistry parameters of the control and silkworm powder treated rats were not different, suggesting that silkworm powder did not alter organ system function in male rats and did not exhibit any toxicity in rats.

No evidence for toxicity was observed in acute and subchronic oral toxicity studies of silk peptide administered to rats (Han et al. 2011)

Silk peptide E5K6, a peptide derived from silk fiber of *Bombyx mori*, was administered by gavage at doses of 0, 2000, or 5000 mg/kg/bw to female and male Sprague-Dawley rats in an acute single dose oral toxicity study indicated no evidence of toxicity following 14 days of observation. Silk peptide E5K6 is different from Mori Silk. E5K6 is a hydrolyzed silk protein with a much lower average molecular weight (1.5 kDa) than Mori Silk (up to approximately 500 kDa). No mortalities and no clinical signs related to the administration of silk peptide were reported during the study. Normal body weights and no abnormal gross findings were reported.

Silk peptide E5K6 was administered by gavage to female and male Sprague-Dawley rats (5/sex/group) at doses of 0, 500, 1000, or 2000 mg/kg·bw/day doses for 90 days in a subchronic oral toxicity study. No mortalities, abnormal clinical signs, changes in body weight or food consumption, ophthalmological findings, hematology and necropsy findings related to the administration of test article were reported. Water consumption in males treated with 1000 or 2000 mg/kg·bw/day was significantly ($P < 0.05$) lower than consumption in the control group in week 4. This was considered unrelated to the administration of silk peptide because it was not dose-dependent, seen in only one sex and at only one time point. Creatine phosphokinase (CPK) was decreased in males treated with greater than 1000 or 2000 mg/kg·bw/day compared to the vehicle control group ($p<0.01$), however this change in blood biochemistry was not dose-related and within the normal range of the reference data. Clear fluid in the uterus of females was reported in several rats across exposure groups, which is often observed in females due to the estrus cyclicity. There were some differences reported in organ weights: greater absolute weight of the prostate gland in the 500 and 1000 mg/kg·bw/day groups, greater relative weight of the prostate gland in the 500 mg/kg·bw/day group, greater relative weight of the kidney in the 1000 and 2000 mg/kg·bw/day treated groups, and greater relative weight of the liver in males in the 500 and 1000 mg/kg·bw/day groups, all compared to the control group ($p < 0.05$). The relative weight of the heart in females in the 2000 mg/kg·bw/day group was significantly less than that of the control group. No treatment-related histopathological findings were reported in any of the treated animals. The authors reported that "the oral NOAEL of Silk peptide E5K6 is greater than 2000 mg/kg·bw/day" in both male and female rats and target organs were not established.

Acute toxicity tests of powdered moth reported no abnormalities (Gao et al 2018)

As summarized in a review of edible insects by Gao et al. (2018), Duan et al. (2000) conducted a study wherein Chinese Kun Ming mice (sex not reported) were gavaged with 10 g/kg/bw of *Bombyx mori* body fluid, 10 g/kg/bw of pupae body fluid, or 0.68 to 17 g/kg/bw graine *[sic]* powder. No toxicity was reported in the maximum tolerated dose acute toxicity tests, and no evidence of genotoxicity was reported in a bone marrow cell micronucleus test (Duan et al., 2000 via Gao et al., 2018). Gao et al (2018) summarized the results of a seven-day acute toxicity test conducted by Gu (2009). Powdered female *Bombyx mori* moths were administered to Institute of Cancer Research (ICR) mice at 83 g/kg/bw. No deaths or abnormalities in organs were observed. One mouse had malaise and one mouse had flatulence; both symptoms disappeared after a couple hours post-administration. As summarized by Gao et al. (2018), Gu (2009) conducted a seven-day acute toxicity test

wherein Sprague-Dawley rats were given 34.4 g/kg/bw of powdered moth and reported no deaths and no abnormalities in organs at the conclusion of the study (Gu 2009 via Gao et al., 2018). The test substances used in these two studies were derived from *Bombyx mori* and contained fibroin as well as other components of *Bombyx mori* in various life stages. These acute toxicity tests showed that the test substances did not result in abnormalities in the test animals.

6.3.3 Tabular Summary of Safety Studies

Studies on Cambridge Crops' Mori Silk are provided in Table 10. Studies investigating related *Bombyx mori*-derived substances are included to support the safety of consumption of silk fibroin and shown in Table 11.

Table 10: Summary of Toxicological Studies Regarding Mori Silk

Study Type	Species/Sex/ Number per Dose Group	Test Material	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks
Genotoxicity and Mutagenicity						
Bacterial reverse mutation test OECD 471	Salmonella Typhimurium strains TA1535, TA1537, TA98, TA100 and E. coli strain WP2 uvrA	Cambridge Crops' Mori Silk	31.6, 100, 316, 1000, 3160, 10,000, 31,600, and 100,000 ug/plate	Precipitation and toxicity were not present in any of the strains at any of the eight doses tested. There was no concentration-related or substantial test substance related increases in the number of revertant colonies observed in all strains in both the absence and presence of S9 metabolic activation.	Yigit et al. 2021, Appendix G1	Cambridge Crops test substance did not show evidence for mutagenicity with respect to bacterial revertants under the conditions of the study.
Mammalian erythrocyte micronucleus test OECD 474	Mouse/MF/10	Cambridge Crops' Mori Silk	Oral gavage; 1000 mg/kg·bw/day for 2 days	Fibroin did not induce a statistically significant increase in frequency of micronucleated reticulocytes in male or female mice. The frequency of reticulocyte and micronucleated normochromatic erythrocytes did not differ significantly after treatment with the test substance.	Yigit et al. 2021, Appendix G2	Cambridge Crops test substance did not show evidence for genotoxicity with respect to micronucleus induction under the conditions of the study.
Repeated Dose Oral Toxicity Studies						
14-day range-finding study OECD 407	Rat/MF/10	Cambridge Crops' Mori Silk	Oral gavage; 0, 125, 250, 500 mg/kg·bw/day for 14 days	The test substance had no observed effect on mortality, body weight, body weight gain, food consumption, food efficiency, or clinical observations. No pathological abnormalities nor macroscopic findings at necropsy were reported.	Yigit et al. 2021, PSL 2020a, Appendix G3	Cambridge Crops test substance did not show evidence for oral toxicity under the conditions of the study.

Table 10: Summary of Toxicological Studies Regarding Mori Silk

Study Type	Species/Sex/ Number per Dose Group	Test Material	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks
28-day oral toxicity study OECD 407	Rat/MF/10	Cambridge Crops' Mori Silk	Oral gavage; 0, 125, 250, 500 mg/kg·bw/day for 28 days	The test substance had no observed effect on mortality, body weight, body weight gain, food consumption, food efficiency, or clinical observations. No pathological abnormalities nor macroscopic findings at necropsy were reported.	Yigit et al. 2021, PSL 2020b, Appendix G4	Cambridge Crops test substance did not show evidence for oral toxicity under the conditions of the study.

Table 11: Summary of Toxicological Studies Regarding other *Bombyx mori*-derived Substances*

Study Type	Species/Sex/ Number per Dose Group	Test Material	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks
Genotoxicity and Mutagenicity						
Bacterial reverse mutation test OECD 471	Salmonella Typhimurium TA100, TA1535, TA98, TA1537 and E. coli WP2 uvrA	Silkworm extract powder	10, 50, 150, 500, 1500, and 5000 mg/plate	No increases of the means of revertant per plate for all doses of test substance for all strains both in the presence and absence of S9 metabolic activation.	Heo et al. 2013	Silkworm extract powder did not show evidence for mutagenicity with respect to bacterial revertants under the conditions of the study.
Mammalian erythrocyte micronucleus test OECD 474	Mouse/M/6	Silkworm extract powder	Oral gavage; 0, 1250, 2500, 5000 mg/kg·bw for 2 consecutive days	The frequency of micronucleated bone marrow polychromatic erythrocytes was not increased at any of the doses tested. No significant differences in PCE:RBC ratio at any of the doses tested. No mortality or abnormalities were reported.	Heo et al. 2013	Silkworm extract powder did not show evidence for genotoxicity with respect to micronucleated erythrocytes under the conditions of the study.

Table 11: Summary of Toxicological Studies Regarding other *Bombyx mori*-derived Substances*

Study Type	Species/Sex/ Number per Dose Group	Test Material	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks
Chromosomal aberration test OECD 473	Chinese hamster lung cells	Silkworm extract powder	0, 150, 275, 300, 550, 600, 900, 700, and 1100 ug/mL for 6 hours with 18-hour recovery period 0, 150, 300, 600 and 700 ug/mL for 24 hours with no recovery period	The number of aberrant metaphases in all of the treatment series in the presence and absence of the metabolic activation system were not significantly increased.	Heo et al. 2013	Silkworm extract powder did not show evidence for genotoxicity with respect to chromosome aberrations under the conditions of the study.
Single Dose Toxicity Studies						
Acute toxicity test	Rat/MF/10	Silkworm extract powder	Oral gavage; 0, 1250, 2500, 5000 mg/kg	No deaths or treatment-related changes were reported in body weight; no gross findings were recorded at necropsy.	Heo et al. 2013	Silkworm extract powder did not show evidence for toxicity at doses up to 5000 mg/kg.
Acute toxicity study	Rat/MF/10	Silk peptide E5K6	Oral gavage; 0, 2000, 5000 mg/kg·bw	No mortalities and no clinical signs related to the administration of silk peptide were reported during the study. Normal body weights and no abnormal gross findings were reported.	Han et al. 2011	Silk peptide E5K6 did not show evidence for toxicity under the conditions of the study.
Repeated Dose Oral Toxicity Studies						
14-day oral toxicity study	Rat/M/6	Silkworm powder	Oral gavage; 0, 1000, 1500, 2000 mg/kg·bw for 14 days	No treatment-related mortalities, signs of toxicity or changes in body weights of control or treated rats were reported.	Rattana et al. 2017	Silkworm powder did not show evidence for oral toxicity under the conditions of the study.
4-week range-finding study OECD 408	Rat/MF/10	Silkworm extract powder	0, 250, 500, 1000, 2000 mg/kg·bw/day	No toxic effects observed at any dose.	Heo et al. 2013	Silkworm extract powder did not show evidence for oral toxicity under the conditions of the study.

Table 11: Summary of Toxicological Studies Regarding other *Bombyx mori*-derived Substances*

Study Type	Species/Sex/ Number per Dose Group	Test Material	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks
Subacute toxicity test	Rat/M/6	Silkworm powder	2000 mg/kg·bw every two days for 6 weeks	The body weights among control and silkworm powder treated rats were not significantly different. Blood glucose levels and blood chemistry parameters of the control and silkworm powder treated rats were not different.	Rattana et al. 2017	Silkworm extract powder did not show evidence for oral toxicity under the conditions of the study.
90-day oral toxicity study OECD 408	Rat/MF/10	Silkworm extract powder	Oral gavage; 0, 500, 1000, 2000 mg/kg·bw/day for 90 consecutive days	No significant changes related to silkworm extract powder exposure were reported in body weights, food and water consumption, ophthalmological findings, hematological values, or histopathological findings. Minor changes in absolute and relative organ weight were not considered treatment-related by the study authors.	Heo et al. 2013	Silkworm extract powder did not show evidence for oral toxicity under the conditions of the study.
90-day oral toxicity study OECD 408	Rat/MF/10	Silk peptide E5K6	0, 500, 1000, 2000 mg/kg·bw/day for 90 days	No mortalities, abnormal clinical signs, changes in body weight or food consumption, ophthalmological findings, hematology and necropsy findings related to the administration of test article were reported. Water consumption and creatine phosphokinase differed from control but was determined by authors as not dose-related. No treatment-related histopathological findings were reported.	Han et al. 2011	Silk peptide E5K6 did not show evidence for oral toxicity under the conditions of the study. The authors reported that the NOAEL is greater than 2000 mg/kg·bw/day for Silk peptide E5K6 in male and female rats.

Note: *Studies investigating related *Bombyx mori*-derived substances are included to support the safety of consumption of silk fibroin.

6.4 Assessment of Potential Allergenicity

Instances of Silk Allergy

There are no instances in the published literature of a reaction to silk fibroin by the oral ingestion route. No evidence was found to suggest that the silk fibroin components (heavy chain, light chain, P25 or sericin) are allergens or that they bind immunoglobulin E (IgE) from allergic subjects. Pupae of the silkworm have been consumed by humans for years, including in North America (see **Part 5**). There are reports of anaphylaxis following consumption of silkworm and silkworm pupae (Feng et al. 2018, Ji et al. 2008); however, silk fibroin is derived from the cocoon, not the silkworm or pupae.

Processed silk from the cocoons of *Bombyx mori* have been used to produce surgical implant devices and sutures that remain safely in human recipients for years without allergic reactions or other adverse health effects (**Part 6.5**). Certain silk implants have caused short-lived inflammatory responses, such as subcutaneous silk-gels (4-week inflammatory response) and woven silk meshes (7-day inflammatory response) (Thurber et al., 2015). This inflammatory response is not expected to be relevant to Cambridge Crops application of ingested proteins.

Proteins of Allergenic Interest

Cambridge Crops and Dr. Richard Goodman of the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln investigated the source organism (*Bombyx mori*) and source product (*Bombyx mori* cocoons) for potential allergens in order to conclude little to no allergenicity in Mori Silk with confidence. The AllergenOnline.org (AOL) database and the World Health Organization/International Union of Immunological Societies (WHO/IUIS) allergen nomenclature only identified one allergen in *Bombyx mori* silkworms: arginine kinase. In Liu et al. (2009), in vitro serum tests demonstrated that arginine kinase was linked to patients' allergic responses to silkworm extracts. A search of PubMed by Dr. Goodman suggested that there may be additional allergens within the silkworm itself that are not reported in the AOL or WHO/IUIS databases. Based on the literature suggesting possible allergens other than arginine kinase in the silkworm as well as literature describing possible allergens existing in arthropods similar to *Bombyx mori* silkworms, four potential allergens were identified for further investigation regarding allergenicity within the silkworm pupae, cocoons, degummed fibroin (Cambridge Crops' in-process fibroin prior to solubilization by salt solution), and Cambridge Crops' final product, Mori Silk. Together, these four potential allergens are (1) arginine kinase, (2) tropomyosin, (3) chitinase, and (4) paramyosin. The four proteins will be referred to as "Proteins of Allergenic Interest" for the remainder of the GRN. Thioredoxin has been demonstrated to have IgE binding from other insects – but not from *Bombyx mori* silkworms – and was thus used as a positive control in the analyses. Dr. Goodman discusses his findings in detail in **Appendix E**.

Proteomics Evaluation of Bombyx mori components and Mori Silk

To determine whether any of the four Proteins of Allergenic Interest are present in Mori Silk, Cambridge Crops commissioned a mass spectrometry analysis from the Harvard University Center for Mass Spectrometry. Samples of *Bombyx mori* pupae, cocoon, degummed fibroin (Cambridge Crops in-process fibroin prior to solubilization) and Mori Silk powder were analyzed, with the raw data being sent to Dr. Philip Johnson of the Department of Food Science and Technology at the University of Nebraska-Lincoln.

