Annex 3

SOY LEGHEMOGLOBIN PREPARATION: AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY PRE-DOSING ESTROUS CYCLE DETERMINATION

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

DATA REQUIREMENT

OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 407): Health Effects, *Repeated Dose 28-Day Oral Toxicity Study in Rodents* (2008).

US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 4. a. *Subchronic Toxicity Studies with Rodents* (2007).

STUDY NUMBER

44856

PERFORMING LABORATORY

Product Safety Labs
2394 US Highway 130
Dayton, New Jersey 08810

STUDY COMPLETION DATE

July 26, 2017

STUDY DIRECTOR

Jayson Chen, PhD

SPONSOR

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CERTIFICATIONS

We, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected during the study.

(b) (6)

Jayson Chen, PhD
Study Director
Product Safety Labs

July 26, 2017
Date

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Odete Mendes, DVM, PhD, DACVP, DABT
Director of Toxicology and Pathology
Product Safety Labs

26 July 2017
Date

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STUDY INFORMATION

Protocol No.:	P703.02 IMP
Test Substances:	Soy Leghemoglobin Preparation
Lot #s:	PP-PGM2-16-088-301
Physical Descriptions:	Red/brown powder
Date Test Substance Received:	July 20, 2016
PSL IDs:	160720-5R
PSL Study Number:	44856
Sponsor:	Impossible Foods Inc. 525 Chesapeake Dr. Redwood City, CA 94063
Study Initiated-Completed:	February 2, 2016 – (see report cover page)
In-Life Study Initiated-Completed:	February 8 – March 23, 2016

KEY PERSONNEL

Product Safety Labs:

President:	Daniel J. Merkel, BS, MBA
Director of Toxicology and Pathology:	Odete Mendes, DVM, PhD, DACVP, DABT
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Technical Writing Supervisor:	Celeste Dunn, AS

The following was responsible for analytical chemistry:

Test substance and dietary analysis	Impossible Foods Inc. 525 Chesapeake Dr. Redwood City, CA 94063
P.I. (chemical analysis):	Rachel Fraser, PhD

The following were responsible for the histological slide preparation and pathology evaluations:

Histological slides preparation:	Histoserv, Inc. 19526 Amaranth Drive, Germantown, MD 20874
P.I. (slide preparation):	Pratiba Vohra
Histological slide evaluation by:	Regan Path/Tox Services 1457 Township Rd. 853 Ashland, OH 44805
P.I. (pathology):	Karen Regan, DVM, DACVP, DABT

1. OBJECTIVE

The objective of this study was to evaluate the potential reproductive toxicity (estrous cycle and reproductive organ histopathology) of Soy Leghemoglobin Preparation in female rats continuously exposed to the test substance in the diet for at least 28 days. Additionally, estrous cycle evaluation was also performed for 14 days prior to dosing.

2. SUMMARY

Four groups of adult Crl: Sprague-Dawley CD® IGS rats (15/group) were maintained on diets calculated to provide target dose levels of 512, 1024, and 1536 mg/kg/day Soy Leghemoglobin Preparation, which correspond to 250, 500, and 750 mg/kg/day of active ingredient (Soy Leghemoglobin).

The animals were observed for viability, signs of gross toxicity, and behavioral changes at least once daily during the study and weekly during the test substance exposure period for a battery of detailed clinical observations. Body weight and food consumption measurements were collected throughout the study and used to calculate the mean overall daily intake of test substance. Gross necropsies and histological evaluation of selected organs and tissues were performed on study animals.

The test substance was considered to be homogenously distributed in the diet preparations at all study concentrations. Animals were considered to have received target dietary concentrations of Soy Leghemoglobin Preparation.

There were no mortalities during the course of the study. There were no clinical observations attributable to the administration of Soy Leghemoglobin Preparation. Mean estrous cycles for female rats in Groups 2-4 were comparable to control Group 1 values throughout the study. There were no changes in body weight, body weight gain, food consumption, and food efficiency attributable to Soy Leghemoglobin Preparation administration.

There were no macroscopic and microscopic observations or organ weight changes attributed to the Soy Leghemoglobin Preparation administration.

Under the conditions of this study and based on the toxicological endpoints evaluated, administration of Soy Leghemoglobin Preparation at dose levels up to 1536 mg/kg/day or 750 mg/kg/day in active ingredient (Soy Leghemoglobin) did not cause an effect in the estrous cycle of female Sprague Dawley rats.

3. TEST SUBSTANCE

A. Source

The test substance was provided by the Sponsor.

B. Identification

The test substances were identified using the following information provided by the Sponsor and Product Safety Labs (PSL) identification number.

Test Substance: Soy Leghemoglobin Preparation
PSL ID: 160720-5R
Lot #: PP-PGM2-16-088-301
Physical Description: Red/brown powder
Composition: Soy Leghemoglobin 48.82%

Storage Conditions: Frozen
Expiration Date: Not Applicable

Documentation of the methods of synthesis, fabrication, or derivation of the test substance is retained by the Sponsor.

C. Analysis

The test substance, as received, was expected to be stable for the duration of the study. The Sponsor was responsible for all analytical work required to characterize the neat test substance and validate its stability. Stability of the test substance in the dietary matrix and that of the concentration of the test substance in the test diets was determined to be stable over 10 days in a previous toxicity study (Product Safety Labs, 2017).

D. Hazards

Appropriate routine safety precautions were exercised in the handling of the test substances.

4. GENERAL TEST SYSTEM PARAMETERS

A. Animal Requirements

4.A.1 Number of Animals: 60

4.A.2 Number of Groups: 4 (3 dose levels per sex + 1 control group per sex)

4.A.3 Number of Animals per Group: 15

4.A.4 Sex: Female; nulliparous and non-pregnant.

4.A.5 Species/Strain: CRL Sprague-Dawley CD® IGS rats

4.A.6 Age/Weight: Seven to eight weeks at initiation; the weight variation did not exceed ± 20% of the mean weight for each sex.

4.A.7 Supplier: Charles River Laboratories, Inc. Rats were shipped in filtered cartons by truck.

On February 2, 2017, sixty-four (64) CRL Sprague-Dawley CD® IGS rats (females) arrived from Charles River Laboratories, with an assigned birth date December 16, 2016. The rats were designated by the supplier to be approximately six to seven weeks of age upon arrival.

B. Test System Justification

The Sprague Dawley® rat is the system of choice because, historically, it has been a preferred and commonly used species for dietary toxicity tests. The current state of scientific knowledge does not provide acceptable alternatives to the use of live animals to accomplish the objective of this study.

C. Animal Husbandry

4.C.1 Housing

The animals were individually housed in suspended stainless steel cages which conform to the size recommendations in the latest *Guide for the Care and Use of Laboratory Animals* (Natl. Res. Council, 2011). Litter paper placed beneath the cage was changed at least three times/week. The animal room had a 12-hour light/dark cycle and was kept clean and vermin free.

4.C.2 Animal Room Temperature and Relative Humidity Ranges

19-23°C and 35-56%

4.C.3 Acclimation

The animals were conditioned to the housing facilities for five days prior to testing. Body weights and clinical observations were recorded at least two times prior to study start.

4.C.4 Feed

2016CM Envigo Teklad Global Rodent Diet® (Envigo Teklad, Inc.) was stored in a dedicated temperature and humidity monitored feed storage site and available *ad libitum* during acclimation and study Days 0-13. Test diets were prepared as described in Section 6.B using 2016CM certified Envigo Teklad Global Rodent Diet® and were available *ad libitum* during at least study Days 14-42.

4.C.5 Water

Filtered tap water was available *ad libitum* from an automatic watering access system. Water analysis was conducted by Precision Analytical Services, Inc., Toms River, NJ and South Brunswick Municipal Water Supply, South Brunswick, NJ.

4.C.6 Contaminants

There are no known contaminants reasonably expected to be found in the food or water that would interfere with the results of this study. Routine analysis consisting of each lot of feed used in this study was received from Envigo Teklad, Madison, WI. Water analysis was conducted periodically and the records are kept on file at Product Safety Labs. The date of the most recent analysis is reported in Appendix B.

D. Identification

4.D.1 Cage

Each cage was identified by a cage card indicating at least the study number, dose level, group assignment, individual animal identification, and sex of the animals.

4.D.2 Animal

Each animal was given a sequential number in addition to being uniquely identified with a Monel® self-piercing stainless steel ear tag.

5. EXPERIMENTAL DESIGN

A. Route of Administration

The test substances were administered in the diet.

B. Justification of Route of Administration

The dietary route of administration was selected by the Sponsor. This route of administration is recommended in the referenced guidelines (Section 8.C.) and a potential route of human exposure.

C. Control of Bias

Animals were randomly assigned, stratified by body weight, to test groups.

D. Dose Levels

Fifteen female rats were randomly assigned to each of the following test groups:

Group	No. Animals/ Group (F)	Target Exposure of Active Ingredient (mg/kg/day)	Target Dietary Dose Level of Test Substance (mg/kg/day)[a]
1	15	Basal Diet Control 0	0
2	15	Low Dose 250	512
3	15	Intermediate Dose 500	1024
4	15	High Dose 750	1536

[a] Based on 48.82% active ingredient (AI, Soy Leghemoglobin) of Soy Leghemoglobin Preparation (Lot # PP-PGM2-16-088-301).

E. Justification of Dose Level Selection

The Sponsor, in consultation with the Study Director and based on a 28-day dietary toxicity study (Product Safety Labs, 2017), selected target dietary dose levels of 512, 1024 and 1536 mg/kg/day that correspond to target dose levels of 250, 500 and 750 mg/kg/day of the active ingredient, Soy Leghemoglobin. To maintain target dietary dose levels throughout the study, concentrations in the test diets were calculated based on the most recent group body weight and food consumption data. Diets for females at each dietary dose level were made separately each week.

6. GENERAL PROCEDURES

A. Selection of Animals

Sixty (60) healthy female rats were used on test. Animals were selected for this study on the basis of adequate body weight gain, absence of clinical signs of disease or injury, and a body weight within ±20% of the mean within a sex. Selected rats were distributed by randomization according to stratification by body weight so that there was no statistically significant difference among group body weight means within a sex. The animals weighed 157-204 grams and were approximately seven to eight weeks of age at initiation of dosing. The rats used on test were randomly distributed, stratified by body weight, among the dose and control groups on the day of study start.

B. Diet Preparation and Sampling

6.B.1 Diet Preparation

The test substances were processed to decrease particle size using a grinder and then added to 2016CM Envigo Teklad Global Rodent Diet® and thoroughly mixed in a high-speed mixer. Control diet (Basal Diet) was mixed under the same conditions as the diets prepared with the test substance. All diets were kept frozen following preparation, unless presented to the test animals on the same day as diet preparation. All diets were prepared approximately weekly.