While arginine kinase was determined to be present in the silkworm itself, none of the four proteins of allergenic interest were found at any detectable limits in Cambridge Crops' degummed fibroin *or* Mori Silk. Thioredoxin, the positive control, was also not found in the degummed fibroin or Mori Silk. As such, under the standardized identification criteria set by the Human Proteome Organization (HUPO)-Proteomics Standards Initiative (PSI), none of the "Proteins of Allergenic Interest" were found at any detectable limits in the Mori Silk product. Further details on the analysis and conclusions are provided in Dr. Johnson's report (**Appendix F**).

Comparison between proteins that have been identified as being present in fibroin to proteins of known and putative allergens or toxic proteins

The amino acid (AA) sequences of the four fibroin proteins, three of which (fibroin heavy chain, fibroin light chain and fibroin P25) form the majority of Mori Silk, were compared to known and putative allergen sequences. The AA sequence comparisons did not uncover matches that suggest any reasonable level of possible allergenicity. A summary of the bioinformatics analysis, interpretations and conclusions by Dr. Goodman are presented below and in full in **Appendix E** and are published in Yigit et al. (2021.).

The four primary components in fibroin: fibroin heavy chain, fibroin light chain, P25 and sericin protein amino acid sequences were used to search for identity matches to allergens and for toxins. The sequence of Ber e 1 from *Bertholletia excelsa* (Brazil nut) was used as an allergen control, and the complete ricin protein from *Ricinus communis* was used as a control for the toxin searches.

In order to compare the fibroin amino acid sequences with the sequences of known allergens, the AllergenOnline database (AOL) was used. The primary amino acid sequences of fibroin were queried in AOL using FASTA3, a computer algorithm that provides similar local alignments and results if the appropriate scoring matrices and criteria are used. None of the full-length searches of the fibroin proteins resulted in >50% identity, and thus no concerns of allergenicity or potential cross-reactivity.

The Codex Alimentarius Commission for Food Safety procedures for evaluating new proteins in foods recommend looking for proteins with more than 35% identity to segments of 80 or more amino acids of allergens or putative allergens. A protein is identified as a putative allergen either if it contains at least eight contiguous exact amino acid matches, or if it has at least 35% sequence similarity within an 80 amino acid (AA) window when compared with known allergens. Based on the recommendation of Codex (2003), an 80 AA search in the AOL database was conducted. This short segment search is utilized as it may identify structural motifs shorter than the intact protein that might contain a conformational IgE binding epitope. It would also identify potentially cross-reactive proteins that are not true homologues of an allergen with significant local identities but may be an immunological target for IgE antibodies in those with allergies to the matched allergen.

Per Codex guidelines, matches >35% over 80 AA may be considered significant. The results yielded a few potentially significant matches for fibroin and sericin (details in **Appendix E**). However, given the overall short alignments and level of identity matches, the matches were dissimilar and unlikely to share IgE epitopes for productive binding and stimulation of cross-reactive IgE binding. Dr. Goodman concluded no concerns of potential allergic reactions or cross-reactivity.

Lastly, Dr. Goodman conducted an exact 8 contiguous amino acid search between fibroin components and any known allergens. None of the fibroin components (heavy chain, light chain, P25, sericin) had a match of 8 AA to any allergen in AOL.

Because the AOL database may not have every possible allergen identified, an additional search of the complete protein database of NCBI ("Entrez Protein") using BLASTP searches looking for high identity matches to "allergens" or "allergenic proteins" was used as a double check. Using the BLASTP algorithm (similar to FASTA3), the fibroin components and control proteins were run against the entire Entrez Protein database. Entire matched sequences were reviewed. Dr. Goodman found no sequences within any of the fibroin or sericin sequences that would be of allergenic or toxic concern.

Though there were 16 matches where there was >35% identity over 80 AA, potentially prompting further investigation into allergy using serum IgE tests, there are no clear risks for those allergic to matched allergens. This is due to the overall low identity matches and dissimilarity of proteins. The alignments were from the highly repetitive region of fibroin, with scattered low identities across broad regions demonstrate the coincidental nature of the matches. There are no common findings for allergic cross-reactivity of proteins from ragweed pollen to bovine collagen to soybean seed storage protein, fish parasite (*Anisakis simplex),* fungal protease, corn and wheat proteins including chitinase and glutenins. Results from the sliding 80mer matching program demonstrates that alignments are scattered across segments of the individual allergenic proteins that would require conformational changes in the proteins to have significant matches. Each match was carefully examined and compared to the fibroin protein. Dr. Goodman concluded that based on the protein structures it is extremely unlikely that any antibody would demonstrate cross-reactivity between fibroin and the identified allergen.

These comparisons and analyses were provided to the members of the GRAS Expert Panel. These analyses and findings were subsequently published in Yigit et al. (2021) and discussed at the Food Allergy and Anaphylaxis Meeting during the European Academy of Allergy & Clinical Immunology Annual Meeting (October 16, 2020) and the International Association for Food Protection Annual Meeting (October 28, 2020). At FDA's request, Dr. Goodman provided further and additional analysis on the potential allergenicity of silk fibroin using other databases based in different Support Vector Machine (SVM) algorithms and predictions.

To that end, Dr. Goodman utilized the AllergenFP 1.0[17] and AllerCATPro[18] databases in addition to the AOL database. Dr. Goodman also identified longer predicted peptides following pepsin digestion of the four proteins for pH 1.3 and pH 2 using PeptideCutter[19] and compared these sequences using the above bioinformatics tools. These alternative comparisons, with or without the use of predicted pepsin fragments, do not provide information that shows any additional possible risks of allergenicity from consumption of Mori Silk.

In sum, Dr. Goodman determined that the Mori Silk product does not contain allergens or potentially IgE-cross-reactive proteins within the realm of functional sequence alignments.

The GRAS Expert Panel agreed that there is no allergenic concern over the consumption of Mori Silk (Appendix A).

[17] AllergenFP 1.0 uses auto-cross covariance (ACC) transformation.
[18] AllerCATPro uses five databases in combination and results are similar to those from AllergenOnline.
[19] PeptideCutter is a protein characterization software: https://web.expasy.org/peptide_cutter/peptidecutter_instructions.html

6.5 Safety Information for Non-Food Uses of Silk Protein

Silk protein is used in biomaterials and approved medical devices

Silk proteins are currently used in other non-oral applications including as a biomaterial in sutures, surgical scaffolds, and wound healing.

Silk was first utilized as a suture in 1869. In the 1960s, Ethicon Inc. patented silk proteins for use in silk sutures (Holland et al., 2019). Since 1980, premarket approvals have been issued for silk sutures in medical applications. Nonabsorbable silk surgical sutures are included as a "General and Plastic Surgery Device" in 21 CFR 878.5030 since 1993.

In 2013, FDA approved a 510(k) premarket submission for SERI® (currently Allergan®) for the use of a silk-fibroin based surgical scaffold. It was launched in the US market for use in humans shortly thereafter.

Other countries have also approved the use of silk fibroin for use in humans. For example, the Ministry of Food and Drug Safety in South Korea approved *Bombyx mori* silk fibroin as a silk patch for ear drum perforations (Tympasil®, Daewoong-Bio) (Lee et al, 2015). Further, the China Food and Drug Administration approved the use of a silk film as a wound dressing for clinical use (Sidaiyi®, Suzhou Soho Biomaterial Science and Technology Co., Ltd) (Song et al. 2018).

Dermal exposure due to cosmetic use: 2015 Cosmetic Ingredient Review Panel Safety Assessment

In 2016[20], the Cosmetic Ingredient Review (CIR) panel met and published a "Safety Assessment of Silk Proteins as Used in Cosmetics" that reviewed the use of ten silk proteins as conditioning and bulking agents in cosmetic products: Fibroin, Hydrolyzed Fibroin, Hydrolyzed Sericin, Hydrolyzed Silk, MEA-Hydrolyzed Silk, Sericin, Silk, Silk Extract, Silk Powder, and Silkworm Cocoon Extract. The CIR described composition of silk, fibroin, and sericin, and the available safety information in the literature.

The CIR noted the use of silk proteins in cosmetics such as powders and hairspray, as well as non-cosmetics. Based on the FDA's Voluntary Cosmetic Registration Program (VCRP) and an industry survey, the CIR noted fibroin is not being used in cosmetics. However, hydrolyzed silk and silk powder (each containing fibroin as a component of silk) are used in 675 and 177 formulations, respectively. Silk powder has been used at a max concentration of 1.4% in leave-on products. Hydrolyzed silk and silk extract in hairspray are used at maximum concentrations up to 0.024% and 0.0036%.[21]

The CIR also searched for and reviewed related literature on toxicity, sensitization, and immunological responses. The CIR reported no notable gross lesions or deaths in an acute dermal toxicity study of silk protein film following OECD Test Guideline 402 wherein rats were exposed dermally to one application of the film for 24 hours and observed for a total of 14 days. Two animal studies reported no skin irritation in response to silk protein film exposure to the skin.

[20] The Cosmetic Ingredient Review Expert Panel for Cosmetic Ingredient Safety reviews ingredients regularly; an update was published in 2020 (Johnson et al. 2020).

[21] The CIR noted that 95-99% of droplets/particles have aerodynamic equivalent diameters >10 µm, and therefore would be deposited in the nasopharyngeal and bronchial regions and not be respirable to any appreciable amount.

The CIR did not find literature on toxicokinetics, repeated dose toxicity, reproductive and developmental toxicity, genotoxicity, and carcinogenicity studies for silk or fibroin protein.[22]

6.6 Summary of Safety Information

The available scientific data supports the conclusion that Cambridge Crops Mori Silk is safe and suitable for use as a food ingredient.

Mori Silk exhibited no evidence of genotoxicity or mutagenicity based on results from an in vitro Ames assay and an in vivo micronucleus assay. The safety of Mori Silk was evaluated in a GLP-compliant repeated dose 28-day oral toxicity study consistent with OECD Test Guideline No. 407 and the US FDA Toxicological Principles (RedBook 2000).[23] The doses tested were determined in a 14-day dose range-finding oral toxicity test. The highest dose tested in both studies, 500 mg/kg·bw/day, was based on the solubility of Mori Silk and using a once-per-day-gavage administration. Mori Silk did not elicit any signs of toxicity at the highest dose tested and the NOAEL was reported to be 500 mg/kg·bw/day for both sexes. The highest dose tested was set at 500 mg/kg·bw/day due to limitations of solubility of the test substance and limiting gavage to once per day (Yigit et al. 2021).

Because there were no adverse effects observed at the highest dose (Yigit et al. 2021), the upper limit of tolerable intake is not known. Therefore, margins between exposure in the 28-day repeated dose oral toxicity study in rats (the NOAEL) and EDIs of Mori Silk by humans are compared. These margins provide information on the ratio between the highest intakes identified in the 28-day oral toxicity animal study (which had no observed adverse effects) and the conservative estimates of Mori Silk intakes for various segments of the U.S. population (assuming *every* piece of food consumed within the categories are coated with Mori Silk). In comparing the reported NOAEL of 500 mg/kg·bw/day with the mean EDIs of Mori Silk by age group, the margins of safety range from 110 – 740. The range of margins are adequate to ensure safety of consumption of Mori Silk based on the readily digestible nature of the protein, the lack of any indication of adverse health effects in toxicology studies, and the lack of allergenic potential.

The intakes of Mori Silk are very likely to be significantly less than the EDIs presented herein because this assessment uses a conservative approach that assumes each food item consumed by an individual from each of the five food categories is coated with Mori Silk, and that none of the Mori Silk is washed off at any point during storage or handling. In addition, a conservative approach was used to estimate the amount of Mori Silk deposited on food items (as described in **Part 3.2**). Furthermore, there were no adverse effects observed at the highest dose tested in an oral toxicity study, therefore the upper limit of tolerable intake is unknown.

The safety of the proposed uses of Mori Silk as a food ingredient was determined based on the results of properly designed and executed toxicological studies and other evidence. There are further sources of supportive safety data for the intended uses of Mori Silk. First, Mori Silk consists primarily of fibroin, a well-characterized silk protein which has a history of safe human exposure from approved *in vivo* medical device use and cosmetic use in the United States. Second, silkworm and silkworm-derived food products including fibroin powder have

[22] The CIR reviewed sericin studies that covered toxicity (evidence for cytotoxicity in one study, proliferation in a rat insulinoma cell line), skin depigmentation, lack of inflammatory responses, and fibroblast proliferation

[23] Duration of oral toxicity study was decided during a pre-consultation notification meeting with FDA on September 10, 2019 and subsequent communications.

a history of safe human consumption for thousands of years in countries with diverse populations. Third, silkworm-derived medical products have been approved by regulatory bodies for *in vivo* use around the world, including silk sutures and surgical scaffolds by FDA with no recalls for any safety reasons. Mori Silk is readily digestible, contains no known allergens, shows no evidence of genotoxicity or mutagenicity, and there were no adverse effects reported at the highest dose achievable in a repeated dose 28-day toxicity study. These data support the conclusion that the proposed food ingredient uses of Mori Silk as an edible food coating are safe.

6.7 Conclusion of GRAS Status

On the basis of scientific procedures and the evidence presented herein, Cambridge Crops concluded that the intended uses on fruits, vegetables, cheese, fish, and select processed foods – i.e., hard candy, candy bars (including chocolate bars), nuts, cookies, cereal or meal bars, and chewing gum – of Mori Silk, manufactured consistent with cGMP and meeting the specifications presented herein, are safe and suitable and GRAS.

PART 7. LIST OF SUPPORTING DATA AND INFORMATION

7.1 Acronyms and Abbreviations

AA	amino acid
AOAC	Association of Analytical Chemists
BAM	Bacteriological Analytical Manual
CASRN	Chemical Abstracts Service Registry Number
CFR	Code of Federal Regulations
cfu	colony-forming units
cGMP	current Good Manufacturing Processes
CIR	Cosmetic Ingredient Review
ECHA	European Chemicals Agency
EDI	estimated daily intake
EPA	Environmental Protection Agency
FARRP	Food Allergy Research and Resource Program
FDA	Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FOIA	Freedom of Information Act
g	gram(s)
GLP	Good Laboratory Practice
GRAS	Generally Recognized As Safe
GRN	Generally Recognized As Safe Notice
HUPO	Human Proteome Organization
ICP-OES	inductively coupled plasma optical emission spectrometry
ICR	Institute of Cancer Research
IgE	immunoglobulin E
kDA	kilodalton(s)
LOAEL	lowest-observed-adverse-effect-level
LOD	limit of detection
mg	milligram(s)
microgram	ug
mL	milliliter(s)

NHANES	National Health and Nutrition Examination Survey
NOAEL	no-observed-adverse-effect-level
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
PCE:RBC	polychromatic erythrocyte/red blood cell
ppmw	parts per million by weight
PPRTV	Provisional Peer-Reviewed Toxicity Value
PSI	Proteomics Standards Initiative
PSL	Product Safety Labs
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SVM	support vector machine

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In accordance with 21 CFR §170.255, all references cited in this document are generally available. All references in this document are either published as noted in Part 7.2 or are provided as appendices in Part 7.3.

7.3 Appendices

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APPENDIX A
GRAS EXPERT PANEL CONSENSUS STATEMENT

Expert Panel Consensus Statement on the Generally Recognized as Safe Status of Proposed Uses of Cambridge Crops' Mori Silk

Introduction

Ramboll Environment and Health (Ramboll), on behalf of Cambridge Crops, Inc. (Cambridge Crops) convened a panel of experts (Expert Panel), qualified by their scientific training and experience to evaluate the safety of food ingredients.[1] The Expert Panel included Joseph Borzelleca, Ph.D., John Erdman, Ph.D., Rick Goodman, Ph.D., and Steve Taylor, Ph.D.; and Duncan Turnbull, DPhil, DABT, served as an advisor to the Panel.

The Expert Panel was tasked with determining the safety, suitability and the Generally Recognized as Safe (GRAS) status of Mori Silk, a silk protein intended to be used as an edible coating on food items, manufactured using *Bombyx mori* cocoons.

Independently and collectively, the Expert Panel critically evaluated the available information presented in documents prepared and presented by Ramboll and other materials deemed appropriate and necessary for this review. This information included the description of the substance (including the identity and physical and chemical properties), analyses demonstrating and confirming the purity and manufacturing consistency of the product, the characterization of Mori Silk, and product specifications. A critical overview about the history of use, intended conditions of use and levels of use, its regulatory status, and anticipated exposures or intake, product stability and safety assessment of Cambridge Crops' Mori Silk were provided to and reviewed by the Expert Panel.

Following its independent and collective critical evaluation of the available information, the Expert Panel, convened on 31 March 2020. Following the discussion, the Expert Panel unanimously agreed to the conclusions described herein. A summary of the basis for these conclusions follows.

Description of Mori Silk, the Manufacturing Process, and Product Specifications

The substance in this GRAS determination is silk protein, primarily consisting of silk fibroin, as extracted from *Bombyx mori* cocoons through a relatively simple process with commonly found salts. The Chemical Abstracts Service Registry Number for fibroin is 90070-76-5. This silk protein will be marketed by Cambridge Crops under the trade name Mori Silk.

The silkworm cocoon naturally contains fibroin protein that is intermingled with sericin in the cocoon fibers. Silk fibers are composed of two proteins (fibroin fibers held together by sericin proteins like a glue or coat). Cambridge Crops isolates fibroin through the removal of sericin by boiling. Fibroin is then solubilized in a salt solution, and then the salt is removed by traditional means, leaving Mori Silk in solution. No antibiotics are used in the production process of Mori Silk. Only food or pharmaceutical grade chemicals and processing aids are used in the manufacture of Mori Silk.

Fibroin's amino acid composition consists primarily of glycine (approximately 42-46%), alanine (approximately 29-31%), and serine (approximately 9-12%). The identity of Mori Silk is confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showing a smear of molecular weights at or around 460 kDa and amino acid analysis showing a distinct amino acid profile to that described in literature.

To ensure that a consistent food-grade ingredient is produced, Cambridge Crops has established specifications for their Mori Silk. The chemical, physical and microbiological specifications are presented in **Table 1**. Three batches were analyzed for chemical and microbiological parameters listed in the specifications including metals. The powder product is made by dehydrating the solution.

[1] Curricula Vitae presented in **Appendix A1.**

Table 1. Specifications of Cambridge Crops' Mori Silk		
Parameter	**Specification**	**Method**
Protein	4-7%wt (40-70 mg/g)	AOAC 992.15
Fat	<0.1%wt	AOAC 922.06
Carbohydrates	<0.1%wt	Calculation
Ash	<0.1%wt	AOAC 920.153
Lithium	<75 ppmw	ICP-OES
Arsenic	<5 ppmw	ICP-OES
Lead	<5 ppmw	ICP-OES
Escherichia coli	<10 cfu/g	AOAC 991.14
Listeria monocytogenes	Negative/25g	AOAC 070702
Salmonella	Negative/25g	AOAC 2009.03
Note: Specifications are for Mori Silk solution or Mori Silk powder reconstituted at 5% weight/volume in water (50 mg Mori Silk in 1 mL potable water, mixed or stirred to solution). Abbreviations: AOAC = Association of Analytical Chemists; cfu = colony-forming units; g = gram(s); ICP-OES = inductively coupled plasma optical emission spectrometry; mg = milligram(s); ppmw = parts per million by weight; wt = weight		

Stability tests confirmed that Mori Silk powder is stable after 317 days when stored under standard conditions of 25°C and 65% humidity as demonstrated in a four-week long test under accelerated conditions (60°C and 30% relative humidity).

History of Exposure and Use

Outside of the U.S., fibroin-containing *Bombyx mori* silkworms and silkworm-derived products, including fibroin powder, are eaten frequently and by people of all age groups. Most populations that consume silkworm-derived products reside primarily in East and Southeast Asian countries. Silkworm consumption dates back to 5,000 years ago in China. Silkworms and silkworm-derived food products exist and are sold in the United States. However, consumption is likely well below rates of consumption in other parts of the world.

Silk fibroin is a well-characterized protein approved for uses in various industries including healthcare and cosmetics: Silk fibroin was first utilized as a suture in the 1800s, and nonabsorbable silk surgical sutures designed to be used *in vivo* are included as a "General and Plastic Surgery Device" in 21 CFR §878.5030 since 1993. Silk fibroin was approved by United States Food and Drug Administration (FDA) as a surgical scaffold to be used *in vivo* in a 510(k) premarket submission by SERI Surgical (Allergan) in 2013 (K123128).

Intended Use: Proposed Uses and Estimated Daily Intakes

Cambridge Crops intends to use Mori Silk to extend the shelf life of foods. Specifically, it intends to use Mori Silk with fruits, vegetables, cheese, candy and processed foods, whole meat, ground meat, processed meat, and fish for ingestion by the general population. Those food categories represent approximately 653 grams of food per day at the 95% percentile of intake among adults. The concentration of Mori Silk on foods is dependent on the food item itself. Mori Silk will be applied to foods in three ways, depending on the food product: (1) wetting the food in a container with Mori Silk solution, (2) mixing Mori Silk in with the food, or (3) spraying the food with a Mori Silk solution.

Estimated daily intake of Mori Silk was based on the following conservative assumptions:

- 50% of foods are coated with a concentration of 110 mg Mori Silk per kg food and 50% of foods are coated with the highest applicable concentration of 880 mg Mori Silk per kg food.
- Every piece of food in the eight food categories eaten by individuals surveyed by NHANES is coated with Mori Silk.
- No rinsing or washing of Mori Silk occurred at any stage in the supply chain.

The estimated daily intake of Mori Silk on foods is presented in **Table 2.** Assuming the 95th percentile food consumption rate, an average adult weighing 83.4 kg would consume 364 mg of Mori Silk each day. An adult consuming the median amount of food per day would consume 136 mg.

Table 2. Estimated Daily Intake of Mori Silk as a Food Coating by Age Group

Population	*n*	g **food** consumed / kg bodyweight / day		mg **Mori Silk** / kg bodyweight / day	
		Median	95th Percentile	Median	95th Percentile
Infants 0-11 months	304	5.52	21.84	2.74	10.83
Toddlers 12-35 months	800	10.81	26.21	5.36	13.00
Children 3-11 years	2,943	6.65	18.37	3.30	9.11
Teenagers 12-19 years	2,399	3.22	9.34	1.60	4.63
Adults 18-79 years	9,874	3.29	8.81	1.63	4.37
All	16,085	3.57	11.58	1.77	5.74

Abbreviations: bw = body weight, g = gram(s), kg = kilogram(s), mg = milligram(s)

Intended Effect

Cambridge Crops' Mori Silk is intended to be used as a coating on various foods including fruits, vegetables, meat, cheeses, and candy to preserve food in accordance with allowed mechanisms described in 21 CFR 170.3(o) including as a surface finishing agent, a substance used to increase palatability, preserve gloss, and inhibit discoloration of foods, including glazes, polishes, waxes, and protective coatings (21 CFR 170.3(o)(30)).

Safety Assessment

Silkworms, pupae, and cocoons have been eaten in many countries for centuries, particularly in Asia. Though there is no large-scale consumption noted in the United States, the safety of consuming silkworm and silkworm-derived products is supported not only by scientific procedures, but also by the many consumption uses in other parts of the world. In addition, silk fibroin products have been approved by FDA and other regulating bodies around the world for use in humans.

Analysis of allergenic potential

There are no published instances of allergenic episodes from the consumption of silk fibroin. As a precaution, potential allergens within the silkworms itself were researched. The AllergenOnline.org (AOL) database and the World Health Organization/International Union of Immunological Societies (WHO/IUIS) allergen nomenclature only identified one allergen in *Bombyx mori* silkworms: arginine kinase. A search of PubMed by Dr. Richard Goodman of the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska-Lincoln suggested that there may be additional allergens within the silkworm itself that are not reported in the AOL or WHO/IUIS databases. Between potential allergens identified in literature, and potential allergens existing in similar arthropods to *Bombyx mori* silkworms, it was decided that there may be four potential allergens within the silkworm itself: arginine kinase, tropomyosin, chitinase, and paramyosin.

Mori Silk samples were then run under mass spectrometry. It was determined that using the standardized identification criteria set by the Human Proteome Organization (HUPO)-Proteomics Standards Initiative (PSI), none of the four proteins of allergenic interest were found at any detectable limits in Cambridge Crops degummed fibroin or Mori Silk.

Absorption, distribution, metabolism and excretion of silk fibroin

In general, digestion of dietary proteins like silk fibroin begins in the stomach and continues into the lumen of the duodenum. Within the digestive tract of humans, proteins are broken down via hydrolysis primarily into free amino acids. Approximately 10% of the proteins in the gastrointestinal tract do not undergo hydrolysis and are excreted in the feces. Cambridge Crops demonstrated in an *in vitro* digestibility study in human simulated gastric fluid that Mori Silk was rapidly digested in pepsin at pH 2.0 at a ratio of 10 units pepsin per ug Mori Silk within two minutes.

Toxicological Studies

The toxicological studies performed with Cambridge Crops Mori Silk demonstrate the safety of the substance. A repeated dose 28-day oral toxicity study with Cambridge Crops Mori Silk demonstrate that it is safe for consumption by rats at levels up to 500 mg/kg·bw/day. The authors concluded a No-Observed-Adversed-Effect-Level (NOAEL) of 500 mg/kg·bw/day, the highest dose tested.

Cambridge Crops Mori Silk is neither mutagenic nor genotoxic as demonstrated in an *in vitro* Ames assay and an *in vivo* micronucleus assay. Not only that, but other *Bombyx mori*-derived test substances have been shown to be neither mutagenic nor genotoxic as demonstrated in several publications. Further, acute and subchronic repeated dose oral toxicity tests demonstrated that various *Bombyx mori*-derived test substances are not toxic. *Bombyx mori*-derived silk and silk fibroin also have been safely used as biomaterial in sutures and surgical meshes, and wound dressing, and as an additive in cosmetics. Silkworms and silkworm products containing fibroin have a long history of consumption in East and Southeast Asia for thousands of years. Combined with the evidence that Mori Silk is readily digestible, contains no known allergens, and that there were no adverse effects observed at the highest dose achievable in a 28-day toxicity study, the available data support the conclusion that the proposed food ingredient uses of Mori Silk as an edible food coating is safe.

Conclusions

We, the members of the Expert Panel, have independently and collectively, critically evaluated the available information on the silk protein (Mori Silk) manufactured by Cambridge Crops, Inc. using *Bombyx mori* cocoons (as presented in this dossier prepared by Ramboll on behalf of Cambridge Crops and summarized herein).

We unanimously conclude that the proposed uses of Cambridge Crops' Mori Silk as a coating on foods, manufactured consistent with current Good Manufacturing Practice and meeting the food grade specifications presented herein, are safe and suitable.

We further unanimously conclude that the proposed uses of Cambridge Crops' Mori Silk, manufactured consistent with current Good Manufacturing Practice and meeting the food grade specifications presented herein, are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other experts, qualified by scientific training and experience, and evaluating the same data and information, would concur with these conclusions.

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APPENDIX A1
GRAS EXPERT PANEL
CURRICULA VITAE

83 Pages of Curriculum Vitae removed in accordance with the Privacy Act of 1974.

APPENDIX B
AMINO ACID PROFILE OF FIBROIN AND MORI SILK

Table B1. Amino Acid Composition: Cambridge Crops Mori Silk Compared With Fibroin and Sericin from Literature

	Fibroin										Mori Silk[d]	Sericin	
Amino Acid[b] (mol %)	Asakura et al. 2002	Qi et al. 2017	Asakura et al. 2015[c]	Sashina et al. 2006 (total)[g]	Sashina et al. 2006 (heavy chain)[g]	Sashina et al. 2006 (light chain)[g]	Zhou et al. 2001[c,e]	Kaplan and McGraith 2012	Mondal et al. 2007[f]	Wray et al. 2011[a]	Cambridge Crops	Wray et al. 2011[a]	Kaplan and McGraith 2012
Glycine	42.9	43	46	42.9	49.4	10	45.9	42.9	44.6	45.7	43.5-46.1	19.0	13.5
Alanine	30.0	30	30	30.0	29.8	16.9	30.3	30.0	29.4	30.6	30.2-31.7	6.4	5.8
Serine	12.2	12	12	12.2	11.3	7.9	12.1	12.2	12.1	8.8	7.3-10.3	24.2	34.0
Tyrosine	4.8	5.3	5.3	4.8	4.6	3.4	5.3	4.8	5.17	NR	4.9-5.5	NR	3.6
Valine	2.5	NR	NR	2.5	2	7.4	1.8	2.5	2.20	NR	2.1-2.4	NR	2.9
Aspartic Acid/Asparagine	NR	NR	NR	1.9	0.65	15.4	NR	1.9	1.30	NR	1.2-1.9	NR	14.6
Glutamic Acid/Glutamine	NR	NR	NR	1.4	0.7	8.4	NR	1.4	1.02	NR	0.9-1.5	NR	6.2

[a]Wray et al. (2011) did not report the full amino acid composition, but rather focused on the amino acids that had the greatest changes in percent composition between fibroin and sericin.

[b]Any amino acids that comprised less than 1% in fibroin are not listed here

[c]Zhou et al. (2001) and Asakura et al. (2015) specifically reported the amino acid composition of fibroin's heavy chain.

[d]Range for all samples tested. Triplicates of degummed fibroin, Mori Silk solution, and Mori Silk powder were analyzed

[e]Qi et al. (2017), Zhou et al. (2001) and Kaplan and McGraith (2012) did not specify the basis for the percentages reported.

[f]Mondal et al. (2007) reported percentages based on residues via Robson 1985.

[g]Sashina et al. (2006) reported total as well as for the heavy chain and light chain separately.

Abbreviations: NR = not reported

Table B2. Average amino acid composition of Cambridge Crops products compared with fibroin solution

	Degummed fibroin		Mori Silk Powder		Mori Silk Solution		Fibroin Solution*
Amino acid proportion (mole %)	150-minute boil	30-minute boil	150-minute boil	30-minute boil	150-minute boil	30-minute boil	
Glycine	44.72	43.69	45.60	45.29	45.94	44.97	44.47
Alanine	30.89	30.28	31.45	31.31	31.56	31.14	30.46
Serine	9.98	4.24	9.52	7.43	9.50	8.65	10.15
Tyrosine	5.05	5.30	5.36	5.30	5.27	5.27	5.28
Valine	2.18	2.25	2.15	2.38	2.10	2.26	2.18
Aspartic Acid/Asparagine	1.53	1.78	1.26	1.90	1.18	1.80	1.65
Glutamic acid	1.14	1.29	0.99	1.43	0.91	1.35	1.23

Notes: Degummed fibroin is in-process fibroin, prior to solubilization step; boil times denote time during degumming step.