6.B.2 Diet Presentation

The control diet was presented to all animals on Days 0-13 of the study. On study Day 14, the control and test diets were presented to their respective groups. The diets were replaced concurrently with food consumption measurements on Days 17, 21, 24, 28, 31, 35 and 38. Additional diet was provided as needed throughout the study to ensure *ad libitum* feeding. Animals were exposed to the control or test diets for at least 28 days.

6.B.3 Sampling

The neat test substance and selected prepared diets (at each concentration), were sampled in duplicate. Samples were frozen until analyzed and/or discarded upon completion of the study.

6.B.4 Stability of Test Substance

The neat test substance stability was previously determined to be stable under normal laboratory conditions for the duration of a 28-day study (Product Safety Labs, 2017). At the initial diet preparation, a sample of the test substance (neat) was retained.

6.B.5 Stability in Dietary Matrix

The test substance in the dietary matrix was previously determined to be stable over 10 days in a previous toxicity study (Product Safety Labs, 2017). Stability of the test substance in the dietary matrix was not assessed in this study.

6.B.6 Homogeneity

Samples to evaluate homogeneity of the test substance distribution were collected from the initial diet preparation. Samples were taken from approximately the top, middle and bottom of the diet mixer. Basal diet control samples were collected from the middle of the mixer only. Chemical analysis to verify the diets as homogeneous and of accurate concentration throughout the study was performed by Impossible Foods.

6.B.7 Concentration Verification

Samples for concentration verification were collected as part of the homogeneity analysis during the first week of the study. Diet preparation calculation was verified and test diets were mixed according to PSL's standard operating procedure. Nominal diet concentrations were used to determine the total intake of the test substance for each group.

6.B.8 Sample Preservation

Upon sampling, diet preparations and neat test substance samples were stored frozen. Samples were considered stable from the point at which they were frozen.

6.B.9 Sample Analysis

A single set of the frozen diet samples described above was sent to Impossible Foods for analysis of diet preparation and neat test substance samples. A signed, analytical report was provided to the Study Director. This report included the methodology, pertinent measurements, study results, and tabulated results. All raw data is retained by Impossible Foods. Any remaining sample material was retained at Product Safety Labs until issuance of the final report.

C. Analytical Chemistry

6.C.1 Sample Storage

Upon receipt, all samples were stored and maintained frozen prior to analysis.

6.C.2 Method Validation

Prior to sample analysis, the suitability of the method was demonstrated. Method validation included, but was not limited to determination of linearity, precision, and accuracy.

6.C.3 Reference Substance

An aliquot of the test substance served as the reference standard.

6.C.4 Chemical Analysis

Analytical test methodology was validated by Impossible Foods personnel. Samples were analyzed in replicate. A detailed description of the analytical test method(s) was documented. Any remaining sample material was retained until the issuance of the final report.

6.C.5 Data Reporting

Data was captured on standard raw data sheets and as instrument output, as necessary, and summarized in tabular form.

6.C.6 Analytical Report and Records to be Maintained

A signed, analytical report was provided to the Study Director. This report included the methodology, pertinent measurements, study results, and tabulated results. All raw data was maintained by Impossible Foods. The analytical report was incorporated into the main study report.

D. Clinical Observations

All animals were observed at least twice daily for viability. Cage-side observations of all animals were performed daily during the study. All findings were recorded.

On Day 14 and approximately weekly thereafter, a detailed clinical observation was conducted while handling the animal, generally occurring on days that the animals were weighed and food consumption measurements taken. Potential signs noted included, but were not limited to: changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Likewise, changes in gait, posture, and response to handling, as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling), or bizarre behavior (e.g., self-mutilation, walking backwards) were also recorded. The date and clock time of all observations and/or mortality checks were recorded.

E. Estrous cycles

Estrous cycle in the female rats was determined daily on Days 0-13 and Days 29-42 of the study, by vaginal lavage to evaluate for regular cyclicity. A vaginal lavage was also performed at termination to determine the stage of estrous cycle at sacrifice. Cytological evaluation was performed without knowledge of treatment group assignment.

F. Body Weight and Body Weight Gain

Individual body weights were recorded at least two times during acclimation. Test animals were weighed on Day 0 (prior to study start) and approximately weekly thereafter (intervals of 7 days ±

1). The animals were also weighed prior to sacrifice. Body weight gain was calculated for selected intervals and for the study overall.

G. Food Consumption, Food Efficiency, and Dietary Intake of Soy Leghemoglobin Preparation

Individual food consumption was measured and recorded on Days 3, 7, 10, 14, 17, 21, 24, 28, 31, 35 and 38 and at the end of the study. Food efficiency and dietary intake of the test substance (mg/kg/day) were also calculated and reported.

H. Terminal Sacrifice and Histopathology

6.H.1 Scheduled Sacrifice

At terminal sacrifice, all survivors were euthanized by exsanguination under isoflurane anesthesia.

Plasma samples were collected from all animals, at their respective sacrifice, and stored frozen (approximately -80°C) for future possible analysis.

All animals in the study were subjected to a necropsy, which included examination of the external surface of the body, all orifices, musculoskeletal system, and the thoracic, abdominal and cranial cavities and their contents. The following tissues (of all animals sacrificed by design) were weighed wet as soon as possible after dissection to avoid drying:

uterus	ovaries with oviducts (combined)

The following organs and tissues from all animals were preserved in 10% neutral buffered formalin for possible future histopathological examination:

vagina[1]	uterus	cervix
ovaries	oviducts	

6.H.2 Histopathology

Histological examination was performed on the preserved organs and tissues of animals from both the control and high dose groups (Groups 1 and 4, respectively). The fixed tissues were trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin (HE), and examined by light microscopy. Slide preparation was performed by Histoserv, Inc. and histological assessment, by a board-certified veterinary pathologist, was performed by Regan Path/Tox Services (Amendment 1). Prior to data recording, initial histopathological evaluation was performed without knowledge of treatment group assignment.

7. STATISTICAL ANALYSIS

Product Safety Labs performed statistical analysis of all data collected during the in-life phase of the study as well as organ weight data. The use of the word "significant" or "significantly" indicates a statistically significant difference between the control and the experimental groups. Significance was judged at a probability value of $p<0.05$.

Mean and standard deviations were calculated for all quantitative data (e.g., weekly body weights, daily bodyweight gains, daily food consumption, food efficiency, daily dietary intake, organ weights, organ-to-body weight ratios, and estrous cycles). If warranted by sufficient group

[1] Anterior-most portion of the vagina.

sizes, data within groups were evaluated for homogeneity of variances and normality by Bartlett's test (Bartlett, 1937). Where Bartlett's test indicated homogeneous variances, treated and control groups were compared using a one-way analysis of variance (ANOVA). When one-way analysis of variance was significant, a comparison of the treated groups to control by Dunnett's test (Dunnett, 1964, 1980) for multiple comparisons was performed. Where variances were considered significantly different by Bartlett's test, groups were compared using a non-parametric method (Kruskal-Wallis non-parametric analysis of variance; Kruskal and Wallis, 1952). When non-parametric analysis of variance was significant, comparison of treated groups to control was performed using Dunn's test (Dunn, 1964). Statistical analysis was performed on all quantitative data for in-life and organ weight parameters using Provantis® version 9, Tables and Statistics, Instem LSS, Staffordshire UK.

8. STUDY CONDUCT

A. Testing Facility

In-life	Product Safety Labs 2394 US Highway 130 Dayton, NJ 08810
Test substance and dietary analysis	Impossible Foods Inc. 525 Chesapeake Dr. Redwood City, CA 94063 P.I.: Rachel Fraser, PhD
Histological slide preparation	Histoserv, Inc. 19526 Amaranth Drive, Germantown, MD 20874 P.I.: Pratiba Vohra
Clinical pathology and histopathology evaluation	Regan Path/Tox Services 1457 Township Rd. 853 Ashland, OH 44805 P.I.: Karen Regan, DVM, DACVP, DABT

B. GLP Compliance

This study was not performed in full compliance with GLP standards, but was conducted in a GLP-compliant facility.

C. Test Procedure Guidelines

This study design conformed to the following guidelines:

- OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 407): Health Effects, *Repeated Dose 28-Day Oral Toxicity Study in Rodents* (2008).
- US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 4. a. *Subchronic Toxicity Studies with Rodents* (2007).

9. FINAL REPORT AND RECORDS TO BE MAINTAINED

The original, signed report and paper raw data will be sent to the Sponsor. A copy of this signed report, together with a copy of the protocol and all raw data generated at PSL, will be maintained in the Product Safety Labs archives.

The following records are maintained:

A. Information on test substance includes the following:

Storage Disposition
Usage

B. Information on animals includes the following:

Receipt, date of birth	Food consumption
Initial health assessment	Individual necropsy records
Dosing	Histopathology data
Body weights	Selected organ weights
Cytology data	

C. All other records that would demonstrate adherence to the protocol.

Prepared slides and pathology data is maintained by Product Safety Labs. Test substance and dietary analysis data are maintained by Impossible Foods Inc. 525 Chesapeake Dr. Redwood City, CA 94063.

Any electronic raw data generated by the Test Site was maintained in accordance to the Test Site SOPs.

10. PROTOCOL AND PROTOCOL AMENDMENT

See Appendix A for the Protocol and Protocol Amendment.

11. RESULTS

A. Test Substance and Diet Analysis (Tables 1A-B, Appendix C)

The test substance was considered to be homogenously distributed in the diet preparations at all study concentrations. Animals were considered to have received target dietary concentrations of Soy Leghemoglobin Preparation.

11.A.1 Analysis of Soy Leghemoglobin in the Neat Test Substance

Neat test substance samples from the initial diet preparation (Study Day 14) were analyzed for the active ingredient Soy Leghemoglobin. The result of the analysis of neat Soy Leghemoglobin Preparation was 102.7% (Table 1A, Appendix C).

11.A.2 Homogeneity

Homogeneity analysis of the initial diet preparation (Study Day 14) resulted in a relative standard deviation (RSD) of 3.22, 2.27, and 0.60% for Groups 2-4, respectively. Average percent of target concentrations in the top, middle, and bottom samples were 93.80, 93.89, and 93.91% of the diet preparations for target concentrations of 512, 1024, and 1536 mg/kg/day Soy Leghemoglobin Preparation, which correspond to 250, 500, and 750 mg/kg/day of active ingredient (Soy Leghemoglobin) for Groups 2-4, respectively (Table 1B, Appendix C). The test substance was considered to be homogenously distributed in the diet preparations at all study concentrations. Animals were considered to have received target dietary concentrations of Soy Leghemoglobin Preparation

B. Mortality and Clinical Observations (Tables 2 and 3, Appendix D-F)

There were no mortalities during the course of the study. There were no clinical observations attributable to the administration of Soy Leghemoglobin Preparation.

The fate of all animals is presented in Appendix M.

Incidental in-life clinical observations included slight to moderate alopecia of the abdomen, back, and left/right flank/forepaw in 4/15 Group 1, 3/15 Group 2, 3/15 Group 3, and 1/15 Group 4 animals and a broken upper right incisor in 1/15 Group 2 animals.

Corresponding findings during detailed clinical observations included hair loss in 3/15 Group 1, 3/15 Group 2, 2/15 Group 3, and 1/15 Group 4 animals.

C. Estrous cycles (Table 4; Appendices G)

Mean estrous cycles for female rats in Groups 2-4 were comparable to control Group 1 values throughout the study.

Mean number of estrous cycles for Groups 1-4 females were 1.9, 2.4, 2.3, and 2.1, respectively, prior to test substance adminstration (Days 0-13) and 2.3, 1.9, 2.1, and 2.1, respectively, during test substance administration (Days 29-42).

D. Body Weight and Body Weight Gain (Tables 5 and 6; Appendices H and I)

There were no changes in body weight and body weight gain attributable to Soy Leghemoglobin Preparation administration.

Mean weekly body weights and daily body weight gain for female rats in Groups 2-4 were comparable to control Group 1 throughout the study, with the exception of an incidental significant increase ($p<0.05$) in mean daily body weight gain for Group 2 animals on Days 21-28.

E. Food Consumption, Food Efficiency, and Dietary Intake of Soy Leghemoglobin Preparation (Tables 7-9; Appendices J-L)

There were no changes in food consumption and food efficiency attributable to Soy Leghemoglobin Preparation administration.

Mean daily food consumption and food efficiency for female rats in Groups 2-4 were comparable to the control Group 1 throughout the study, with the exception of an incidental significant increase ($p<0.05$) in mean food efficiency for Group 2 animals on Days 21-28.

Mean overall active ingredient dietary intake was calculated based on body weight and food consumption measurements collected throughout the study. For Soy Leghemoglobin Preparation target doses of 512, 1024, and 1536 mg/kg/day that correspond to active ingredient concentrations of 250, 500, and 750 mg/kg/day, the calculated nominal dietary intake levels (Days 14-42) were 513.0, 1016.5, and 1512.5 mg/kg/day for the female rats, which correspond to active ingredient concentrations of 250, 496, and 738 mg/kg/day.

F. Sacrifice, Macroscopic Observations, and Histopathology (Tables 10-13; Appendix N-R)

There were no macroscopic and microscopic observations or organ weight changes attributed to the Soy Leghemoglobin Preparation administration.

11.F.1 Macroscopic

There were no test substance-related macroscopic observations.

The only macroscopic observations were urinary bladder thickened (size recorded as 13 x 5 x 8 mm) and urolith present for 4F 7047, and uterus fluid-filled for animals 1F 7003, 1F 7013, 2F 7026, 3F 7040, 4F 7053, and 4F 7060. Per protocol, the urinary bladder was not saved for microscopic examination. Fluid-filled uterus correlated with the proestrus

stage of the estrous cycle as determined from the microscopic examination; dilated/fluid-filled uterus is a normal physiologic change at to proestrus stage of the estrous cycle.

11.F.2 Microscopic

11.F.2.1 Blind Evaluation

Estrous cyclicity was comparable across all test groups.

11.F.2.2 Unblinded Evaluation

There were no test substance-related changes in microscopic observations.

11.F.3 Organ Weights and Ratios

There were no test substance-related organ weights and organ-to-body weight ratio findings.

Mean absolute and relative organ-to-body weights for female rats in Groups 2-4 were comparable to control Group 1 throughout the study.

12. CONCLUSION

Under the conditions of this study and based on the toxicological endpoints evaluated, administration of Soy Leghemoglobin Preparation at dose levels up to 1536 mg/kg/day or 750 mg/kg/day in active ingredient (Soy Leghemoglobin) did not cause an effect in the estrous cycle of female Sprague Dawley rats.

13. REFERENCES

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TABLE 1A: CHEMICAL ANALYSIS RESULTS

Result for Neat Test Substance Sample

Sample Name	Sampling Day[1]	Measured Recovery (%)
NT-1 A	Day 0	102.7%

[1] Day relative to initial dietary preparation (Study Day 14).

TABLE 1B: CHEMICAL ANALYSIS RESULTS

Results for Homogeneity of Dietary Preparation

Day[1]	Group	Sample Location	Target Concentration (ppm)[2]	Measured Concentration (ppm)	% of Target[3]	Average % of Target	RSD (%)
0	1	Middle	0	ND	NA	NA	NA
	2	Top	5863	5545	94.57%	93.80%	3.22%
		Middle		5649	96.36%		
		Bottom		5304	90.46%		
	3	Top	11354	10633	93.65%	93.89%	2.27%
		Middle		10433	91.89%		
		Bottom		10915	96.13%		
	4	Top	16936	16008	94.52%	93.91%	0.60%
		Middle		15882	93.78%		
		Bottom		15822	93.42%		

NA = Not Applicable; ND = Not Detected

[1] Day relative to initial dietary preparation (Study Day 14).

[2] Concentrations calculated using Study Day 14 group animal body weights, food consumption, and target dose levels.

[3] % of Target = Measured Conc. (ppm) / Target Conc. (ppm) x 100.

TABLE 2: SUMMARY OF IN-LIFE CLINICAL OBSERVATIONS

Day numbers relative to Start Date

Sex: Female

	0 mg/kg/day	512 mg/kg/day	1024 mg/kg/day	1536 mg/kg/day
Alopecia				
Number of Observations	75	56	15	19
Number of Animals	4	3	3	1
Days from - to	6 43	0 43	5 43	21 39
Broken Tooth				
Number of Observations	.	8	.	.
Number of Animals	.	1	.	.
Days from - to	.	35 42	.	.

TABLE 3: SUMMARY OF DETAILED CLINICAL OBSERVATIONS

SUMMARY OF DETAILED CLINICAL OBSERVATIONS

Days 14, 21, 28, 35, and 42

Group	1	2	3	4
Dose Level (mg/kg/day)	0	512	1024	1536
Number of Animals in Group	6	6	6	6
Observations During Removal From Cage And Handling	Score[1]			
Handling Reactivity	0	0	0	0
Vocalization	0	0	0	0
Palpebral Closure	0	0	0	0
Lacrimation	0	0	0	0
Eyes	0	0	0	0
Mucous Membranes	0	0	0	0
Salivation	0	0	0	0
Emaciation	0	0	0	0
Piloerection	0	0	0	0
Fur/Skin	3(3)	3(3)	2(3)	1(3)
Muscle Tone	0	0	0	0
Respiratory Pattern	0	0	0	0
Open Field Observations				
Activity/Arousal	0	0	0	0
Convulsions	0	0	0	0
Tremors	0	0	0	0
Posture	0	0	0	0
Gait	0	0	0	0
Locomotion	0	0	0	0
Vocalizations	0	0	0	0
Defecation	0	0	0	0
Urination	0	0	0	0
Unusual Behaviors	0	0	0	0
Pupillary Response				
Pupillary Reflex	0	0	0	0

[1] An entry of 0 indicates that all animals in the group appeared normal when evaluated for the specified observation, or that all animals did not exhibit the specific clinical sign. An entry greater than 0 indicates the number of animals in the group that exhibited the specific clinical sign. A number in the parenthesis (if present) represents the score given for the observed clinical sign.

TABLE 4: SUMMARY OF ESTROUS CYCLES

Number of Cycles

Sex: Female Day(s) Relative to Start Date		0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
0 → 13	Mean	1.9	2.4	2.3	2.1
	SD	0.5	0.6	0.6	0.5
	N	15	15	15	15

Statistical Test: Generalised Anova/Ancova Test Transformation: Identity (No Transformation)

Number of Cycles

Sex: Female Day(s) Relative to Start Date		0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
29 → 42	Mean	2.3	1.9	2.1	2.1
	SD	0.6	0.5	0.3	0.4
	N	15	15	15	15

Statistical Test: Generalised Anova/Ancova Test Transformation: Identity (No Transformation)

TABLE 5: SUMMARY OF MEAN WEEKLY BODY WEIGHTS

Bodyweight (g)

Sex: Female Day(s) Relative to Start Date		0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
0	Mean	179.7 R[1]	180.1	179.5	180.7
	SD	13.5	13.0	14.0	13.8
	N	15	15	15	15
7	Mean	198.9 I[2]	198.5	200.2	201.0
	SD	14.0	13.8	14.7	14.9
	N	15	15	15	15
14	Mean	213.7 I[2]	212.7	212.9	215.6
	SD	15.3	14.5	17.1	18.4
	N	15	15	15	15
21	Mean	228.3 I[2]	224.5	223.2	230.1
	SD	17.8	18.3	19.5	22.2
	N	15	15	15	15
28	Mean	235.7 I[2]	237.1	233.7	239.0
	SD	19.5	20.4	21.6	27.6
	N	15	15	15	15
35	Mean	240.7 I[2]	243.3	242.5	247.7
	SD	21.2	21.0	23.0	27.9
	N	15	15	15	15
42	Mean	249.9 I[2]	251.7	252.5	257.3
	SD	22.4	22.7	22.4	28.6
	N	15	15	15	15
43	Mean	249.9 I[2]	253.1	253.3	259.0
	SD	21.8	23.1	22.9	29.6
	N	15	15	15	15

Statistical Test: Generalised Anova/Ancova Test Transformation: Automatic

1 [R - Automatic Transformation: Rank]
2 [I - Automatic Transformation: Identity (No Transformation)]

TABLE 6: SUMMARY OF MEAN DAILY BODY WEIGHT GAIN

Mean Daily Body Weight Gain (g/day)

Sex: Female Day(s) Relative to Start Date		0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
0 → 7	Mean	2.75 [1]	2.63	2.96	2.90
	SD	1.02	0.59	1.07	1.07
	N	15	15	15	15
7 → 14	Mean	2.10 [1]	2.03	1.81	2.09
	SD	0.77	0.69	0.89	0.88
	N	15	15	15	15
14 → 21	Mean	2.10 [1]	1.68	1.48	2.08
	SD	1.05	1.08	0.99	0.83
	N	15	15	15	15
21 → 28	Mean	1.05 [R2]	1.81 [d3]	1.50	1.27
	SD	0.61	0.56	1.04	1.09
	N	15	15	15	15
28 → 35	Mean	0.72 [R2]	0.88	1.27	1.24
	SD	0.68	0.51	0.62	1.10
	N	15	15	15	15
35 → 42	Mean	1.31 [1]	1.21	1.42	1.38
	SD	0.69	0.85	0.82	0.69
	N	15	15	15	15
0 → 42	Mean	1.67 [R2]	1.70	1.74	1.82
	SD	0.38	0.34	0.41	0.48
	N	15	15	15	15