*One sample of fibroin obtained from Advanced BioMatrix, California (value reported in table is not an average)

Cambridge Crops powder

CC-30-powder-1
Mass Hydrolyzed=2.3mg
Final Vol=20mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	1.02	0.12	1.87	2.84	0.18	2.05
Thr	0.37	0.04	0.68	0.91	0.06	0.66
Ser	4.00	0.35	7.32	8.40	0.69	6.05
Glx	0.74	0.10	1.36	2.31	0.13	1.66
Pro	0.34	0.03	0.62	0.80	0.06	0.57
Gly	24.80	1.42	45.43	34.18	4.31	24.63
Ala	17.08	1.21	31.29	29.32	2.97	21.12
Val	1.30	0.13	2.38	3.10	0.23	2.23
Ile	0.38	0.04	0.70	1.04	0.07	0.75
Leu	0.31	0.03	0.57	0.84	0.05	0.61
Tyr	2.87	0.47	5.25	11.30	0.50	8.14
Phe	0.54	0.08	0.99	1.92	0.09	1.38
His	0.06	0.01	0.11	0.19	0.01	0.14
Lys	0.15	0.02	0.27	0.46	0.03	0.33
Arg	0.36	0.06	0.67	1.37	0.06	0.99
Cysteic acid	0.06	0.01	0.10	0.14	0.01	0.10
Met sulfone	0.07	0.01	0.12	0.22	0.01	0.16
Trp	0.15	0.03	0.27	0.65	0.03	0.47
Total	**54.59**	**4.14**				
Total mg		**1.66**				
% Protein		**72.00**				**72.04**

CC-30-powder-2
Mass Hydrolyzed=2.7mg
Final Vol=20mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	1.37	0.16	1.94	2.94	0.20	2.34
Thr	0.51	0.05	0.72	0.96	0.08	0.77
Ser	5.31	0.46	7.49	8.60	0.79	6.85
Glx	1.06	0.14	1.50	2.55	0.16	2.03
Pro	0.34	0.03	0.47	0.61	0.05	0.48
Gly	32.04	1.83	45.21	34.03	4.75	27.10
Ala	22.23	1.58	31.36	29.40	3.29	23.41
Val	1.69	0.17	2.38	3.11	0.25	2.48
Ile	0.54	0.06	0.77	1.14	0.08	0.91
Leu	0.44	0.05	0.62	0.93	0.07	0.74
Tyr	3.76	0.61	5.30	11.40	0.56	9.08
Phe	0.65	0.10	0.92	1.78	0.10	1.42
His	0.08	0.01	0.11	0.20	0.01	0.16
Lys	0.20	0.03	0.28	0.47	0.03	0.37
Arg	0.41	0.06	0.57	1.18	0.06	0.94
Cysteic acid	0.07	0.01	0.10	0.13	0.01	0.10
Met sulfone	0.08	0.01	0.11	0.19	0.01	0.15
Trp	0.11	0.02	0.15	0.37	0.02	0.29
Total	**70.87**	**5.38**				
Total mg		**2.15**				
% Protein		**79.60**				**79.64**

CC-30-powder-3
Mass Hydrolyzed=3.3mg
Final Vol=30mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	1.03	0.12	1.90	2.87	0.19	2.15
Thr	0.38	0.04	0.70	0.93	0.07	0.69
Ser	4.04	0.35	7.48	8.57	0.73	6.40
Glx	0.77	0.10	1.43	2.42	0.14	1.81
Pro	0.21	0.02	0.38	0.49	0.04	0.36
Gly	24.43	1.39	45.23	33.96	4.44	25.36
Ala	16.89	1.20	31.28	29.24	3.07	21.83
Val	1.28	0.13	2.37	3.09	0.23	2.31
Ile	0.44	0.05	0.81	1.20	0.08	0.90
Leu	0.34	0.04	0.64	0.95	0.06	0.71
Tyr	2.89	0.47	5.35	11.48	0.53	8.57
Phe	0.53	0.08	0.99	1.91	0.10	1.43
His	0.06	0.01	0.11	0.19	0.01	0.14
Lys	0.15	0.02	0.27	0.46	0.03	0.35
Arg	0.30	0.05	0.55	1.13	0.05	0.84
Cysteic acid	0.05	0.01	0.10	0.13	0.01	0.10
Met sulfone	0.07	0.01	0.12	0.21	0.01	0.16
Trp	0.17	0.03	0.31	0.75	0.03	0.56
Total	**54.01**	**4.11**				
Total mg		**2.46**				
% Protein		**74.70**				**74.67**

CC-150-powder-1
Mass Hydrolyzed=2.9mg
Final Vol=30mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	0.69	0.08	1.25	1.92	0.14	1.68
Thr	0.37	0.04	0.67	0.91	0.08	0.78
Ser	5.25	0.46	9.50	11.05	1.09	9.46
Glx	0.54	0.07	0.97	1.68	0.11	1.44
Pro	0.14	0.01	0.25	0.32	0.03	0.28
Gly	25.23	1.44	45.68	34.82	5.22	29.81
Ala	17.37	1.24	31.45	29.85	3.59	25.55
Val	1.18	0.12	2.14	2.83	0.24	2.42
Ile	0.31	0.04	0.56	0.85	0.06	0.73
Leu	0.23	0.03	0.41	0.63	0.05	0.54
Tyr	2.91	0.48	5.27	11.48	0.60	9.83
Phe	0.42	0.06	0.77	1.50	0.09	1.29
His	0.09	0.01	0.16	0.29	0.02	0.25
Lys	0.10	0.01	0.18	0.30	0.02	0.26
Arg	0.20	0.03	0.36	0.75	0.04	0.64
Cysteic acid	0.03	0.00	0.06	0.09	0.01	0.07
Met sulfone	0.05	0.01	0.08	0.14	0.01	0.12
Trp	0.13	0.02	0.24	0.59	0.03	0.50
Total	**55.23**	**4.14**				
Total mg		**2.48**				
% Protein		**85.60**				**85.60**

CC-150-powder-2
Mass Hydrolyzed=3.4mg
Final Vol=30mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	0.97	0.11	1.26	1.94	0.17	1.98
Thr	0.52	0.05	0.68	0.92	0.09	0.94
Ser	7.34	0.64	9.53	11.07	1.30	11.28
Glx	0.76	0.10	0.98	1.69	0.13	1.73
Pro	0.25	0.02	0.32	0.41	0.04	0.42
Gly	35.19	2.01	45.67	34.90	6.24	35.46
Ala	24.23	1.72	31.44	29.83	4.28	30.40
Val	1.66	0.16	2.15	2.85	0.29	2.90
Ile	0.37	0.04	0.48	0.73	0.07	0.74
Leu	0.28	0.03	0.37	0.55	0.05	0.56
Tyr	4.14	0.68	5.37	11.70	0.73	11.93
Phe	0.54	0.08	0.71	1.39	0.10	1.41
His	0.13	0.02	0.17	0.31	0.02	0.31
Lys	0.14	0.02	0.18	0.30	0.02	0.31
Arg	0.28	0.04	0.36	0.76	0.05	0.77
Cysteic acid	0.00	0.00	0.00	0.00	0.00	0.00
Met sulfone	0.07	0.01	0.09	0.16	0.01	0.17
Trp	0.18	0.03	0.23	0.58	0.03	0.59
Total	**77.06**	**5.77**				
Total mg		**3.46**				
% Protein		**101.90**				**101.90**

CC-150-powder-3
Mass Hydrolyzed=2.7mg
Final Vol=20mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	0.95	0.11	1.28	1.96	0.14	1.63
Thr	0.51	0.05	0.69	0.93	0.08	0.77
Ser	7.11	0.62	9.52	11.05	1.05	9.17
Glx	0.76	0.10	1.01	1.74	0.11	1.45
Pro	0.31	0.03	0.42	0.54	0.05	0.45
Gly	33.92	1.94	45.46	34.57	5.03	28.70
Ala	23.48	1.67	31.46	29.78	3.48	24.72
Val	1.61	0.16	2.16	2.85	0.24	2.37
Ile	0.36	0.04	0.49	0.72	0.05	0.61
Leu	0.28	0.03	0.37	0.56	0.04	0.46
Tyr	4.07	0.66	5.45	11.84	0.60	9.83
Phe	0.53	0.08	0.70	1.38	0.08	1.15
His	0.11	0.02	0.15	0.28	0.02	0.23
Lys	0.13	0.02	0.17	0.30	0.02	0.25
Arg	0.27	0.04	0.37	0.77	0.04	0.64
Cysteic acid	0.00	0.00	0.00	0.00	0.00	0.00
Met sulfone	0.05	0.01	0.07	0.12	0.01	0.10
Trp	0.18	0.03	0.24	0.58	0.03	0.48
Total	**74.63**	**5.60**				
Total mg		**2.24**				
% Protein		**83.00**				**83.00**

CC-30-sol-1
Initial Vol (µL)=50
Final Vol (µL)=30000

Amino acid	nm/50µL	ugr/50µL	mole %	weight %
Asx	**1,085**	**0.125**	**1.79**	**2.72**
Thr	0.455	0.046	0.75	1.00
Ser	5.242	0.457	8.64	9.93
Glx	0.819	0.106	1.35	2.30
Pro	0.117	0.011	0.19	0.25
Gly	27.288	1.558	44.99	33.88
Ala	18.896	1.344	31.15	29.21
Val	1.374	0.136	2.26	2.96
Ile	0.431	0.049	0.71	1.06
Leu	0.342	0.039	0.56	0.84
Tyr	3.195	0.521	5.27	11.34
Phe	0.608	0.089	1.00	1.95
His	0.071	0.010	0.12	0.21
Lys	0.189	0.024	0.31	0.53
Arg	0.326	0.051	0.54	1.11
Cysteic acid	0.038	0.004	0.06	0.09
MetSO2	0.059	0.008	0.10	0.17
Trp	0.119	0.022	0.20	0.48
Total	**60.66**	**4.60**		
Total µg		**2759.8**		
Conc. (µg/µL)		**55.2**		

CC-30-sol-2
Initial Vol (µL)=50
Final Vol (µL)=30000

Amino acid	nm/50µL	ugr/50µL	mole %	weight %
Asx	**1.126**	**0.13**	**1.76**	**2.67**
Thr	0.482	0.049	0.75	1.00
Ser	5.593	0.487	8.72	10.02
Glx	0.835	0.108	1.30	2.22
Pro	0.157	0.015	0.24	0.31
Gly	28.859	1.648	45.01	33.91
Ala	19.958	1.419	31.13	29.20
Val	1.447	0.143	2.26	2.95
Ile	0.451	0.051	0.70	1.05
Leu	0.353	0.040	0.55	0.82
Tyr	3.439	0.561	5.36	11.55
Phe	0.587	0.086	0.92	1.78
His	0.074	0.010	0.12	0.21
Lys	0.198	0.025	0.31	0.52
Arg	0.328	0.051	0.51	1.05
Cysteic acid	0.038	0.004	0.06	0.08
MetSO2	0.060	0.008	0.09	0.16
Trp	0.129	0.024	0.20	0.50
Total	**64.11**	**4.86**		
Total µg		**2916.0**		
Conc. (µg/µL)		**58.3**		

CC-30-sol-3
Initial Vol (µL)=50
Final Vol (µL)=30000

Amino acid	nm/50µL	ugr/50µL	mole %	weight %
Asx	**1.13**	**0.128**	**1.84**	**2.80**
Thr	0.465	0.047	0.77	1.03
Ser	5.18	0.451	8.60	9.87
Glx	0.851	0.11	1.41	2.40
Pro	0.188	0.018	0.31	0.40
Gly	27.064	1.545	44.91	33.81
Ala	18.759	1.334	31.13	29.18
Val	1.357	0.134	2.25	2.94
Ile	0.432	0.049	0.72	1.07
Leu	0.339	0.038	0.56	0.84
Tyr	3.119	0.509	5.18	11.14
Phe	0.599	0.088	0.99	1.93
His	0.071	0.010	0.12	0.21
Lys	0.189	0.024	0.31	0.53
Arg	0.323	0.050	0.54	1.10
Cysteic acid	0.038	0.004	0.06	0.09
MetSO2	0.058	0.008	0.10	0.17
Trp	0.121	0.023	0.20	0.49
Total	**60.26**	**4.57**		
Total µg		**2742.3**		
Conc. (µg/µL)		**54.8**		

CC-150-sol-1
Initial Vol (µL)=50
Final Vol (µL)=30000

Amino acid	nm/50µL	ugr/50µL	mole %	weight %
Asx	0.646	0.074	1.18	1.82
Thr	0.36	0.036	0.66	0.89
Ser	5.093	0.444	9.30	10.86
Glx	0.507	0.065	0.93	1.60
Pro	0.076	0.007	0.14	0.18
Gly	25.238	1.441	46.08	35.27
Ala	17.336	1.233	31.65	30.17
Val	1.156	0.115	2.11	2.80
Ile	0.301	0.034	0.55	0.83
Leu	0.215	0.024	0.39	0.60
Tyr	2.829	0.462	5.17	11.30
Phe	0.458	0.067	0.84	1.65
His	0.083	0.011	0.15	0.26
Lys	0.123	0.016	0.23	0.39
Arg	0.191	0.030	0.35	0.73
Cysteic acid	0.012	0.001	0.02	0.03
MetSO2	0.041	0.005	0.08	0.13
Trp	0.102	0.019	0.19	0.46
Total	**54.77**	**4.09**		
Total µg		**2451.4**		
Conc. (µg/µL)		**49**		

CC-150-sol-2
Initial Vol (µL)=50
Final Vol (µL)=30000

Amino acid	nm/50µL	ugr/50µL	mole %	weight %
Asx	0.705	0.081	1.16	1.79
Thr	0.397	0.04	0.66	0.89
Ser	5.769	0.502	9.52	11.11
Glx	0.536	0.069	0.88	1.53
Pro	0.132	0.013	0.22	0.28
Gly	27.806	1.588	45.90	35.10
Ala	19.143	1.361	31.60	30.09
Val	1.266	0.125	2.09	2.77
Ile	0.325	0.037	0.54	0.81
Leu	0.242	0.027	0.40	0.61
Tyr	3.214	0.525	5.31	11.60
Phe	0.438	0.065	0.72	1.43
His	0.088	0.012	0.15	0.27
Lys	0.135	0.017	0.22	0.38
Arg	0.218	0.034	0.36	0.75
Cysteic acid	0.015	0.002	0.02	0.03
MetSO2	0.046	0.006	0.08	0.13
Trp	0.101	0.019	0.17	0.41
Total	**60.57**	**4.52**		
Total µg		**2713.7**		
Conc. (µg/µL)		**54.3**		

CC-150-sol-3
Initial Vol (µL)=50
Final Vol (µL)=30000

Amino acid	nm/50µL	ugr/50µL	mole %	weight %
Asx	0.76	0.087	1.21	1.87
Thr	0.43	0.043	0.69	0.93
Ser	6.067	0.528	9.68	11.28
Glx	0.57	0.074	0.91	1.57
Pro	0.096	0.009	0.15	0.20
Gly	28.727	1.640	45.84	35.02
Ala	19.702	1.401	31.44	29.91
Val	1.325	0.131	2.11	2.80
Ile	0.332	0.038	0.53	0.80
Leu	0.243	0.027	0.39	0.59
Tyr	3.340	0.545	5.33	11.64
Phe	0.458	0.067	0.73	1.44
His	0.088	0.012	0.14	0.26
Lys	0.138	0.018	0.22	0.38
Arg	0.224	0.035	0.36	0.75
Cysteic acid	0.015	0.002	0.02	0.03
MetSO2	0.046	0.006	0.07	0.13
Trp	0.104	0.019	0.17	0.41
Total	**62.66**	**4.68**		
Total µg		**2810.4**		
Conc. (µg/µL)		**56.2**		