Statistical Test: Generalised Anova/Ancova Test Transformation: Automatic

1 [I - Automatic Transformation: Identity (No Transformation)]
2 [R - Automatic Transformation: Rank]
3 [d - Test: Dunn 2 Sided p < 0.05]

TABLE 7: SUMMARY OF MEAN DAILY FOOD CONSUMPTION

Mean Daily Food Consumption (g/day)

Sex: Female Day(s) Relative to Start Date		0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
3 → 7	Mean	18.23 [R1]	18.75	18.93	18.55
	SD	3.93	2.03	2.07	2.06
	N	15	15	15	15
7 → 10	Mean	19.38 [L2]	17.82	18.20	19.13
	SD	3.40	2.00	2.09	2.38
	N	15	15	15	15
10 → 14	Mean	19.28 [L2]	19.18	19.97	19.93
	SD	2.25	1.50	3.30	2.68
	N	15	15	15	15
14 → 17	Mean	20.31 [I3]	19.36	19.20	19.11
	SD	2.55	2.64	2.79	2.15
	N	15	15	15	15
17 → 21	Mean	20.52 [I3]	20.72	19.80	20.37
	SD	1.44	2.48	2.28	2.60
	N	15	15	15	15
21 → 24	Mean	19.11 [I3]	19.80	18.60	18.20
	SD	1.97	1.75	2.45	2.86
	N	15	15	15	15
24 → 28	Mean	20.33 [I3]	20.07	20.17	20.13
	SD	2.44	2.18	2.80	3.09
	N	15	15	15	15
28 → 31	Mean	19.11 [I3]	19.58	20.13	19.56
	SD	2.38	1.73	2.14	2.54
	N	15	15	15	15
31 → 35	Mean	19.47 [L2]	19.05	19.23	19.02
	SD	3.48	1.97	2.42	2.63
	N	15	15	15	15
28 → 35	Mean	19.31 [L2]	19.28	19.62	19.25
	SD	2.88	1.81	2.18	2.53
	N	15	15	15	15
35 → 38	Mean	20.16 [I3]	20.29	20.27	20.13
	SD	2.27	2.43	2.35	2.61
	N	15	15	15	15
3 → 38	Mean	19.59 [I3]	19.47	19.47	19.44
	SD	1.73	1.76	2.16	2.17
	N	15	15	15	15

Statistical Test: Generalised Anova/Ancova Test Transformation: Automatic

1 [R - Automatic Transformation: Rank]
2 [L - Automatic Transformation: Log]
3 [I - Automatic Transformation: Identity (No Transformation)]

TABLE 8: SUMMARY OF MEAN FOOD EFFICIENCY[1]

[1] Food efficiency = Mean Daily Body Weight Gain / Mean Daily Food Consumption

Food Efficiency

Sex: Female Day(s) Relative to Start Date		0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
0 → 7	Mean	0.149 [1]	0.142	0.157	0.157
	SD	0.053	0.026	0.052	0.050
	N	15	15	15	15
7 → 14	Mean	0.110 [1]	0.108	0.092	0.106
	SD	0.041	0.032	0.044	0.040
	N	15	15	15	15
14 → 21	Mean	0.100 [1]	0.081	0.074	0.103
	SD	0.042	0.046	0.045	0.035
	N	15	15	15	15
21 → 28	Mean	0.052 [R2]	0.091 [d3]	0.074	0.062
	SD	0.029	0.027	0.049	0.051
	N	15	15	15	15
28 → 35	Mean	0.037 [1]	0.046	0.064	0.062
	SD	0.035	0.026	0.029	0.050
	N	15	15	15	15
35 → 42	Mean	0.065 [1]	0.059	0.070	0.069
	SD	0.031	0.040	0.042	0.032
	N	15	15	15	15
0 → 42	Mean	0.085 [R2]	0.087	0.089	0.093
	SD	0.017	0.012	0.016	0.017
	N	15	15	15	15

Statistical Test: Generalised Anova/Ancova Test Transformation: Automatic

1 [I - Automatic Transformation: Identity (No Transformation)]
2 [R - Automatic Transformation: Rank]
3 [d - Test: Dunn 2 Sided p < 0.05]

TABLE 9: SUMMARY OF MEAN DAILY DIETARY INTAKE OF SOY LEGHEMOGLOBIN PREPARATION

Dietary Intake Variable (mg/kg/day)

Sex: Female Day(s) Relative to Start Date		0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
14 → 17	Mean	0.0	535.0	1023.3	1502.3
	SD	0.0	76.8	121.0	131.2
	N	15	15	15	15
17 → 21	Mean	0.0	528.0	1036.3	1574.2
	SD	0.0	43.0	76.0	96.8
	N	15	15	15	15
21 → 24	Mean	0.0	505.5	974.7	1406.2
	SD	0.0	33.3	108.4	153.1
	N	15	15	15	15
24 → 28	Mean	0.0	515.0	1059.3	1603.1
	SD	0.0	38.2	100.6	181.1
	N	15	15	15	15
28 → 35	Mean	0.0	503.5	1028.6	1536.3
	SD	0.0	29.2	60.9	118.8
	N	15	15	15	15
35 → 38	Mean	0.0	540.1	1062.1	1609.2
	SD	0.0	64.6	99.0	109.5
	N	15	15	15	15
14 → 42	Mean	0.0	513.0	1016.5	1512.5
	SD	0.0	31.5	60.9	77.7
	N	15	15	15	15

TABLE 10: SUMMARY OF NECROPSY OBSERVATIONS

Removal Reason: ALL	Female			
	0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
Number of Animals	15	15	15	15
Number of Completed Animals	15	15	15	15
urinary bladder				
thick				1
urolith				1
uterus				
Submitted	15	15	15	15
fluid filled	2	1	1	2

TABLE 11: SUMMARY OF MEAN TERMINAL BODY AND ORGAN WEIGHTS

Sex: Female Day(s) Relative to Start Date			0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
Terminal BW (g)		Mean	249.9	253.1	253.3	259.0
		SD	21.8	23.1	22.9	29.6
		N	15	15	15	15
Ovaries with Oviducts Wt (g)		Mean	0.1311 [1]	0.1343	0.1234	0.1370
		SD	0.0174	0.0209	0.0128	0.0156
		N	15	15	15	15
Uterus Wt (g)		Mean	0.604 [R2]	0.547	0.570	0.703
		SD	0.221	0.102	0.162	0.223
		N	15	15	15	15

1 [I - Automatic Transformation: Identity (No Transformation)]
2 [R - Automatic Transformation: Rank]

TABLE 12: SUMMARY OF MEAN ORGAN-TO-BODY WEIGHT RATIOS[1]

[1] [organ weight/body weight] x 1000

Sex: Female Day(s) Relative to Start Date			0 mg/kg/day Group 1	512 mg/kg/day Group 2	1024 mg/kg/day Group 3	1536 mg/kg/day Group 4
Ovaries with oviducts/TBW (Ratio)		Mean	0.5270 [1]	0.5325	0.4886	0.5332
		SD	0.0733	0.0772	0.0467	0.0667
		N	15	15	15	15
Uterus /TBW (Ratio)		Mean	2.412 [R2]	2.166	2.276	2.745
		SD	0.841	0.390	0.731	0.957
		N	15	15	15	15

1 [I - Automatic Transformation: Identity (No Transformation)]
2 [R - Automatic Transformation: Rank]

TABLE 13: SUMMARY OF BLIND HISTOPATHOLOGY ESTROUS CYCLE EVALUATION

SUMMARY OF ESTROUS CYCLES

Blind Histopathological Determination (Day 43)

Stage	Number of Animals per Stage of Estrous Cycle			
	Group 1 0 mg/kg/day	Group 2 512 mg/kg/day	Group 3 1024 mg/kg/day	Group 4 1536 mg/kg/day
Proestrus	3/15	1/15	2/15	5/15
Estrus	3/15	2/15[1]	3/15	3/15
Metestrus	6/15	2/15	1/15	2/15
Diestrus	3/15	10/15	9/15	5/15

[1] Animal 7027 appeared to have a prolonged estrus (PE) based on morphology of the ovaries (large atretic follicles, multiple CLs at a similar age/ stage of atresia) and the presence of squamous epithelial metaplasia in the uterus.

APPENDIX A: PROTOCOL AND PROTOCOL AMMENDMENTS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Product Safety Labs

28-Day Dietary Toxicity Study
Protocol #: P703.02 IMP
PSL ID: 160720-5R
Study No: 44856

SOY LEGHEMOGLOBIN PREPARATION: AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY PRE-DOSING ESTRUS CYCLE DETERMINATION

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

PSL PROTOCOL NO.

P703.02 IMP

PERFORMING LABORATORY

Product Safety Labs
2394 US Highway 130
Dayton, New Jersey 08810

PSL STUDY NUMBER

44856

STUDY DIRECTOR

Jayson Chen, PhD

SPONSOR

Impossible Foods Inc.
525 Chesapeake Dr.
Redwood City, CA 94063

Product Safety Labs

28-Day Dietary Toxicity Study
Protocol #: P703.02 IMP
PSL ID: 160720-5R
Study No: 44856

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Product Safety Labs

28-Day Dietary Toxicity Study
Protocol #: P703.02 IMP
PSL ID: 160720-5R
Study No: 44856

1. TITLE OF STUDY: SOY LEGHEMOGLOBIN PREPARATION: AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY PRE-DOSING ESTRUS CYCLE DETERMINATION

2. OBJECTIVE

The objective of this study is to evaluate the potential reproductive toxicity (estrus cycle and reproductive organ histopathology) of Soy Leghemoglobin Preparation in female rats continuously exposed to the test substance in the diet for at least 28 days. Additionally, estrus cycle evaluation with also be performed for 14 days prior to dosing.

3. STUDY DIRECTOR

Jayson Chen, PhD
Study Director
Tel: 732-438-5100 x1582
Email: JaysonChen@ProductSafetyLabs.com

4. NAME AND ADDRESS OF THE TESTING FACILITY

Product Safety Labs (PSL)
2394 US Highway 130
Dayton, NJ 08810
Tel: 732-438-5100

5. SPONSOR

Impossible Foods Inc.
525 Chesapeake Dr.
Redwood City, CA 94063

6. SPONSOR REPRESENTATIVE

Rachel Fraser, PhD
Impossible Foods Inc.
525 Chesapeake Dr.
Redwood City, CA 94063
Email: rachel.fraser@impossiblefoods.com

7. DATES

Proposed In-Life Start Date: February 8, 2017

Proposed Experimental Termination Date: March 23, 2017

8. TEST SUBSTANCE

8.A Source

The test substance will be provided by the Sponsor.

8.B Identification

The test substance will be identified using the following information provided by the Sponsor and Product Safety Labs (PSL) identification number.

Test Substance: Soy Leghemoglobin Preparation
PSL ID: 160720-5R
Lot #: PP-PGM2-16-088-301
Physical Description: Red/brown powder
Composition: Soy Leghemoglobin 48.82%
Storage Conditions: Frozen
Expiration Date: Not Applicable

Documentation of the methods of synthesis, fabrication, or derivation of the test substance is retained by the Sponsor.

8.C Analysis

The test substance, as received, is expected to be stable for the duration of the study. The Sponsor will be responsible for all analytical work required to characterize the neat test substance and validate its stability. Stability of the test substance in the dietary matrix and that of the concentration of the test substance in the test diets was determined to be stable over 10 days in a previous toxicity study[1].

8.D Hazards

Appropriate routine safety precautions will be exercised in the handling of the test substance unless otherwise indicated by the Sponsor.

9. GENERAL TEST SYSTEM PARAMETERS

9.A Animal Requirements

9.A.1 Number of Animals: 60

9.A.2 Number of Groups: 4 (3 dose levels + 1 control group)

9.A.3 Number of Animals per Group: 15

9.A.4 Sex: Female; females will be nulliparous and non-pregnant.

9.A.5 Species/Strain: CRL Sprague-Dawley CD® IGS rats

9.A.6 Age/Weight: Seven to eight weeks at initiation; the weight variation will not exceed ± 20% of the mean weight for each sex.

9.A.7 Supplier: Charles River Laboratories, Inc. Rats will be shipped in filtered cartons by airfreight and/or truck.

9.B Test System Justification

The Sprague-Dawley® rat is the system of choice because, historically, it has been a preferred and commonly used species for dietary toxicity tests. The current state of scientific knowledge does not provide acceptable alternatives to the use of live animals to accomplish the objective of this study.

[1] Product Safety Labs (2016) Soy Leghemoglobin Preparation: a 28-day Dietary Study in Rats. PSL Study #43160 *(Report in preparation)*

9.C Husbandry

9.C.1 Housing

The animals will be individually housed in suspended stainless steel cages which conform to the size recommendations in the latest *Guide for the Care and Use of Laboratory Animals*[1]. Litter paper placed beneath the cage will be changed at least three times/week. The animal room will have a 12-hour light/dark cycle and will be kept clean and vermin free. Environmental controls are set to maintain temperature and relative humidity ranges of 21 ± 2°C and 30-70%, respectively. Observed ranges will be documented in the raw data.

9.C.2 Acclimation

The animals will be conditioned to the housing facilities for at least five days prior to testing. Body weights and clinical observations will be recorded at least two times prior to study start.

9.C.3 Feed

2016 Certified Envigo Teklad Global Rodent Diet® (Envigo Teklad, Inc.) will be stored in a dedicated temperature and humidity monitored feed storage site and available *ad libitum* during acclimation and study Days 0-13. Test diets will be prepared as described in Section 11.B using 2016 Certified Envigo Teklad Global Rodent Diet® and will be available *ad libitum* during at least study Days 14-42.

9.C.4 Water

Filtered tap water will be available *ad libitum* from individual bottles attached to the cages or from an automatic watering access system. Water analysis is conducted by Precision Analytical Services, Inc., Toms River, NJ and South Brunswick Municipal Water Supply, South Brunswick, NJ.

9.C.5 Contaminants

There are no known contaminants reasonably expected to be found in the food or water that would interfere with the results of this study. Routine analysis consisting of each lot of feed used in this study will be received from Envigo Teklad, Madison, WI. Water analysis is conducted periodically and the records are kept on file at Product Safety Labs. The date(s) of the most recent analyses will be reported in the final report.

9.D Identification

9.D.1 Cage

Each cage will be identified by a cage card indicating at least the study number, dose level, group assignment, individual animal identification, and sex of the animal.

9.D.2 Animal

Each animal will be given a sequential number in addition to being uniquely identified with a Monel® self-piercing stainless steel ear tag.

[1] National Research Council. (2011). *Guide for the Care and Use of Laboratory Animals* (8th ed.). Washington, DC: The National Academies Press.

10. EXPERIMENTAL DESIGN

10.A Route of Administration

The test substance will be administered in the diet.

10.B Justification of Route of Administration

The dietary route of administration was selected by the Sponsor. This route of administration is recommended in the referenced guidelines (Section 14.C) and a potential route of human exposure.

10.C Control of Bias

Animals will be randomly assigned to test groups according to PSL SOP #714.

10.D Dose Levels

Fifteen female rats will be randomly assigned to each of the following test groups:

Group	No. Animals/ Group (F)	Target Exposure of Active Ingredient (mg/kg/day)	Target Dietary Dose Level of Test Substance (mg/kg/day)*
1	15	Basal Diet Control 0	0
2	15	Low Dose 250	512
3	15	Intermediate Dose 500	1024
4	15	High Dose 750	1536

* Based on 48.82% active ingredient (AI, Soy Leghemoglobin) of Soy Leghemoglobin Preparation (Lot # PP-PGM2-16-068-301).

10.E Justification of Dose Level Selection

The Sponsor, in consultation with the Study Director and based on a 28-day dietary toxicity study[1], selected target dietary dose levels of 512, 1024 and 1536 mg/kg/day that correspond to target dose levels of 250, 500 and 750 mg/kg/day of the active ingredient, Soy Leghemoglobin. To maintain target dietary dose levels throughout the study, concentrations in the test diets will be calculated based on the most recent group body weight and food consumption data. Diets for females at each dietary dose level will be made separately each week.

11. GENERAL PROCEDURES

11.A Selection of Animals

Sixty (60) healthy female rats will be used on test. Animals will be selected for this study on the basis of adequate body weight gain, absence of clinical signs of disease or injury, and a body weight within ±20% of the mean. Selected rats will be distributed by randomization according to stratification by body weight so that there will be no statistically significant difference among group body weight means.

11.B Dose Preparations and Procedures

11.B.1 Diet Preparations (PSL SOP #605)

The test substance will be processed, as needed, to decrease particle size using a grinder and then added to 2016 Certified Envigo Teklad Global Rodent Diet® and thoroughly mixed in a high-speed mixer. Control diet (Basal Diet) will be mixed under the same conditions as the diets prepared with the test substance. All diets will be kept frozen following preparation, unless presented to the test animals on the same day as diet preparation. All diets will be prepared approximately weekly or more frequently, as needed.

11.B.2 Diet Presentation

The control diet will presented to all animals on Days 0-13 of the study. On study Day 14, the control and test diets will be presented to their respective groups. The diets will be replaced concurrently with food consumption measurements on Days 17, 21, 24, 28, 31, 35 and 38. Additional diet may be provided as needed throughout the study to ensure *ad libitum* feeding. Animals will be exposed to the control or test diets for at least 28 days.

11.B.3 Sampling (PSL SOP #607)

The neat test substance and selected prepared diets (at each concentration), will be sampled in duplicate. Samples will be frozen until analyzed and/or may be discarded upon completion of the study.

11.B.4 Stability of Test Substance

The neat test substance was previously determined to be stable under normal laboratory conditions for the duration of a 28-day study[3]. At the initial diet preparation, a sample of the test substance (neat) will be retained.

11.B.5 Stability in Dietary Matrix

The test substance in the dietary matrix was previously determined to be stable over 10 days in a previous toxicity study[3]. Stability of the test substance in the dietary matrix will not be assessed in this study.

11.B.6 Homogeneity

Samples to evaluate homogeneity of the test substance distribution will be collected from the initial diet preparation. Samples will be taken from approximately the top, middle and bottom of the diet mixer. Basal diet control samples will be collected from the middle of the mixer only. Chemical analysis will verify the diets as homogeneous and of accurate concentration throughout the study.

11.B.7 Concentration Verification

Samples for concentration verification will be collected as part of the homogeneity analysis during the first week of the study. Diet preparation calculation will be verified and test diets will be mixed according to PSL's standard operating procedure. Nominal diet concentrations will be used to determine the total intake of the test substance for each group.

11.B.8 Sample Preservation

Upon sampling, diet preparations and neat test substance will be stored frozen. Samples will be considered stable from the point at which they are frozen.

11.B.9 Sample Analysis

A single duplicate of the frozen diet samples described above will be sent to Impossible Foods for analysis of diet preparation and neat test substance samples. A signed, analytical report will be provided to the Study Director. This report will include the methodology, pertinent measurements, study results, and tabulated results. All raw data will be retained by Impossible Foods. Any remaining sample material will be retained at Product Safety Labs until issuance of the final report.

11.C Analytical Chemistry

11.C.1 Sample Storage

Upon receipt, all samples will be stored and maintained frozen prior to analysis.

11.C.2 Method Validation

Prior to sample analysis, the suitability of the method will be demonstrated. Method validation will include, but is not limited to determination of linearity, precision and accuracy.

11.C.3 Reference Substance

An aliquot of the test substance will serve as the reference standard.

11.C.4 Chemical Analysis

Analytical test methodology will be validated by Impossible Foods personnel. Samples will be analyzed in replicate. A detailed description of the analytical test method(s) will be documented. Any remaining sample material will be retained until the issuance of the final report.

11.C.5 Data Reporting

Data will be captured on standard raw data sheets and as instrument output, as necessary, and summarized in tabular form.

11.C.6 Analytical Report and Records to be Maintained

A signed, analytical report will be provided to the Study Director. This report will include the methodology, pertinent measurements, study results, and tabulated results. All raw data will be maintained by Impossible Foods. The analytical report will be incorporated into the main study report.

11.D Clinical Observations

All animals will be observed at least twice daily for viability. Cage-side observations of all animals will be performed daily during the study. All findings will be recorded.

On Day 14 and approximately weekly thereafter, a detailed observation will be conducted (PSL SOP #726) while handling the animal, generally on days that the animals are weighed and food consumption measurements are taken. Potential signs noted should include, but not be limited to: changes in skin, fur, eyes, and mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). Likewise, changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g., excessive grooming, repetitive circling), or bizarre behavior (e.g., self-mutilation, walking backwards) should also be recorded. The date and clock time of all observations and/or mortality checks will be recorded.