CC-30-df-1
Mass Hydrolyzed=2.4mg
Final Vol=20mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	1.28	0.15	1.90	2.86	0.21	2.45
Thr	0.58	0.06	0.87	1.14	0.10	0.98
Ser	6.82	0.59	10.17	11.56	1.14	9.90
Glx	0.92	0.12	1.38	2.32	0.15	1.99
Pro	0.35	0.03	0.52	0.66	0.06	0.56
Gly	29.16	1.67	43.48	32.38	4.86	27.75
Ala	20.25	1.44	30.20	28.01	3.38	24.00
Val	1.54	0.15	2.29	2.96	0.26	2.54
Ile	0.53	0.06	0.79	1.17	0.09	1.00
Leu	0.45	0.05	0.66	0.98	0.07	0.84
Tyr	3.48	0.57	5.20	11.05	0.58	9.48
Phe	0.55	0.08	0.83	1.59	0.09	1.36
His	0.26	0.04	0.39	0.69	0.04	0.59
Lys	0.18	0.02	0.27	0.45	0.03	0.39
Arg	0.40	0.06	0.59	1.21	0.07	1.04
Cysteic acid	0.06	0.01	0.08	0.11	0.01	0.10
Met sulfone	0.07	0.01	0.10	0.18	0.01	0.15
Trp	0.19	0.03	0.28	0.67	0.03	0.58
Total	**67.07**	**5.14**				
Total mg		**2.06**				
% Protein		**85.70**				**85.69**

CC-150-df-1
Mass Hydrolyzed=5.4mg
Final Vol=50mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	0.96	0.11	1.64	2.47	0.18	2.04
Thr	0.47	0.05	0.79	1.05	0.09	0.87
Ser	5.94	0.52	10.14	11.58	1.10	9.57
Glx	0.70	0.09	1.19	2.02	0.13	1.67
Pro	0.19	0.02	0.33	0.41	0.04	0.34
Gly	25.89	1.48	44.21	33.11	4.79	27.38
Ala	17.86	1.27	30.50	28.44	3.31	23.52
Val	1.31	0.13	2.23	2.90	0.24	2.40
Ile	0.40	0.04	0.68	1.01	0.07	0.83
Leu	0.32	0.04	0.55	0.81	0.06	0.67
Tyr	3.05	0.50	5.21	11.14	0.56	9.21
Phe	0.50	0.07	0.85	1.65	0.09	1.36
His	0.14	0.02	0.25	0.44	0.03	0.37
Lys	0.16	0.02	0.27	0.45	0.03	0.37
Arg	0.31	0.05	0.53	1.08	0.06	0.89
Cysteic acid	0.06	0.01	0.10	0.13	0.01	0.11
Met sulfone	0.05	0.01	0.09	0.16	0.01	0.13
Trp	0.27	0.05	0.47	1.15	0.05	0.95
Total	**58.57**	**4.47**				
Total mg		**4.47**				
% Protein		**82.70**				**82.69**

CC-30-df-2
Mass Hydrolyzed=2.4mg
Final Vol=20mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	1.25	0.14	1.81	2.72	0.21	2.40
Thr	0.59	0.06	0.86	1.14	0.10	1.00
Ser	7.10	0.62	10.29	11.71	1.18	10.31
Glx	0.88	0.11	1.27	2.15	0.15	1.89
Pro	0.29	0.03	0.42	0.53	0.05	0.47
Gly	30.26	1.73	43.83	32.71	5.04	28.80
Ala	20.86	1.48	30.22	28.08	3.48	24.72
Val	1.56	0.15	2.26	2.92	0.26	2.57
Ile	0.47	0.05	0.68	1.00	0.08	0.88
Leu	0.39	0.04	0.57	0.84	0.07	0.74
Tyr	3.71	0.60	5.37	11.45	0.62	10.08
Phe	0.60	0.09	0.86	1.66	0.10	1.46
His	0.17	0.02	0.24	0.43	0.03	0.38
Lys	0.18	0.02	0.26	0.43	0.03	0.38
Arg	0.36	0.06	0.52	1.07	0.06	0.94
Cysteic acid	0.06	0.01	0.09	0.12	0.01	0.11
Met sulfone	0.09	0.01	0.13	0.21	0.01	0.19
Trp	0.22	0.04	0.34	0.82	0.04	0.72
Total	**69.04**	**5.28**				
Total mg		**2.11**				
% Protein		**88.00**				**88.04**

CC-150-df-2
Mass Hydrolyzed=3.5mg
Final Vol=40mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	0.77	0.09	1.51	2.31	0.18	2.03
Thr	0.39	0.04	0.77	1.03	0.09	0.91
Ser	5.06	0.44	9.89	11.44	1.16	10.08
Glx	0.57	0.07	1.12	1.92	0.13	1.69
Pro	0.25	0.02	0.49	0.64	0.06	0.56
Gly	23.02	1.31	44.96	34.11	5.26	30.04
Ala	15.91	1.13	31.07	29.35	3.64	25.85
Val	1.09	0.11	2.14	2.81	0.25	2.48
Ile	0.29	0.03	0.56	0.84	0.07	0.74
Leu	0.24	0.03	0.46	0.69	0.05	0.61
Tyr	2.53	0.41	4.93	10.70	0.58	9.42
Phe	0.41	0.06	0.80	1.56	0.09	1.37
His	0.12	0.02	0.23	0.41	0.03	0.36
Lys	0.11	0.01	0.22	0.38	0.03	0.34
Arg	0.23	0.04	0.45	0.94	0.05	0.83
Cysteic acid	0.03	0.00	0.06	0.08	0.01	0.07
Met sulfone	0.05	0.01	0.09	0.16	0.01	0.14
Trp	0.13	0.02	0.25	0.62	0.03	0.55
Total	**51.20**	**3.85**				
Total mg		**3.08**				
% Protein		**88.10**				**88.07**

CC-30-df-3
Mass Hydrolyzed=5.9mg
Final Vol=50mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	1.05	0.12	1.63	2.46	0.18	2.06
Thr	0.54	0.05	0.83	1.10	0.09	0.92
Ser	6.62	0.58	10.25	11.71	1.12	9.77
Glx	0.79	0.10	1.22	2.07	0.13	1.73
Pro	0.25	0.02	0.39	0.50	0.04	0.42
Gly	28.47	1.63	44.12	33.03	4.83	27.56
Ala	19.62	1.40	30.41	28.34	3.33	23.64
Val	1.41	0.14	2.19	2.85	0.24	2.37
Ile	0.41	0.05	0.64	0.95	0.07	0.79
Leu	0.33	0.04	0.51	0.75	0.06	0.63
Tyr	3.44	0.56	5.33	11.40	0.58	9.51
Phe	0.60	0.09	0.93	1.79	0.10	1.49
His	0.17	0.02	0.26	0.46	0.03	0.39
Lys	0.15	0.02	0.23	0.39	0.03	0.33
Arg	0.33	0.05	0.51	1.04	0.06	0.87
Cysteic acid	0.05	0.01	0.08	0.11	0.01	0.09
Met sulfone	0.07	0.01	0.11	0.19	0.01	0.16
Trp	0.23	0.04	0.35	0.86	0.04	0.72
Total	**64.53**	**4.92**				
Total mg		**4.92**				
% Protein		**83.40**				**83.43**

CC-150-df-3
Mass Hydrolyzed=2.6mg
Final Vol=20mL

Amino acid	nm/50µL	ugr/50µL	mole %	weight %	µm/mg	%(w/w)
Asx	1.09	0.13	1.45	2.21	0.17	1.93
Thr	0.57	0.06	0.75	1.01	0.09	0.88
Ser	7.49	0.65	9.92	11.48	1.15	10.04
Glx	0.83	0.11	1.11	1.90	0.13	1.66
Pro	0.29	0.03	0.38	0.49	0.04	0.43
Gly	33.99	1.94	44.99	34.12	5.23	29.85
Ala	23.50	1.67	31.11	29.38	3.62	25.71
Val	1.63	0.16	2.16	2.85	0.25	2.49
Ile	0.42	0.05	0.56	0.84	0.07	0.74
Leu	0.34	0.04	0.46	0.69	0.05	0.60
Tyr	3.78	0.62	5.01	10.86	0.58	9.36
Phe	0.57	0.08	0.75	1.46	0.09	1.28
His	0.19	0.03	0.25	0.46	0.03	0.40
Lys	0.17	0.02	0.22	0.38	0.03	0.33
Arg	0.34	0.05	0.45	0.92	0.05	0.81
Cysteic acid	0.04	0.00	0.06	0.08	0.01	0.07
Met sulfone	0.07	0.01	0.09	0.16	0.01	0.14
Trp	0.22	0.04	0.29	0.71	0.03	0.62
Total	**75.54**	**5.69**				
Total mg		**2.27**				
% Protein		**87.50**				**87.49**

Standard 1(AB)
Initial Vol (µL)=50
Final Vol (µL)=30000

Amino acid	nm/50µL	ugr/50µL	mole %	weight %
Asx	0.775	0.089	1.65	2.50
Thr	0.389	0.039	0.83	1.10
Ser	4.768	0.415	10.15	11.64
Glx	0.576	0.074	1.23	2.08
Pro	0.115	0.011	0.25	0.31
Gly	20.884	1.192	44.47	33.42
Ala	14.307	1.017	30.46	28.51
Val	1.022	0.101	2.18	2.84
Ile	0.342	0.039	0.73	1.09
Leu	0.257	0.029	0.55	0.81
Tyr	2.480	0.405	5.28	11.35
Phe	0.435	0.064	0.93	1.80
His	0.092	0.013	0.20	0.35
Lys	0.122	0.016	0.26	0.44
Arg	0.235	0.037	0.50	1.03
Cysteic acid	0.029	0.003	0.06	0.08
MetSO2	0.043	0.006	0.09	0.16
Trp	0.092	0.017	0.20	0.48
Total	**46.96**	**3.57**		
Total µg		**2140.7**		
Conc. (µg/µL)		**42.8**		

Creative Proteomics

45-1 Ramsey Road, Shirley, NY 11967, USA
Tel: 1-631-275-3058 Fax: 1-631-614-7828
www.creative-proteomics.com
info@creative-proteomics.com

Analysis Report

Project Name	Amino Acids Composition Analysis
Sample Description	Silk fibroin
Sample Quantity	20
Order Number	CPMC02132001
Client	
Project Date	*2020-3*
Remark	

45-1 Ramsey Road, Shirley, NY 11967, USA
Tel: 1-631-275-3058 Fax: 1-631-614-7828

Part 1 Sample Preparation

1.1 Samples and Groups

20 silk fibroin samples, for quantitative measurement of amino acids in the collected sample.
Name: Shown in the excel sheet.

1.2 Analytical Procedures

(1) For powder, weigh X mg of sample into hydrolysis tube. For solution, transfer X μL sample into hydrolysis tube; Dry. For DF, crush aliquot of sample with mortar and pestle, weigh X mg of sample into hydrolysis tube.

(2) Soak "PO" samples in 800 μL fresh performic acid overnight; Dry.

(3) Perform parallel base hydrolysis (200 μL 4.2N NaOH 110℃ for 24hrs); Neutralize NaOH with 200 μL 4.2N HCl, add 600 μL Norleu diluent.

(4) Perform liquid phase hydrolysis on "HCl" and "PO" samples (200μL 6N HCl/1% phenol @ 110℃ for 24hrs); Dry.

(5) Add Norleu dilution buffer for a final dilution as indicated.

(6) Vortex; Spin down; Load 50μL

Note: Each 50μL injection = 2.0nmol Norleu.

Part 2 Analytical Results

The chromatograms and results are shown in the PDFs and excel sheet.

APPENDIX C
MORI SILK SPECIFICATIONS AND BATCH DATA

APPENDIX C1
MORI SILK: SPECIFICATIONS

Table C1-1: Specifications of Mori Silk*		
Parameter	Product Specification	
	Specification	Method
Protein	3.5 – 6.0%wt (35 - 60 mg/g)	AOAC 992.15
Ash	<0.1%wt	AOAC 920.153
Fat	<0.1%wt	AOAC 922.06
Carbohydrates	<0.1%wt	Calculation
Arsenic	<1 mg/L	EPA 200.8
Lead	<1 mg/L	EPA 200.8
Escherichia coli	<10 cfu/g	AOAC 991.14
Listeria monocytogenes	Negative/25g	USDA/FSIS MLG 8.05
Salmonella	Negative/25g	AOAC 2009.03
* Mori Silk solution or Mori Silk powder reconstituted at 5% weight/volume in water (50 mg Mori Silk in 1 mL potable water, mixed or stirred to solution).		
Abbreviations: AOAC = Association of Analytical Chemists; cfu = colony-forming units; g = gram(s); ICP-OES = inductively coupled plasma optical emission spectrometry; mg = milligram(s); ppmw = parts per million by weight; wt = weight		

APPENDIX C2
MORI SILK SOLUTION: BATCH DATA

Table C2-1. Analysis of Production Batches of Mori Silk Solution*

Parameter	Specification	Unit	Batches[1,2]			Method
			Batch 342	Batch 337	Batch 335	
			10/24/2020	10/15/2020	10/05/2020	
Specification Parameters						
Protein	3.5 – 6.0 (35 – 60)	%wt (mg/g)	4.15	3.69	5.44	AOAC 992.15
Fat	<0.1	%wt	0.02	0.06	0.05	AOAC 922.06
Carbohydrates	<0.1	%wt	0.1	<0.1	<0.1	Calculation
Ash	<0.1	%wt	0.04	0.04	0.04	AOAC 920.153
Arsenic	<1	mg/L	<0.5 mg/L	<0.5 mg/L	<0.5 mg/L	EPA 200.8
Lead	<1	mg/L	<0.5 mg/L	<0.5 mg/L	<0.5 mg/L	EPA 200.8
Escherichia coli	<10	cfu/g	<10	<10	<10	AOAC 991.14
Listeria monocytogenes	Negative/25g	none	Negative/10g	Negative/10g	Negative/10g	USDA/FSIS MLG 8.05[2]
Salmonella	Negative/25g	none	Negative/10g	Negative/10g	Negative/10g	AOAC 2009.03
Other Parameters						
Calcium	N/A	mg/L	210 mg/L	211 mg/L	216 mg/L	ICP-OES
Magnesium	N/A	mg/L	3.75	1.74	2.54	AOAC 985.01
Mercury	N/A	mg/kg	<0.050	<0.050	<0.050	EPA 7471B
Bacillus cereus	N/A	mpn/g	<3	<3	<3	AOAC 980.31/ISO 7932
Cronobacter	N/A	none	Negative/10g	Negative/10g	Negative/10g	PCR
Enterobacteriaceae	N/A	cfu/g	<10	<10	<10	AOAC 2003.01
Staphylococcus aureus	N/A	cfu/g	<10	<10	<10	AOAC 2003.07
Mold	N/A	cfu/g	<10	<10	<10	BAM Ch. 18
Yeast	N/A	cfu/g	<10	<10	<10	BAM Ch. 18