The Study Director will be promptly notified of severe/remarkable clinical observations, will be advised when an animal is found in a moribund condition, and may authorize euthanasia and necropsy as necessary to avoid the loss of quality data. All such authorizations will be recorded in the raw data.

11.E Estrus Cycles

Estrus in the female rats will be determined daily on Days 0-13 and Days 29-42 of the study, by vaginal lavage to evaluate for regular cyclicity. A vaginal lavage was also performed at termination to determine the stage of estrus at sacrifice. Cytological evaluation will be performed without knowledge of treatment group assignment. At the end of the pre-dosing estrus evaluation, the Study Director will evaluate estrus cycle data and will exclude any study animal considered to exhibit atypical estrus cycling. If necessary, animals may be reassigned to ensure balanced number across groups.

11.F Body Weight and Body Weight Gain

Individual body weights will be recorded at least two times during acclimation. Test animals will be weighed on Day 0 (prior to study start) and approximately weekly thereafter (intervals of 7 days ± 1). The animals will also be weighed prior to sacrifice. Decedents need not be weighed. Body weight gain will be calculated for selected intervals and for the study overall.

11.G Food Consumption, Food Efficiency, and Dietary Intake of Soy Leghemoglobin Preparation

Individual food consumption will be measured and recorded on Days 3, 7, 10, 14, 17, 21, 24, 28, 31, 35 and 38 and at the end of the study. Food efficiency and dietary intake of the test substance (mg/kg/day) will also be calculated and reported.

11.H Terminal Sacrifice and Histopathology

11.H.1 Scheduled Sacrifice

At terminal sacrifice, all survivors will be euthanized by exsanguination under isoflurane anesthesia.

Plasma samples will be collected from all animals, at their respective sacrifice, and stored frozen (approximately -80°C) for future possible analysis. If performed, the description of analysis will be added by amendment.

All animals in the study (excluding decedents) will be subjected to a necropsy, which will include examination of the external surface of the body, all orifices, musculoskeletal system, and the thoracic, abdominal and cranial cavities and their contents. The following tissues (of all animals sacrificed by design) will be weighed wet as soon as possible after dissection to avoid drying:

uterus	ovaries with oviducts (combined)	

The following organs and tissues from all animals will be preserved in 10% neutral buffered formalin for possible future histopathological examination:

vagina	uterus	cervix
ovaries	oviducts	

Additional tissues may be preserved if indicated by signs of toxicity or target organ involvement at the discretion of the Study Director. Tissues may be discarded upon finalization of the study with approval from the Sponsor.

11.H.2 Unscheduled Sacrifice

Any rat that dies or is sacrificed because of a moribund condition will be examined for the cause of death or moribund condition on the day the observation is made. The animal(s) will be evaluated for gross lesions. Organs and tissues will be excised, weighed (except for animals found dead), and preserved as described for those animals sacrificed by design.

11.H.3 Histopathology

Histological examination will be performed on the preserved organs and tissues of animals from both the control and high dose groups (Groups 1 and 4, respectively) and from any animal that dies during the course of the study. These examinations may be extended to other tissues and organs including tissues from the low and intermediate groups at the request of Pathologist, in consultation with the Study Director and Sponsor, to further investigate changes observed in the high dose group. The fixed tissues will be trimmed, processed, embedded in paraffin, sectioned with a microtome, placed on glass microscope slides, stained with hematoxylin and eosin (HE), and examined by light microscopy. Additional special stains can be added based on HE evaluation at the discretion of the study pathologist, in consultation with the Study Director and Sponsor. Slide preparation and histological assessment, by a board-certified veterinary pathologist, will be performed at Histo-Scientific Research Laboratories (HSRL). Prior to data recording, initial histopathological evaluation will be performed without knowledge of treatment group assignment.

12. STATISTICAL ANALYSIS

Product Safety Labs will perform statistical analysis of all data collected during the in-life phase of the study as well as organ weight data, if applicable. The use of the word "significant" or "significantly" indicates a statistically significant difference between the control and the experimental groups. Significance will be judged at a probability value of $p < 0.05$.

Mean and standard deviations will be calculated for all quantitative data (e.g., weekly body weights, daily bodyweight gains, daily food consumption, food efficiency, daily dietary intake, organ weights, organ-to-body weight ratios, and estrus cycles). If warranted by sufficient group sizes, data within groups will be evaluated for homogeneity of variances[1] and normality. Where homogeneous variances and normal distribution is observed, treatment and control groups will be compared using a one-way analysis of variance (ANOVA). When one-way analysis of variance is significant, a comparison of the treated groups to control will be performed with a multiple comparisons test (e.g. Dunnett's test)[2,3]. Where variances are considered significantly different, groups will be compared using a non-parametric method (e.g. Kruskal-Wallis non-parametric analysis of variance)[4]. When non-parametric analysis of variance is significant, a comparison of treated groups to control will be performed (e.g. Dunn's test)[5].

If warranted by sufficient group sizes, the incidence of clinical observations may be evaluated through sequential application of a trend test[6]. Other procedures will be used if appropriate and will be described in the final report.

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[1] Bartlett, M.S. (1937). Properties of sufficiency and statistical tests. *Proceedings of the Royal Society of London, Series A*, 160, 268-282.
[2] Dunnett, C.W. (1980). Pairwise multiple comparisons in the unequal variance case. *J. Amer. Statist. Assoc.*, 75, 796-800.
[3] Dunnett, C.W. (1964). New tables for multiple comparisons with control. *Biometrics*, 482-491.
[4] Kruskal, W.H. and Wallis W.A. (1952). Use of ranks in one-criterion analysis of variance. *J. Amer. Statist. Assoc.*, 47, 583-621.
[5] Dunn, O.J. (1964). Multiple comparisons using rank sums. *Technometrics*, 6, 241-252, 1964.
[6] Agresti, A. (2013). *Categorical Data Analysis* (3rd Edition). John Wiley & Sons, Inc. Hoboken, NJ.

Statistical analysis will be conducted by using one or more of the following software applications: Provantis® version 9, Tables and Statistics, Instem LSS, Staffordshire, UK; INSTAT or Prism Biostatistics, GraphPad Software, San Diego, CA; Statview, version 5, SAS Institute Inc., Cary, NC; and SigmaStat, version 2, Systat Software, San Jose, CA. Other statistical methods will be used if appropriate, at the time of analysis and described in the final report.

13. FINAL REPORT

A signed study report will be provided to the Sponsor. This final report will include the procedures and conclusions drawn by the Study Director. This report will include, but not be limited to, the following information:

- individual animal data (and averages where appropriate) for actual concentration of test substance received;
- time of observation of each abnormal sign and its subsequent course;
- estrus cycle;
- body weights, body weight gain, food consumption, and food efficiency values;
- Selected organ weights and organ-to-body weight ratio;
- necropsy and pathology findings;

14. STUDY CONDUCT

14.A Testing Facility

In-life	Product Safety Labs 2394 US Highway 130 Dayton, NJ 08810
Test substance and dietary analysis	Impossible Foods Inc. 525 Chesapeake Dr. Redwood City, CA 94063 Prospective P.I.: Rachel Fraser, PhD
Histological slide preparation	Histo-Scientific Research Laboratories 5930 Main Street Mount Jackson, VA 22842 P.I. (histology): Craig Zook
Histological slide evaluation	Histo-Scientific Research Laboratories 5930 Main Street Mount Jackson, VA 22842 Prospective P.I. (pathology): Laura E. Elcock, DVM, PhD, DACVP

14.B GLP Compliance

This study will not be performed in full compliance with GLP standards, but will be conducted in a GLP-compliant facility.

14.C Test Procedure Guidelines

This study design is based on the following guidelines:

- OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 407): Health Effects, *Repeated Dose 28-Day Oral Toxicity Study in Rodents* (2008).
- US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 4. a. *Subchronic Toxicity Studies with Rodents* (2007)

15. RECORDS TO BE MAINTAINED

The original signed report and paper raw data will be sent to the Sponsor. A copy of the signed report, together with copy of the protocol and all raw data generated at Product Safety Labs, will be maintained in the Product Safety Labs archives.

The following records will be maintained:

A. Information on test substance will include but not be limited to the following:

Storage	Disposition
Usage	

B. Information on animals will include but not be limited to the following:

Receipt, date of birth	Food consumption
Initial health assessment	Individual necropsy records
Dosing	Histopathology data
Body weights	Selected organ weights
Cytology data	

C. All other records that would demonstrate adherence to the protocol.

Prepared slides and pathology data will be maintained by Product Safety Labs and/or by HSRI., Mount Jackson, VA. Test substance and dietary analysis data will be maintained by Impossible Foods, Inc. 525 Chesapeake Dr. Redwood City, CA 94063.

Any electronic raw data generated by the Test Site will be maintained in accordance to the Test Site SOPs.

16. PROTOCOL AMENDMENTS AND DEVIATIONS

All amendments and/or deviations to this protocol and the reasons therefore, shall be appropriately documented, signed by the Study Director, and described in the final report.

17. DISPOSITION OF TEST SUBSTANCE

A reserve sample of the test substance and records of sample disposition will be maintained at Product Safety Labs. All remaining test substance will be retained for at least one year from receipt, unless otherwise specified by the Sponsor. All remaining test substance will be returned to the Sponsor unless otherwise directed.

Product Safety Labs

28-Day Dietary Toxicity Study
Protocol #: P703.02 IMP
PSL ID: 160720-5R
Study No: 44856

18. PROTOCOL APPROVAL

Signature: (b) (6)
Rachel Fraser, PhD
Sponsor Representative
Impossible Foods Inc.
Date: 2/1/17

Signature: (b) (6)
Jayson Chen, PhD
Study Director
Product Safety Labs
Date: Feb 2, 2017

Signature: (b) (6)
Odete Mendes, DVM, PhD, DACVP, DABT
Director, Toxicology and Pathology
Product Safety Labs
Date: 2 Feb 2017

New Issue: 02/01/17

Product Safety Labs

PROTOCOL AMENDMENT

SOY LEGHEMOGLOBIN PREPARATION:
AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY PRE-DOSING ESTRUS CYCLE DETERMINATION

PROTOCOL NO.: P703.02 IMP **AMENDMENT NO.:** 1

STUDY NO.: 44856 **PSL NO.:** 160720-5R

PROTOCOL SECTION:
1) 11.H.3 Histopathology

Change From:
Slide preparation and histological assessment, by a board-certified veterinary pathologist, will be performed at Histo-Scientific Research Laboratories (HSRL).

Change To:
Slide preparation will be performed by Histoserv Inc. Histological assessment, by a board-certified veterinary pathologist, will be performed at Regan Path/Tox Services.

2) 14.A Testing Facility

Change From:

Histological slide preparation	Histo-Scientific Research Laboratories 5930 Main Street Mount Jackson, VA 22842 P.I. (histology): Craig Zook
Histological slide evaluation	Histo-Scientific Research Laboratories 5930 Main Street Mount Jackson, VA 22842 Prospective P.I. (pathology): Laura E. Elcock, DVM, PhD, DACVP

Change To:

Histological slide preparation	Histoserv, Inc. 19526 Amaranth Drive Germantown, MD 20874 P.I.: Protiba Vohra
Histological slide evaluation	Regan Path/Tox Services 1457 Township Rd. 853 Ashland, OH 44805 P.I.: Karen Regan, DVM, DACVP, DABT

Product Safety Labs

REASON: To change the test facility for the histological slide preparation and evaluation

EFFECTIVE DATE: February 13, 2017

(b) (6)

Jayson Chen
Study Director
Product Safety Labs

Mar 23, 2017
Date

APPENDIX B: FEED AND WATER ANALYSES

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

APPENDIX B: FEED

2016CM

True copy of original
date by _____
date _____

ENVIGO

Teklad Certified Global 16% Protein Rodent Diet (MEAL)

Lot Number	2016CM-020717MA
Date of Manufacture	02/07/17
Report Date	02/20/17

Analysis	Result (%)
Protein	15.90
Fat	3.65
Fiber	3.60
Moisture	12.01
Ash	5.02
Calcium	0.92
Phosphorus	0.70

Laboratory Diet Certification Report

The following data is a consolidation of results obtained from one or more independent testing laboratories. The actual laboratory results are available upon request.

(b) (6)

I have reviewed this document
2017.02.20
10:38:19 -06'00'

Analysis	Result	Units	Established Maximum Concentration
Arsenic	0.12	ppm	1.00
Cadmium	< 0.10	ppm	0.50
Lead	< 0.20	ppm	1.50
Mercury	< 0.05	ppm	0.20
Selenium	0.03	ppm	0.50
Aflatoxin B1, B2, G1, G2	< 5.00	ppb	5.00
Aldrin	< 0.01	ppm	0.03
Lindane	< 0.01	ppm	0.05
Chlordane	< 0.01	ppm	0.05
DDT & related substances	< 0.03	ppm	0.15
Dieldrin	< 0.02	ppm	0.03
Endrin	< 0.02	ppm	0.03
Heptachlor	< 0.01	ppm	0.03
Heptachlor Epoxide	< 0.01	ppm	0.03
Toxaphene	< 0.10	ppm	0.15
PCB's	< 0.10	ppm	0.15
a-BHC	< 0.01	ppm	0.05
b-BHC	< 0.01	ppm	0.05
d-BHC	< 0.01	ppm	0.05
Hexachlorobenzene	< 0.01	ppm	0.03
Mirex	< 0.01	ppm	0.02
Methoxychlor	< 0.05	ppm	0.50
Thimet	< 0.15	ppm	0.50
Diazinon	< 0.14	ppm	0.50
Disulfaton	< 0.15	ppm	0.50
Methyl Parathion	< 0.14	ppm	0.50
Malathion	< 0.14	ppm	0.50
Parathion	< 0.12	ppm	0.50
Thiodan	< 0.02	ppm	0.50
Ethion	< 0.14	ppm	0.50
Trithion	< 0.15	ppm	0.50

Envigo Teklad Diets + Madison WI + envigo.com + tekladinfo@envigo.com + (800) 483-5523

APPENDIX B (cont.): WATER

In March 2017, water was analyzed for contaminants.

LABORATORY: PRECISION ANALYTICAL SERVICES, INC.
726 Bernice Court
Toms River, NJ 08753

Results of water analysis for possible contaminants were acceptable within regulatory standards.

Specialists in Drinking Water Testing Technologies ■ Residential ■ Industrial ■ Municipal

2141 WHITESVILLE ROAD TOMS RIVER, NJ 08755 PHONE 732-914-1515 FAX 732-914-1818

CERTIFICATE OF ANALYSIS

Customer : Product Safety Labs
2394 Route 130
Dayton, NJ 08810

Project ID : 1st Quarter
PAS Project ID : P17-1238

Matrix : Drinking Water
Report Date : 3/24/2017

PAS Sample ID	Client ID	Analysis	Results	Units	PQL	MDL	MCL	Method	Date Sampled	Date Analyzed
P17-1238-01	Room #4	Copper	ND	mg/L	0.050	0.0235	1.30*	SM 3111 B	3/21/17 11:10	3/22/17 15:44
P17-1238-01	Room #4	Zinc	ND	mg/L	0.025	0.0118	5.00**	SM 3111 B	3/21/17 11:10	3/23/17 12:11
P17-1238-01	Room #4	Lead	ND	mg/L	0.002	0.000462	0.015*	SM 3113 B	3/21/17 11:10	3/22/17 15:30
P17-1238-01	Room #4	E. Coli / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:10	3/21/17 16:40
P17-1238-01	Room #4	Total Coliform / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:10	3/21/17 16:40
P17-1238-07	Room #13	Copper	ND	mg/L	0.050	0.0235	1.30*	SM 3111 B	3/21/17 11:20	3/22/17 15:46
P17-1238-02	Room #13	Zinc	ND	mg/L	0.025	0.0118	5.00**	SM 3111 B	3/21/17 11:20	3/23/17 12:13
P17-1238-02	Room #13	Lead	ND	mg/L	0.002	0.000462	0.015*	SM 3113 B	3/21/17 11:20	3/22/17 15:33
P17-1238-07	Room #13	E. Coli / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:20	3/21/17 16:40
P17-1238-02	Room #13	Total Coliform / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:20	3/21/17 16:40
P17-1238-03	Room #10	Copper	ND	mg/L	0.050	0.0235	1.30*	SM 3111 B	3/21/17 11:30	3/22/17 15:49
P17-1238-03	Room #10	Zinc	ND	mg/L	0.025	0.0118	5.00**	SM 3111 B	3/21/17 11:30	3/23/17 12:16
P17-1238-03	Room #10	Lead	ND	mg/L	0.002	0.000462	0.015*	SM 3113 B	3/21/17 11:30	3/22/17 15:39
P17-1238-03	Room #10	E. Coli / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:30	3/21/17 16:40
P17-1238-03	Room #10	Total Coliform / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:30	3/21/17 16:40
P17-1238-04	Room #29 Pressure Station	Copper	0.075	mg/L	0.050	0.0235	1.30*	SM 3111 B	3/21/17 11:40	3/22/17 15:50
P17-1238-04	Room #29 Pressure Station	Zinc	ND	mg/L	0.025	0.0118	5.00**	SM 3111 B	3/21/17 11:40	3/23/17 12:17
P17-1238-04	Room #29 Pressure Station	Lead	ND	mg/L	0.002	0.000462	0.015*	SM 3113 B	3/21/17 11:40	3/22/17 15:43
P17-1238-04	Room #29 Pressure Station	E. Coli / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:40	3/21/17 16:40
P17-1238-04	Room #29 Pressure Station	Total Coliform / Colilert	Absent	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9223 B	3/21/17 11:40	3/21/17 16:40
P17-1238-05	Sipper Bottle	E. Coli	ND	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9221 E + MUG	3/21/17 11:45	3/22/17 11:10
P17-1238-05	Sipper Bottle	Total Coliform	ND	Col/100mL	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9222 B	3/21/17 11:45	3/22/17 11:10
P17-1238-06	Sipper Top	E. Coli	ND	Pres/Abs	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9221 E + MUG	3/21/17 11:45	3/22/17 11:10
P17-1238-06	Sipper Top	Total Coliform	ND	Col/100mL	1 Col/100mL	1 Col/100mL	0 Col/100mL	SM 9222 B	3/21/17 11:45	3/22/17 11:10

Except for the parameters tested, PAS makes no representation as to the fitness or quality of the water sample taken.

MCL = Maximum Contaminant Level
PQL = Practical Quantitation Limit
MDL = Minimum Detection Limit
ND = Analyzed for but not detected
* = Federal Action Level
** = Secondary MCL / Recommended Upper Limit

All samples are analyzed in accordance with New Jersey Department of Environmental Protection protocols.

(b) (6)

Mark D. Feldstein, Lab. Director

room # 7 unavailable for testing due to special procedures in place for specimen dish, recalled to the next room in line
CR 3/31/2017

APPENDIX C: CHEMICAL ANALYSIS REPORT

Submitted By:

Impossible Foods Inc.
525 Chesapeake Dr.
Redwood City, CA 94063

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

IMPOSSIBLE™

Project Title:
Analysis of Samples from Study:
Soy Leghemoglobin Preparation: AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY PRE-DOSING ESTRUS CYCLE DETERMINATION

Sponsor
Impossible Foods Inc.
525 Chesapeake Dr.
Redwood City, CA 94063

ANALYTICAL REPORT

Test Substance:
160720-5R

Author:
Rachel Fraser, PhD

Analytical Report Completion Date:
March 17, 2017

Performing Laboratory:

Analytical Services:
Impossible Foods
525 Chesapeake Dr.
Redwood City, CA, 94063

Project Identification Number:
Impossible Foods Study Number IF-44856

IMPOSSIBLE

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Soy Leghemoglobin Preparation

This analysis was conducted in a non-GLP certified facility. Method validation and sample analysis were performed and documented according to GLP. Characterization of reference substance was documented according to GLP.

Principal Investigator (b) (6) Date: 3/17/17

Name of Signer: Rachel Fraser, PhD

Name of Company: Impossible Foods

IMPOSSIBLE™

SIGNATURE

Soy Leghemoglobin Preparation

I, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected during the study.

(b) (6)

Rachel Fraser, PhD
Principal Scientist
Impossible Foods

3/17/17
Date

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TABLE OF CONTENTS

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STUDY INFORMATION

Protocol No.:	IF-44856
Test Substance:	Soy Leghemoglobin Preparation Lot/Batch #: PP-PGM2-16-088-301
Physical Description:	Red/Brown Powder
Date Test Substance Received:	February 28, 2017
PSL Reference No.:	160720-5R
PSL Study Number:	44856
Sponsor:	Impossible Foods Inc.
Dates of Analysis:	
Analytical Principal Investigator:	Rachel Fraser, PhD
Primary Chemist:	Puja Agrawal, MS

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1. SUMMARY

This report presents the dietary mixture and test substance analysis phase of PSL Study Number 44856: Soy Leghemoglobin Preparation: AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY PRE-DOSING ESTRUS CYCLE DETERMINATION. Samples were collected during the first feed preparation of the dosing phase of the study for neat test substance concentration verification (NT) and feed homogeneity (HO) and concentration verification (performed on the HO samples) and transferred to the analytical laboratory of Impossible Foods. Test article stability (neat and in rat feed) was previously demonstrated in PSL Study Number 43166: Soy Leghemoglobin Preparation: A 28-DAY DIETARY STUDY IN RATS and therefore was not repeated in this study. This method was validated in terms of linearity, specificity, precision, and accuracy. All samples were received frozen and were maintained frozen prior to extraction.