Notes: Protein was provided on a weight basis in mg protein/g sample.
* Mori Silk solution before dehydration
[1] = Values provided with "<" are the limits of detection for those analytes.
[2] = AOAC Certification 070702 issued by AOAC Research Institute to BioControl Systems, Inc. for its Assurance GDS® assay for *Listeria monocytogenes* detection (method BAM Ch. 10 and USDA/FSIS MLG 8.05)

Abbreviations: AOAC = Association of Analytical Chemists; BAM = Bacteriological Analytical Manual; cfu = colony-forming units; g = gram(s); ICP-OES = inductively coupled plasma optical emission spectrometry; LOD = limit of detection; mg = milligram(s); mL = milliliter(s); N/A = not applicable (no specification); PCR = polymerase chain reaction; ppmw = parts per million by weight; USDA/FSIS MLG = United States Department of Agriculture Food Safety and Inspection Service Microbiology Laboratory Guidebook

Alliance Analytical Laboratories, Inc.
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Coopersville, MI 49404
Phone: (616) 837-7670
Fax: (616) 837-7701

TEST RESULTS REPORT

Test methods marked with ^ are accredited under the laboratory's ISO/IEC 17025 accreditation issued by ANSI-ASQ National Accreditation Board. Refer to certificate and scope of accreditation AT-2044

CUSTOMER Cambridge Crops
444 Somerville Ave
Somerville, MA 02143
Email: sezin@cambridgecrops.com;

SAMPLE DESCRIPTION Batch 335 Mori Silk Solution
SD NA

SAMPLE DATE

DATE RECEIVED 11/3/2020

REFERENCE NUMBER 200413: 2041314 **Customer PO**

TEMPERATURE AT RECEIVING

Test Requested	Test Method		Results	Ref Number	Start Date
Ash	AOAC 920.153 Analyst: 42		0.04 %	200003:150	11/10 8:27 AM
Bacillus cereus	AOAC 980.31/ ISO 7932 Analyst: 44		<3 mpn/g		11/4 12:37 PM
BAM:Mold	^BAM Ch. 18 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 4:57 PM
BAM:Yeast	^BAM Ch. 18 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 4:57 PM
Calcium	AOAC 985.01 Analyst: 45		216 mg/L	200010:125	11/5 10:00 AM
Carbohydrates	Calculation Analyst: 04		<0.1 %		11/11 10:56 AM
Cronobacter	PCR Analyst: 43		Negative/10g	Kit Lot Number: GDSCB12191922I	11/4 9:53 AM
Enterobacteriaceae:^AOAC 2003.01	^AOAC 2003.01 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
Escherichia coli - Generic	^AOAC 991.14 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
Fat:Acid Hydrolysis:Cereal	AOAC 922.06 Analyst: 37		0.05 %	200009:75	11/6 12:00 PM
Heavy Metals	TP-A055				

It is the customer's responsibility to evaluate the compliance of these results to any regulatory requirement.
Test results apply to the sample as received.

Reviewed and Approved by: [redacted] Date: 11/11/2020

Andrea Balduck - Laboratory Manager

Date: 11/11/2020

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Coopersville, MI 49404
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TEST RESULTS REPORT

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SAMPLE DESCRIPTION Batch 335 Mori Silk Solution
SD NA

SAMPLE DATE

DATE RECEIVED 11/3/2020

REFERENCE NUMBER 200413: 2041314 **Customer PO**

TEMPERATURE AT RECEIVING

Test Requested	Test Method		Results	Ref Number	Start Date
Arsenic	TP-A055 Analyst: 45		<0.5 mg/L	200010:125	11/5 10:00 AM
Lead	TP-A055 Analyst: 45		<0.5 mg/L	200010:125	11/5 10:00 AM
Listeria monocytogenes	^AOAC 070702 Analyst: 44		Negative/10g	Kit Lot Number: GDSLM03262022E	11/4 11:57 AM
Magnesium ICP	AOAC 985.01 Analyst: 45		2.54 mg/L	200010:125	11/5 10:00 AM
Protein	^AOAC 992.15 Analyst: 37		5.44 %	200009:74	11/6 12:00 PM
Salmonella	^AOAC 2009.03 Analyst: 44		Negative/10g	Kit Lot Number: GDSSL06222011A	11/4 11:57 AM
Solids	AOAC 927.23 Analyst: 37		4.79 %	200009:71	11/5 4:16 PM
Staphylococcus aureus	^AOAC 2003.07 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
T:Lithium	EPA 200.8 Analyst: *212 ID#8001		<0.40 mg/Kg		11/4 12:00 PM *Subcontracted
T:Mercury, other	EPA 7471B Analyst: *212 ID#8001		<0.050 mg/Kg		11/5 12:00 PM *Subcontracted

It is the customer's responsibility to evaluate the compliance of these results to any regulatory requirement.
Test results apply to the sample as received.

Reviewed and Approved by: [redacted] Date: 11/11/2020

Andrea Balduck - Laboratory Manager

Date: 11/11/2020

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CUSTOMER Cambridge Crops
444 Somerville Ave
Somerville, MA 02143
Email: sezin@cambridgecrops.com;

SAMPLE DESCRIPTION Batch 337 Mori Silk Solution
SD NA

SAMPLE DATE

DATE RECEIVED 11/3/2020

REFERENCE NUMBER 200413: 2041315 **Customer PO**

TEMPERATURE AT RECEIVING

Test Requested	Test Method		Results	Ref Number	Start Date
Ash	AOAC 920.153 Analyst: 42		0.04 %	200003:150	11/10 8:27 AM
Bacillus cereus	AOAC 980.31/ ISO 7932 Analyst: 44		<3 mpn/g		11/4 12:37 PM
BAM:Mold	^BAM Ch. 18 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 4:57 PM
BAM:Yeast	^BAM Ch. 18 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 4:57 PM
Calcium	AOAC 985.01 Analyst: 45		211 mg/L	200010:125	11/5 10:00 AM
Carbohydrates	Calculation Analyst: 04		<0.1 %		11/11 10:56 AM
Cronobacter	PCR Analyst: 44		Negative/10g	Kit Lot Number: GDSCB12191922I	11/4 12:12 PM
Enterobacteriaceae:^AOAC 2003.01	^AOAC 2003.01 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
Escherichia coli - Generic	^AOAC 991.14 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
Fat:Acid Hydrolysis:Cereal	AOAC 922.06 Analyst: 37		0.06 %	200009:75	11/6 12:00 PM
Heavy Metals	TP-A055				

It is the customer's responsibility to evaluate the compliance of these results to any regulatory requirement.
Test results apply to the sample as received.

Reviewed and Approved by: _____ Date: 11/11/2020
Andrea Balduck - Laboratory Manager

Date: 11/11/2020

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SAMPLE DESCRIPTION Batch 337 Mori Silk Solution
SD NA

SAMPLE DATE

DATE RECEIVED 11/3/2020

REFERENCE NUMBER 200413: 2041315 **Customer PO**

TEMPERATURE AT RECEIVING

Test Requested	Test Method		Results	Ref Number	Start Date
Arsenic	TP-A055 Analyst: 45		<0.5 mg/L	200010:125	11/5 10:00 AM
Lead	TP-A055 Analyst: 45		<0.5 mg/L	200010:125	11/5 10:00 AM
Listeria monocytogenes	^AOAC 070702 Analyst: 44		Negative/10g	Kit Lot Number: GDSLM03262022E	11/4 11:57 AM
Magnesium ICP	AOAC 985.01 Analyst: 45		1.74 mg/L	200010:125	11/5 10:00 AM
Protein	^AOAC 992.15 Analyst: 37		3.69 %	200009:74	11/6 12:00 PM
Salmonella	^AOAC 2009.03 Analyst: 44		Negative/10g	Kit Lot Number: GDSSL06222011A	11/4 11:57 AM
Solids	AOAC 927.23 Analyst: 37		3.32 %	200009:71	11/5 4:16 PM
Staphylococcus aureus	^AOAC 2003.07 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
T:Lithium	EPA 200.8 Analyst: *212 ID#8001		<0.40 mg/Kg		11/4 12:00 PM *Subcontracted
T:Mercury, other	EPA 7471B Analyst: *212 ID#8001		<0.050 mg/Kg		11/5 12:00 PM *Subcontracted

Reviewed and Approved by: [redacted] Date: 11/11/2020

Andrea Balduck - Laboratory Manager

Date: 11/11/2020

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CUSTOMER Cambridge Crops
444 Somerville Ave
Somerville, MA 02143
Email: sezin@cambridgecrops.com;

SAMPLE DESCRIPTION Batch 342 Mori Silk Solution
SD NA

SAMPLE DATE

DATE RECEIVED 11/3/2020

REFERENCE NUMBER 200413: 2041316 **Customer PO**

TEMPERATURE AT RECEIVING

Test Requested	Test Method		Results	Ref Number	Start Date
Ash	AOAC 920.153 Analyst: 42		0.04 %	200003:150	11/10 8:27 AM
Bacillus cereus	AOAC 980.31/ ISO 7932 Analyst: 44		<3 mpn/g		11/4 12:37 PM
BAM:Mold	^BAM Ch. 18 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 4:57 PM
BAM:Yeast	^BAM Ch. 18 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 4:57 PM
Calcium	AOAC 985.01 Analyst: 45		210 mg/L	200010:125	11/5 10:00 AM
Carbohydrates	Calculation Analyst: 04		<0.1 %		11/11 10:56 AM
Cronobacter	PCR Analyst: 44		Negative/10g		11/4 12:12 PM Kit Lot Number: GDSCB12191922I
Enterobacteriaceae:^AOAC 2003.01	^AOAC 2003.01 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
Escherichia coli - Generic	^AOAC 991.14 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
Fat:Acid Hydrolysis:Cereal	AOAC 922.06 Analyst: 37		0.02 %	200009:75	11/6 12:00 PM
Heavy Metals	TP-A055				

It is the customer's responsibility to evaluate the compliance of these results to any regulatory requirement.
Test results apply to the sample as received.

Reviewed and Approved by: [redacted] Date: 11/11/2020

Andrea Balduck - Laboratory Manager

Date: 11/11/2020

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TEST RESULTS REPORT

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SAMPLE DESCRIPTION Batch 342 Mori Silk Solution
SD NA

SAMPLE DATE

DATE RECEIVED 11/3/2020

REFERENCE NUMBER 200413: 2041316 **Customer PO**

TEMPERATURE AT RECEIVING

Test Requested	Test Method		Results	Ref Number	Start Date
Arsenic	TP-A055 Analyst: 45		<0.5 mg/L	200010:125	11/5 10:00 AM
Lead	TP-A055 Analyst: 45		<0.5 mg/L	200010:125	11/5 10:00 AM
Listeria monocytogenes	^AOAC 070702 Analyst: 44		Negative/10g	Kit Lot Number: GDSLM03262022E	11/4 11:57 AM
Magnesium ICP	AOAC 985.01 Analyst: 45		3.75 mg/L	200010:125	11/5 10:00 AM
Protein	^AOAC 992.15 Analyst: 37		4.15 %	200009:74	11/6 12:00 PM
Salmonella	^AOAC 2009.03 Analyst: 44		Negative/10g	Kit Lot Number: GDSSL06222011A	11/4 11:57 AM
Solids	AOAC 927.23 Analyst: 37		3.70 %	200009:71	11/5 4:16 PM
Staphylococcus aureus	^AOAC 2003.07 Analyst: 44	Log: <1.0	<10 cfu/g		11/4 12:37 PM
T:Lithium	EPA 200.8 Analyst: *212 ID#8001		<0.40 mg/Kg		11/4 12:00 PM *Subcontracted
T:Mercury, other	EPA 7471B Analyst: *212 ID#8001		<0.050 mg/Kg		11/5 12:00 PM *Subcontracted

It is the customer's responsibility to evaluate the compliance of these results to any regulatory requirement.
Test results apply to the sample as received.

Reviewed and Approved by: _____ Date: 11/11/2020

Andrea Balduck - Laboratory Manager

Date: 11/11/2020

AOAC RESEARCH INSTITUTE

CERTIFICATION

AOAC® *Performance Tested*℠

Certificate No.

070702

The AOAC Research Institute hereby certifies that the performance of the test kit known as:

Assurance GDS® for *Listeria monocytogenes*

manufactured by

BioControl Systems, Inc.
12822 SE 32nd Street
Bellevue, WA 98005
USA

This method has been evaluated in the AOAC® *Performance Tested Methods*℠ Program, and found to perform as stated by the manufacturer contingent to the comments contained in the manuscript. This certificate means that an AOAC® Certification Mark License Agreement has been executed which authorizes the manufacturer to display the AOAC *Performance Tested*℠ certification mark along with the statement - "THIS METHOD'S PERFORMANCE WAS REVIEWED BY AOAC RESEARCH INSTITUTE AND WAS FOUND TO PERFORM TO THE MANUFACTURER'S SPECIFICATIONS" - on the above mentioned method for a period of one calendar year from the date of this certificate (January 15, 2016 – December 31, 2016). Renewal may be granted at the end of one year under the rules stated in the licensing agreement.

Deborah McKenzie, Senior Director
Signature for AOAC Research Institute

January 15, 2016
Date

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Internet e-mail: aoacri@aoac.org, * World Wide Web Site: http://www.aoac.org

METHOD AUTHORS
Philip Feldsine, Andrew H. Lienau, Marcus Jucker, and David Kerr

SUBMITTING COMPANY
BioControl Systems, Inc.
12822 SE 32nd St.
Belleuve, WA 98005
USA

KIT NAME(S)
Assurance GDS® for *Listeria monocytogenes*

CATALOG NUMBERS
61010-100, 71010-100

INDEPENDENT LABORATORY
Silliker Inc,
Food Science Center
South Holland, IL
USA

AOAC EXPERTS AND PEER REVIEWERS
Thomas Hammack[1], Elliot Ryser[2], Eddie Richter[3]
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[2] Michigan State University, East Lansing, MI, USA
[3] Richter International, Columbus, OH, USA

APPLICABILITY OF METHOD
Target organism –*Listeria monocytogenes*
Matrices – (25 g) - liquid pasteurized milk, Mexican soft cheese, frankfurter, , ready-to-eat turkey meat, raw green beans, raw fish
(environmental sponge or swab – 100 mL/10 mL BLEB+LiCl+PALCAM, respectfully) stainless steel, rubber and plastic
Performance claims - In this study, we observed 100% inclusivity among the 50 *L. monocytogenes* strains and 100% exclusivity for the 31 potential cross-reacting non-*L. monocytogenes* organisms. In method comparison studies conducted by an independent laboratory as well as internally, GDS test results showed that it was equivalent to the USDA FSIS and FDA BAM reference culture methods for selected foods and environmental surfaces.

REFERENCE METHOD
USDA/FSIS MLG 8.05 *Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, Egg, and Environmental Samples.* (5)
FDA/BAM Chapter 10 *Detection and Enumeration of Listeria monocytogenes in Foods.* (6)

PRINCIPLE OF THE METHOD
GDS is a gene-based assay that incorporates multiple levels of specificity to ensure highly accurate results. The method utilizes proprietary probes and specific primers directed against a highly conserved DNA sequence of the target organism. GDS also utilizes a proprietary device and reagents that concentrate populations of target microorganisms and eliminate potential competitive microflora. The method is designed to be highly selective, and does not detect microorganisms that are potential cross-reactors in antibody-based assays. The proof of the principle was previously demonstrated in the validations of the Assurance GDS for *E. coli* O157:H7 Method (OMA 2005.04), Assurance GDS for Shiga-like Toxin Genes Method (OMA 2005.05) (3) and Assurance GDS for *Salmonella* Method (Certificate of Performance Tested Status No. 050602).

DISCUSSION OF THE VALIDATION STUDY
Results of these studies validated that Assurance GDS for *L. monocytogenes* is rugged, and is inclusive and specific for *L. monocytogenes*. The results also demonstrated that GDS is an effective method for the detection of *L. monocytogenes* in selected foods and environmental surfaces. In the internal method comparison study, GDS and the reference culture methods are statistically not different for the detection of *Listeria monocytogenes* in liquid milk, Mexican soft cheese, ready-to-eat turkey meat, raw green beans, raw fish, rubber, and plastic. The external method comparison study yielded similar results for raw fish and stainless steel. In the internal method comparison study, for Mexican soft cheese (low level), frankfurter (low level), stainless steel (low level) and concrete, results indicated that GDS and reference methods are statistically different, with GDS results recovering higher number of *L. monocytogenes* confirmed positive samples than reference culture method. In this study, we observed 100% inclusivity among the 50 *L. monocytogenes* serovars and 100% exclusivity for the 31 non-*L. monocytogenes* organisms analyzed. Sensitivity rates for the GDS method for food and environmental surface analysis ranged from 83 to 100 percent. The sensitivity rate for the reference method was of a greater range, from 17 to 100 percent.

In this study, all the samples are unmatched, in other words, the primary enrichment used is different between the GDS and the reference methods. This could have attributed to some differences between the test and reference methods. Additionally, the *L. monocytogenes* contamination levels in this study are, by design, quite low and *Listeria* is a relatively slow growing microorganism. Any variation due to non-homogeneity of the inoculum in the samples will be magnified. This hypothesis is supported by an analysis of the food type/level combinations where the two methods yielded different results in the internal study. Of the 14 food type/level combinations where the number of positives was different between GDS and reference, GDS recovered a higher number of positives in 8 food type/levels, while reference method recovered a higher number of positives in 6 of the food type/levels. In absolute terms, GDS recovered 44 more positive samples than reference, while reference recovered 12 more positive samples respectively. Twenty-four of the 44 additional confirmed positive samples versus reference resulted from concrete and stainless steel samples. Overall, data indicate that the enrichment and detection procedure of GDS may be preferred for certain environmental surfaces, particularly stainless steel and concrete, and to a smaller degree, for frankfurter and Mexican soft cheese.

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Table 1. Comparative results for the detection of *L. monocytogenes* in foods and environmental surfaces by the Assurance GDS for *L. monocytogenes* method

Food/surface	Level	cfu/g [a]	No. Test Portions	GDS Presumptive [b]	GDS Confirmed [c]	Reference method [d]	Mantel-Haenszel Chi square [e]	GDS Sensitivity [f], %	GDS False neg [g], %	GDS Specificity [h], %	GDS False pos [i], %	Ref Sensitivity [j], %	Ref False neg [k], %
liquid milk	low	0.023	20	13	13	12	0.10	-	-		-	92%	8%
	control [l]	<0.003	5	0	0	0	-	-	-	100%	0%	-	-
	high	2.4	20	20	20	20	-	100%	0%		-	100%	0%
	control	<0.003	5	0	0	0	-	-	-	100%	0%	-	-
Mexican soft cheese	low	0.092	20	16	16	10	3.86	-	-	-	-	63%	38%
	control	<0.003	5	0	0	0	-	-	-	100%	0%	-	-
	high	0.427	20	20	20	20	-	100%	0%	-	-	100%	0%
	control	<0.003	5	0	0	0	-	-		100%	0%	-	-
frankfurter	low	0.004	20	12	12	2	10.71	-	-	-	-	17%	83%
	control	<0.003	5	0	0	0	-	-	-	100%	0%	-	-
	high	0.023	20	19	16	15	1.52	-	-	-	-	83%	17%
	control	<0.003	5	0	0	0	-	-	-	100%	0%	-	-
ready-to-eat turkey meat	low	0.009	20	10	10	12	0.39	83%	17%		-	-	-
	high	0.092	20	20	20	20	NA	100%	0%		-	100%	0%
	control	<0.003	5	0	0	0	-	-		100%	0%	-	-
raw green beans	low	0.038	20	11	11	13	0.41	85%	15%	-	-	-	-
	control	<0.003	5	0	0	0	-	-		100%	0%	-	-
	high	0.092	20	19	19	20	1.00	95%	5%	-	-	-	-
	control	<0.003	5	0	0	0	-	-		100%	0%	-	-
raw fish	high	0.231	20	17	17	18	0.22	94%	6%		-	-	
	control	<0.003	5	0	0	0	-	-		100%	0%	-	
stainless steel	low	NA	20	20	20	15	5.57	-		-	-	75%	25%
	control	NA	5	0	0	0	-	-		100%	0%	-	-
	high	NA	20	20	20	18	2.05	-		-	-	90%	10%
	control	NA	5	0	0	0	-	-		100%	0%	-	-
rubber	low	NA	20	18	17	20	3.16	85%	15%	-	-	-	-
	control	NA	5	0	0	0	-	-		100%	0%	-	-
concrete	low	NA	20	15	15	7	6.30	-	-	-	-	47%	53%
	high	NA	20	17	17	8	8.42	-	-	-	-	47%	53%
	control	NA	5	0	0	0	-	-	-	100%	0%		-
plastic	low	NA	20	20	17	20	3.16	85%	15%	-	-	-	-
	high	NA	20	20	20	20	-	100%	0%		-	100%	0%
	control	NA	5	0	0	0	-	-		100%	0%	-	-

[a] Most probable number of colony forming units (cfu)/g food.

[b] Number of GDS assay positive samples not considering subsequent confirmations.

[c] Number of GDS assay positive samples subsequently confirmed.

[d] Number of samples positive by Reference.

[e] Mantel-Haenszel chi square for unmatched samples is defined as $((n-1)(ad-bc)^2)/((a+b)(a+c)(b+d)(c+d))$ where a = samples confirmed positive by GDS, b = samples negative by GDS, c = samples positive by Reference, d = samples negative by Reference and $n = a+b+c+d$. A chi square value > 3.84 indicates significance at $P < 0.05$.

[f] GDS sensitivity rate is defined as the number of GDS confirmed positive test results divided by the number of Reference positive test results, expressed as a percentage. Not calculated only where the number of confirmed GDS positive test results is higher than number of Reference positive test results.

[g] GDS false negative rate is 100 - sensitivity rate.

[h] GDS specificity rate is defined as the number of GDS assay negative samples divided by the number of negative samples, expressed as a percentage. Calculated only for control levels with no confirmed positive results.

[i] GDS false positive rate is 100-specificity rate.

[j] Reference sensitivity rate is defined as the number of Reference positive test results divided by the number of GDS confirmed positive test results, expressed as a percentage. Not calculated where the number of Reference positive results is higher than confirmed GDS positive test results.

[k] Reference false negative rate is 100 - Reference sensitivity rate.

[l] Controls are uninoculated test portions.

Table 2A. Organisms included in validation inclusivity of the Assurance GDS for *L. monocytogenes*

Organism	Serotype	Source	Culture #	Isolate source (if known)
Listeria monocytogenes	1	ATCC	19111	poultry
Listeria monocytogenes	2	ATCC	19112	human
Listeria monocytogenes	3	ATCC	19113	human
Listeria monocytogenes	1/2a	ATCC	51772	cheese
Listeria monocytogenes	1/2a	ATCC	51775	dairy product
Listeria monocytogenes	1/2a	ATCC	51773	dairy product
Listeria monocytogenes	1/2a	ATCC	51774	human
Listeria monocytogenes	1/2a	ILSI NA	FSL R2-499	human
Listeria monocytogenes	1/2a	ILSI NA	FSL C1-056	human
Listeria monocytogenes	1/2a	FDA	SEA 3362	breaded fish
Listeria monocytogenes	1/2b	Silliker	unknown	meat
Listeria monocytogenes	1/2b	Silliker	unknown	environmental
Listeria monocytogenes	1/2b	ATCC	51780	dairy product
Listeria monocytogenes	1/2b	ATCC	BAA-839	human
Listeria monocytogenes	1/2b	ILSI NA	FSL J1-177	human
Listeria monocytogenes	1/2b	ILSI NA	FSL R2-503	human
Listeria monocytogenes	1/2c	ATCC	51779	cheese
Listeria monocytogenes	1/2c	ILSI NA	FSL J1-094	human
Listeria monocytogenes	1b	ATCC	43248	guinea pig
Listeria monocytogenes	3a	FDA	unknown	unknown
Listeria monocytogenes	3a	ATCC	51782	dairy product
Listeria monocytogenes	3a	ILSI NA	FSL C1-115	human
Listeria monocytogenes	3b	Health Canada	HPB #60	unknown
Listeria monocytogenes	3b	FDA	H8399	unknown
Listeria monocytogenes	3b	FDA	SE-31	unknown
Listeria monocytogenes	3b	ILSI NA	FSL J1-169	human
Listeria monocytogenes	3c	Health Canada	HPB #61	unknown
Listeria monocytogenes	3c	FDA	H6919	unknown
Listeria monocytogenes	3c	ILSI NA	FSL J1-049	human
Listeria monocytogenes	4ab	ILSI NA	FSL J1-129	human
Listeria monocytogenes	4a	ATCC	19114	animal brain
Listeria monocytogenes	4a	ILSI NA	FSL W1-112	unknown
Listeria monocytogenes	4b	FDA	Scott A	human
Listeria monocytogenes	4b	FDA	SEA 3364	crab meat
Listeria monocytogenes	4b	FDA	SEA 3337	unknown
Listeria monocytogenes	4b	Univ. of Vermont	CRC 6416	unknown
Listeria monocytogenes	4b	ATCC	49594	human
Listeria monocytogenes	4b	ATCC	19115	human
Listeria monocytogenes	4b	ATCC	51776	dairy product
Listeria monocytogenes	4b	ATCC	51777	dairy product
Listeria monocytogenes	4b	ILSI NA	FSL J1-110	food, epidemic
Listeria monocytogenes	4c	ATCC	19116	chicken
Listeria monocytogenes	4c	ILSI NA	FSL W1-110	unknown
Listeria monocytogenes	4c	ILSI NA	FSL W1-111	unknown
Listeria monocytogenes	4d	ILSI NA	FSL J1-107	human
Listeria monocytogenes	4d	ATCC	19117	sheep
Listeria monocytogenes	4e	ATCC	19118	chicken
Listeria monocytogenes	7	Health Canada	HPB #222	unknown
Listeria monocytogenes	7	FDA	H3293	unknown
Listeria monocytogenes		ATCC	15313	rabbit

Table 1E. Organisms included in Validating Exclusivity of the Assurance GDS for *L. monocytogenes*

Organism	Source	Culture #
Bacillus cereus	ATCC	13061
Bacillus licheniformis	ATCC	14580
Bacillus megaterium	ATCC	14581
Bacillus subtilis	ATCC	6051
Bacillus subtilis subsp. *spizizenii*	ATCC	6633
Brevibacterium linens	ATCC	9172
Burkholderia cepacia	ATCC	25416
Candida albicans	ATCC	18804
Corynebacterium pseudodiphtheriticum	ATCC	10700
Escherichia coli	ATCC	25922
Escherichia coli O157:H7	ATCC	35150
Enterococcus faecalis	ATCC	33186
Enterococcus faecium	ATCC	19434
Erysipelothrix rhusiopathiae	ATCC	19414
Jonesia denitrificans	ATCC	14870
Kurthia zopfii	ATCC	33403
Lactobacillus casei	ATCC	393
Listeria grayi	ATCC	25400
Listeria grayi subsp *murrayi*	ATCC	25401
Listeria innocua	ATCC	33090
Listeria ivanovii	ATCC	49953
Listeria seeligeri	ATCC	51334
Listeria welshimeri	ATCC	49591
Propionibacterium acidipropionici	ATCC	4965
Pseudomonas aeruginosa	ATCC	10145
Staphylococcus aureus	ATCC	49444
Staphylococcus epidermidis	ATCC	14990
Staphylococcus saprophyticus	ATCC	15305
Streptococcus bovis	ATCC	9809
Streptococcus equi subsp. *equi*	ATCC	9528
Streptococcus pyogenes	ATCC	49399

Table 6. Comparative results for the detection of *L. monocytogenes* in foods and environmental surfaces by the Assurance GDS for *L. monocytogenes* method

Food/surface	Level	cfu/g [a]	No. Test Portions	GDS Presumptive [b]	GDS Confirmed [c]	Reference method [d]	Mantel-Haenszel Chi square [e]	GDS Sensitivity [f], %	GDS False neg [g], %	GDS Specificity [h], %	GDS False pos [i], %	Ref Sensitivity [j], %	Ref False neg [k], %
stainless steel	low	NA	20	15	15	12	1.00	-	-	-	-	80%	20%
	control [l]	NA	5	0	0	0	-	-	-	100%	0%	-	-
	high	NA	20	20	20	20	-	100%	0%	-	-	100%	0%
	control	NA	5	0	0	0	-	-		100%	0%	-	-
raw fish	low	0.015	20	12	9	9	0.00	100%	0%	-	-	100%	0%
	control	<0.003	5	0	0	0	-	-		100%	0%	-	-
	high	0.023	20	9	8	9	0.10	89%	11%	-	-	-	-
	control	<0.003	5	0	0	0	-			100%	0%	-	-

[a] Most probable number of colony forming units (cfu)/g food.

[b] Number of GDS assay positive samples not considering subsequent confirmations.

[c] Number of GDS assay positive samples subsequently confirmed.

[d] Number of samples positive by Reference.

[e] Mantel-Haenszel chi square for unmatched samples is defined as $((n-1)(ad-bc)^2)/((a+b)(a+c)(b+d)(c+d))$ where a = samples confirmed positive by GDS, b = samples negative by GDS, c = samples positive by Reference, d = samples negative by Reference and n = a+b+c+d. A chi square value > 3.84 indicates significance at $P < 0.05$.

[f] GDS sensitivity rate is defined as the number of GDS confirmed positive test results divided by the number of Reference positive test results, expressed as a percentage. Not Calculated only where the number of confirmed GDS positive test results is higher than number of Reference positive test results.

[g] GDS false negative rate is 100 - sensitivity rate.

[h] GDS specificity rate is defined as the number of GDS assay negative samples divided by the number of negative samples, expressed as a percentage. Calculated only for control levels with no confirmed positive results.

[i] GDS false positive rate is 100-specificity rate.

[j] Reference sensitivity rate is defined as the number of Reference positive test results divided by the number of GDS confirmed positive test results, expressed as a percentage. Not calculated where the number of Reference positive results is higher than confirmed GDS positive test results.

[k] Reference false negative rate is 100 - Reference sensitivity rate.

[l] Uninoculated samples used as controls.

ORIGINAL CERTIFICATION DATE
July 6, 2007

METHOD MODIFICATION RECORD

1. **January 2016**

Under this AOAC® *Performance Tested*℠ License Number, 070702 this method is distributed by:
None

CERTIFICATION RENEWAL RECORD
Renewed annually through December 2016

SUMMARY OF MODIFICATION

1. **Hold time change from 15 to 20 minutes**

Under this AOAC® *Performance Tested*℠ License Number, 070702 this method is distributed as:
None

APPENDIX C3
MORI SILK POWDER

Alliance Analytical Laboratories, Inc.
179 West Randall Street
Coopersville, MI 49404
Phone: (616) 837-7670
Fax: (616) 837-7701

TEST RESULTS REPORT

Test methods marked with ^ are accredited under the laboratory's ISO/IEC 17025 accreditation issued by ANSI-ASQ National Accreditation Board. Refer to certificate and scope of accreditation AT-2044

CUSTOMER	Cambridge Crops 444 Somerville Ave Somerville, MA 02143 Email: sezin@cambridgecrops.com;
SAMPLE DESCRIPTION	Powder 5gr SD NA
SAMPLE DATE	
DATE RECEIVED	2/14/2020
REFERENCE NUMBER	200063: 2006302 **Customer PO**
TEMPERATURE AT RECEIVING	

Test Requested	Test Method	Results	Ref Number	Start Date
Protein	^AOAC 992.15 Analyst: 37	98.60 % Protein determined from 18.44% Nitrogen and collagen conversion factor of 5.55. Adjusted for 3.63% moisture.		

It is the customer's responsibility to evaluate the compliance of these results to any regulatory requirement.
Test results apply to the sample as received.

Reviewed and Approved by: [redacted] Date: 2/24/2020

Audrey Monroe - Laboratory Director

Date: 2/24/2020

APPENDIX D
MORI SILK STABILITY STUDY

I. Study Preparation

1. Experimental Design:
    - 1.1 Prepare the required powder amount aliquoted into chosen packaging materials and the environment for the stability study.
        - 1.1.1 All samples are stored at 60°C, 30% RH
        - 1.1.2 Packaging materials to be tested are compostable plant fiber cup, metallized mylar, and PLA container.
            - 1.1.2.1 Compostable Plant Fiber Container purchased from World Centric, SKU CU-SC-U2
            - 1.1.2.2 Metallized Mylar Container purchased from QQStudio, Product Number ASIN B071VDZXWX
            - 1.1.2.3 Compostable PLA Souffle Container purchased from World Centric, SKU CP-CS-2S
    - 1.2 Sampling time and duration
        - 1.2.1 Studies will span 4 weeks (28 days)
        - 1.2.2 Testing will be taken on Day 0, Day 7, Day 14, Day 21, and Day 28
    - 1.3 The amount of silk is calculated for the entirety of the stability study
        - 1.3.1 1 gram of silk powder is aliquoted into each packaging unit
        - 1.3.2 5 packaging units per packaging category (Compostable Plant Fiber Cup; Metallized Mylar; and PLA Container)
            - 1.3.2.1 4 * 5 = 20 samples per packaging category
2. All samples stored in an environmental chamber at 60°C and 30% RH until sampling.
3. Complete Day 0 testing for each sample in each package.
    a. Stability study testing will consist concentration measurement centrifuged and non-centrifuged samples using the BCA protein assay after solubilization of 50mg of the sample in 1ml (5% w/v). Testing should be completed according to Version 1.0 of the "Silk Concentration Measurement Using BCA Protein Assay" protocol, listed in detail under Section V.

II. Samples Tested at Day 7, 14, 21, and 28

1. Solutions prepared
    - i. 50mg of each sample weighed (n=5 per group) and placed in an Eppendorf tube.
    - ii. 1 mL of Milli-Q (MQ) water added; Eppendorf tube vortexed for 20 seconds for full reconstitution.
2. Concentration tested using BCA Protein assay
    - 3.1 500 µL of each stability study sample transferred from Eppendorf tubes into a new microcentrifuge tube.
        - 3.1.1 Microcentrifuge tubes labeled using the same nomenclature as the original stability study sample tube, adding "C" to indicate centrifugation.
    - 3.2 For each sample, concentration tested using the BCA protein assay for 2 replicates of both centrifuged and non-centrifuged samples (4 samples total) according to the protocol "Silk Concentration Measurement Using BCA Protein Assay" as described in Section V.

III. Data Analysis

1. Each sample concentration recorded and averaged for each sampling point (each week)
    a. Centrifuge and non-centrifuged data treated as separate groups
2. After week 4 (end of study), data (n=5 data points) plotted for respective packaging unit on a separate graph that shows the trend from Week 1-4 and delta between centrifuge and non-centrifuged.

IV. Results

Concentrations were verified at each timepoint for each container type and compared to a standard. Averages between each measurement were taken after centrifuging each sample. The standard BCA assay has a margin of error (MOE) of +/- 10mg/ml. Table 2 shows those results:

Week	Compostable PLA Cups (mg/ml)	Metallized Mylar Bag (mg/ml)	Compostable Plant Fiber Cups (mg/ml)	Standard (Advanced BioMatrix) (mg/ml)	Standard error (mg/ml)
t=0	49.59	49.59	49.59	50	+/- 10
Week 1	52.13	52.78	54.55	50	+/- 10
Week 2	47.27	47.57	54.05	50	+/- 10
Week 3	56.21	53.95	53.95	50	+/- 10
Week 4	45.38	56.73	56.73	50	+/- 10

Table 2. Average BCA Concentrations

The results indicate that the silk powder remained stable in all of the storage containers at high temperature and humidity. The concentration over the course of the four-week study did not exhibit an overall decrease. While there is a week to week movement both up and down for the concentrations, this is due to inherent inconsistencies in the BCA assay and are all within an expected margin of error. The general trend for all samples showed consistent concentration measurements within the expected range for the BCA assay.

Due to the fact that the samples do not exhibit an overall decrease in concentration over time nor an increase in concentration difference between the centrifuged and non-centrifuged samples, it is concluded that the powder remained stable in all of the tested storage containers throughout the four-week study at high temperature and humidity.

V. BCA Protocol

Materials and Equipment

1. Advanced BioMatrix (AB) silk fibroin solution, 50 mg/mL

2. Thermo Scientific BCA Reagent A
3. Thermo Scientific BCA Reagent B
4. Milli-Q (MQ) water
5. Microcentrifuge tubes
6. Microcentrifuge tube racks
7. 96-well microplates
8. 50 mL centrifuge tube
9. Multichannel pipette reservoirs
10. 200 µL multichannel pipette
11. Micropipettes + tips
12. Centrifuge
13. Vortex mixer
14. SPECTRAmax™ 250 microplate spectrophotometer (incubator set to 37°C)

Procedure

1. Microplate Reader Setup
   1.1. Set the plate reader temperature to 37°C.

2. Protein Standard Preparation
   2.1. Thaw one microcentrifuge tube of AB silk fibroin solution over ice.
   2.2. Label 6 sets of 3 microcentrifuge tubes from #1 to #6.
   2.3. In Tube #1, add 50 µL of original AB silk fibroin solution + 450 µL of MQ water. Vortex 5 times in 1-second intervals.
      2.3.1. **Note:** This 1:10 dilution is necessary to create the remaining dilutions in the protein standard. A 1:10 dilution is too concentrated for the BCA assay to yield accurate results and ultimately is not plated.
   2.4. In Tube #2, add 100 µL from Tube #1 + 400 µL of MQ water. Vortex.
   2.5. In Tube #3, add 375 µL from Tube #2 + 225 µL of MQ water. Vortex.
   2.6. In Tube #4, add 400 µL from Tube #3 + 100 µL of MQ water. Vortex.
   2.7. In Tube #5, add 200 µL from Tube #4 + 300 µL of MQ water. Vortex.
   2.8. In Tube #6, add 125 µL from Tube #5 + 375 µL of MQ water. Vortex.
   2.9. Repeat steps 1.3-1.8 for all 3 replicates of tubes 1-6.

Table 1. Final AB silk concentrations prepared from serial dilutions

Tube Number	1	2	3	4	5	6
Silk Concentration (mg/mL)	5.0	1.0	0.625	0.5	0.2	0.05

3. Silk Powder Sample
   3.1. For each sample of interest, label 8 microcentrifuge tubes with its identifier (e.g., container type and replicate number).
      3.1.1. Label 4 of the tubes "1:10" to indicate a 1 to 10 dilution.
         3.1.1.1. Label 2 of these tubes with a "C" to indicate centrifuged samples.
         3.1.1.2. Place these "1:10" tubes in a microcentrifuge tube rack.

3.1.1.3. **Note:** The 1:10 dilutions are necessary to accurately create the following 1:100 dilutions. Note that a 1:10 dilution is too concentrated for the BCA assay to yield accurate results and ultimately is not plated.

3.1.2. Label the other 4 tubes "1:100" to indicate a 1 to 100 dilution.

3.1.2.1. Label 2 of these tubes with a "C" to indicate centrifuged samples.

3.1.2.2. Place these "1:100" tubes in a **separate** microcentrifuge tube rack. See Figure 1.

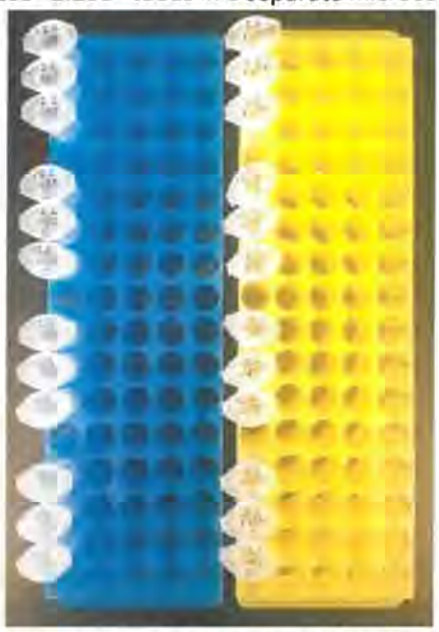

Figure 1. Labeled tubes for unknown sample dilutions with 1:10 and 1:100 tubes places in separate tube racks.

3.2. Add 450 µL of MQ water to all labeled tubes.

3.3. Invert the container holding the sample of interest 5 times, or until it is well-mixed.

3.4. Transfer 50 µL of the sample into each of its 2 pre-labeled "1:10" tubes (**without** the "C"); close the lids.

3.5. For each unknown, transfer 250 µL of the sample of interest into a clean microcentrifuge tube.

3.6. Label the tube(s) with a sample identifier (e.g., batch number) and a "C" to indicate that it will be centrifuged.

3.7. Centrifuge the tube(s) for 3 minutes at 15,000 rpm.

3.8. Transfer 50 µL from the top portion of the centrifuged tube into each of its 2 pre-labeled "1:10, C" tubes; close the lids. Ensure not to transfer any aggregates formed during centrifugation.

3.9. With all the "1:10" tubes (with closed lids) vortex each of the samples for 10 seconds.

3.10. Transfer 50 µL from each "1:10" tube into its corresponding "1:100" tube; close the lids.

3.11. With all the "1:100" tubes (with closed lids) vortex each of the samples for 10 seconds.

4. Working Reagent (WR) Preparation

4.1. Use the following formula to determine the total volume of WR required:

$$\left(\begin{matrix}\#\ of\\ controls\end{matrix} + \begin{matrix}\#\ of\\ standards\end{matrix} + \begin{matrix}\#\ of\\ unknowns\end{matrix}\right) \times \left(\begin{matrix}\#\ of\\ replicates\end{matrix}\right) \times \left(\begin{matrix}volume\ of\ WR\\ per\ sample\end{matrix}\right) + 2\ mL = \begin{matrix}total\ volume\ of\\ WR\ required\end{matrix}$$

4.1.1. # of controls = 1 (water)
4.1.2. # of standards = 5 (tubes 2-6 of the serially diluted AB silk)
4.1.3. # of unknowns = variable
4.1.4. # of replicates = 3
4.1.5. Volume of WR per sample = 0.260 mL
4.1.6. +2 mL to ensure there is enough solution

4.2. To prepare the WR, mix 50 parts BCA Reagent A with 1 part BCA Reagent B (50:1, Reagent A:B). Use the following formulas to calculate the volumes of BCA Reagents A and B required:

$$\begin{pmatrix} total\ volume\ of\ WR \\ required \end{pmatrix} \div 51 = \begin{matrix} volume\ of \\ BCA\ Reagent\ B \end{matrix}$$

$$\begin{pmatrix} volume\ of \\ BCA\ Reagent\ B \end{pmatrix} \times 50 = \begin{matrix} volume\ of \\ BCA\ Reagent\ A \end{matrix}$$

4.3. Add *volume of BCA Reagent A* to a centrifuge tube of appropriate size.
4.4. Add *volume of BCA Reagent B* to the same centrifuge tube.
4.5. Gently invert the WR 10 times or until well-mixed.
4.5.1. **Note:** Throughout the assay, be sure not to contaminate the WR. Small amounts of protein will lead to color development.

5. Assay Protocol
5.1. Arrange all replicates of silk standard tubes 2-6 and all replicates of the 1:100 (**not** 1:10) sample dilutions in tube racks in the same order that they will be placed into the 96-well microplate. Order them top to bottom, keeping replicates together, and **take a picture**. Note that each column of the 96-well microplate has 8 wells. See Figure 2.

Figure 2. Silk standards and 1:100 unknowns arranged in the same order that they will be placed into the 96-well microplate

5.2. Pipette 9 µL of each replicate of silk standards and 1:100 dilutions to the center of the microplate well.
5.2.1. **Note:** Add samples directly to the center of the well and avoid touching the sides of the well.
5.2.2. **Note:** Precision pipetting is essential. Small errors when pipetting account for large errors when measuring the absorbance.

5.3. Pipette 9 µL of MQ water into 3 more wells.
5.4. Add 260 µL of the WR to each well.
  5.4.1. Invert the WR tube 5 times.
  5.4.2. Pour some of the WR into a new multichannel pipette reservoir.
  5.4.3. Using the multichannel pipette, add 130 µL **two times** to each column of sample wells.
  5.4.4. For the final row of samples if not 8, carefully remove unneeded tips by hand and proceed as above.
  5.4.5. **Note:** Take care not to touch the pipette tip to the solution in each well. If this occurs, discard the pipette tips. If no contamination occurs, the same tips can be used throughout the addition of WR.
  5.4.6. **Note:** Add WR to all wells within 5 minutes. Color development begins as soon as the WR is added.
5.5. Place the microplate into the microplate reader and click the "Mix" button in the software 5 times.
5.6. Incubate the plate at 37°C for exactly 30 minutes in the microplate reader; click the "Mix" button every 15 minutes.
5.7. Cool plate at room temperature (RT) for exactly 5 minutes. Turn off the temperature control on the microplate reader.
  5.7.1. **Note:** Because the BCA Assay does not reach a true end point, color development will continue even after cooling to RT. The absorbance increases at a rate of ~0.25% per minute at RT.
5.8. Measure the absorbance of the standards, unknown samples, and water controls at 562nm on the plate reader.
5.9. Export the data as a text file and transfer to another computer for analysis in Microsoft Excel.
5.10. Prepare a linear standard curve by plotting the average water-corrected 562nm value for each silk standard vs. its concentration (mg/mL). Use the standard curve to determine the protein concentration of each unknown sample.
  5.10.1. **Note:** The Excel file should automatically subtract the average 562nm absorbance value of the water replicates from the 562nm absorbance value of all silk standards and unknown sample replicates.

References

1. Advanced BioMatrix Silk Fibroin Solution Usage Guide
2. Pierce® Microplate BCA Protein Assay Kit – Reducing Agent Compatible Instructions
3. Pierce® BCA Protein Assay Kit – Reducing Agent Compatible Instructions