Samples:

Neat test substance for concentration verification: Week 1

NT 1 A

Initial (Day 0) Dietary Samples for Concentration Verification and Homogeneity (T = top, M = middle, B = bottom):

HO 1 A M
HO 2 A T
HO 3 A M
HO 4 A B
HO 5 A T
HO 6 A M
HO 7 A B
HO 8 A T
HO 9 A M
HO 10 A B

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2. PROCEDURE FOR THE DETERMINATION OF SOY LEGHEMOGLOBIN PREPARATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

A. Reference Standard

Note: The neat test substance was used as the reference standard. No purity correction was applied. Results were reported as test substance concentration (versus active ingredient concentration).

Name: Soy Leghemoglobin Preparation
Lot/Batch #: PP-PGM2-16-088-301
PSL No.: 160720-5R
Purity: 48.82%
Exp. Date: June 2017
Supplied by: Impossible Foods Inc

B. Method Validation

Linearity, system suitability, specificity, precision, and accuracy (spike recovery) determinations were performed prior to analysis.

Stock Standard Solution: A standard solution was prepared by weighing 0.1 grams of reference standard into a 50 mL polypropylene tube, diluting with 25 g of Lysis Reagent, shaking for 60 minutes, and mixing well.

2.B.1 Detector Linearity: The linearity of detector response was assessed using reference substance solutions targeted to bracket the expected concentrations for the analyte.

Linearity Standard Preparation: Five standard solutions with concentrations ranging from approximately 0.125 to 2 mg/g (LIN 1 - LIN 5) were prepared by preparing individual dilutions of the stock standard solution in Lysis Reagent by weight and mixing well. Linearity solution shelf life is 3 days at 4C or 12 months at -80C.

Linear regression of the analyte peak gave coefficients of determination (R^2) of 0.9998, which were considered acceptable.

2.B.2 System Suitability: Five replicate injections of the mid-point linearity solution (LIN 3-1) produced relative standard deviations for this study of 0.4-0.7% for peak response and 0.1% for retention time.

2.B.3 Specificity: Specificity was demonstrated by the absence of significant interferences in replicate linearity (LIN 1-A) and control feed samples (HO 1 AM-1). Background was <5% of the lowest standard signal.

2.B.4 Accuracy (Spike recovery) and Precision:

Duplicate QC stock solutions were prepared by weighing approximately 0.5 gram of a control sample (HO 1 AM) into separate 50 mL polypropylene centrifuge tubes, adding 1.25 g (QC Low) or 2.5 g (QC High) of STD1 stock standard solution, and adding 8.75 g (QC High) or 7.5 g (QC High) of Lysis Reagent into each tube. Each mixture was capped and placed in a mechanical shaker for 60 minutes. The solutions were allowed to settle for 30 minutes and filtered using a 0.2µm 96-well filter plates. Filtrate was

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collected in 96-well conical bottom plate for HPLC analysis.

Chromatography of the working QC solutions demonstrated accuracy (% recovery) to be 96.4% for QC Low and 98.3% for QC High. The %RSD was 1.4% for QC Low and 0.3% for QC High for precision.

C. Analysis by High Performance Liquid Chromatography (HPLC)

2.C.1 Standard Preparation: The linearity solutions were injected and used for interpolation of assay results. The result is shown in 2.B.1.

Note: All diet samples were removed from the freezer and allowed to equilibrate to room temperature before weighing.

2.C.2 Test Sample Preparation for Neat Test Substance: Samples were prepared in triplicate. Approximately 0.1 g of the test substance was weighed into 50 mL polypropylene centrifuge tubes, diluted with 25 g lysis reagent, and placed in a mechanical shaker for 60 minutes. Secondary dilutions were performed as necessary. Samples were mixed well and filtered using a 0.2µm 96-well filter plates. Filtrate was collected in 96-well conical bottom plate for HPLC analysis. Filtrate shelf life is 3 days at 4C or 12 months at -80C.

2.C.3 Sample Preparation for Dietary Samples: Each sample was prepared in triplicate. Approximately 0.5 g of a sample was weighed into a 50 mL polypropylene centrifuge tube and diluted with Lysis Reagent as necessary (higher concentration samples had a higher dilution). The solution was capped and placed in a mechanical shaker for 60 minutes. The solutions were allowed to settle for 30 minutes and filtered using a 0.2µm 96-well filter plates. Filtrate was collected in 96-well conical bottom plate for HPLC analysis. Filtrate shelf life is 3 days at 4C or 12 months at -80C.

2.C.4 Analysis: At the beginning of the analysis, the instrument was equilibrated until it gave a stable, consistent baseline. The standards and samples were injected at consistent time intervals in order to maintain a steady baseline. A solvent blank and standards were run; all samples were injected in singlet except for the spike recovery samples, which were injected in duplicate.

2.C.5 Calculations: Results were determined as follows:

$$\text{Calculated Conc. (mg/g)} = \frac{\text{Peak Area - Intercept}}{\text{Slope}}$$

$$\text{Dose Conc. (ppm)} = \frac{\text{Calc. Conc. (mg/g) x Extraction Buffer Wt. (g) x 1000}}{\text{Sample weight (g)}}$$

$$\text{Theoretical Spike Conc. (mg/g)} = \frac{\text{Wt. of Std. (g)}}{\text{Extraction Buffer Wt. (g)}} \times \text{Std. Conc. (mg/g)}$$

$$\text{Final Conc. (mg/g)} = \text{Theoretical Spike Conc. (mg/g) x Wt. of Sample Aliquot (g) / Final Wt (g)}$$

$$\text{\% Recovery} = \frac{\text{Calc. Conc. (mg/g)}}{\text{Final Conc.(mg/g)}} \times 100$$

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$$\% \text{ Signal / Background} = \frac{\text{Avg. LIN 1-A area response}}{\text{Avg. Control area response}} \times 100$$

% Target = Dose Conc. (ppm) / Corrected Dose Level (ppm) x 100

3. RESULTS

A summary of the analytical chemistry results is presented in Table 1A-B. HPLC operating conditions are presented in Table 2. The analytical method passed all validation parameters (linearity, system suitability, specificity, precision, and accuracy) and results are reported in Table 3. Detailed results of neat test article concentration verification and feed homogeneity and concentration verification are presented in Tables 4-5. Chromatograms are maintained in the raw data but were not included in this report.

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TABLE 1A: CHEMICAL ANALYSIS RESULTS

Result for Neat Test Substance Sample

Sampling Day	Measured Recovery (%)
Day 0	102.7%

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TABLE 1B: CHEMICAL ANALYSIS RESULTS

Results for Homogeneity of Dietary Preparations

Day[1]	Group	Sample Location	Target Concentration (ppm)	Measured Concentration (ppm)	% of Target[2]	Average % of Target	RSD (%)
0	1	Middle	0	ND	NA	NA	NA
	2	Top	5863	5545	94.57%	93.80%	3.22%
		Middle		5649	96.36%		
		Bottom		5304	90.46%		
	3	Top	11354	10633	93.65%	93.89%	2.27%
		Middle		10433	91.89%		
		Bottom		10915	96.13%		
	4	Top	16936	16008	94.52%	93.91%	0.60%
		Middle		15882	93.78%		
		Bottom		15822	93.42%		

NA = Not Applicable; ND = Not Detected

[1] Day relative to initial dietary preparation.

[2] % of Target = Measured Conc. (ppm) / Target Conc. (ppm) x 100.

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TABLE 2: HPLC OPERATING CONDITIONS

Instrument		Agilent 1100 Series HPLC System, with DAD	
Column		Waters Acquity xBridge BEH125 SEC, 7.8 x 150 mm ID 3.5µm	
Flow rate (mL/min)		0.86	
Injection Volume (µL)		25	
Wavelength (nm)		405	
Column Temperature (°C)		Ambient	
Tray Temperature (°C)		4	
Run time (min)	Flow rate (ml/min)	HPLC-Grade Water (%)	50 mM Potassium Phosphate pH 7.4, 5 mM Sodium Chloride (%)
0-14.00 min	0.86	0	100
14.01-19.00 min	0.86	100	0
19.01 to 30.00 min	0.86	0	100

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TABLE 3: METHOD VALIDATION RESULTS

Linearity

(Analyzed on 03/15/2017)

Sample ID	Peak Area	Theoretical Concentration (mg/g)
Lin 1	11.996	0.134
	12.259	0.131
Lin 2	24.822	0.264
	24.928	0.264
Lin 3	49.916	0.540
	51.512	0.517
Lin 4	100.749	1.044
	101.589	1.036
Lin 5	199.076	2.066
	199.105	2.058
Slope:		96.983
Intercept:		-0.512
Correlation Coefficient (r):		1.000

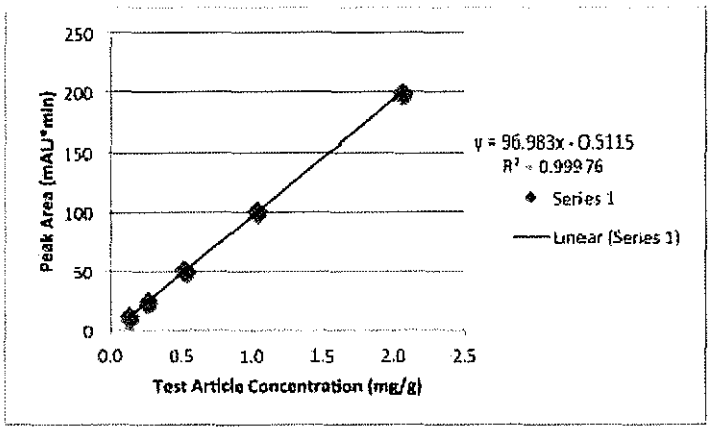

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TABLE 3 (cont.): METHOD VALIDATION RESULTS

System Suitability

(Analyzed on 03/15/2017)

Sample ID	Theoretical Conc. (mg/g)	Retention time (min)	Peak Area
LIN 3-1	0.540	4.369	50.067
		4.370	50.093
		4.370	50.097
		4.377	50.038
		4.368	50.531
Average		4.371	50.165
STDEV		0.004	0.206
%RSD		0.1%	0.4%

Accuracy and Precision

(Analyzed on 03/15/2017)

Sample Name	Theoretical Conc. (mg/g)	Peak Area	Calculated Conc. (mg/g)	% Recovery	Average % Recovery (SD / %RSD)
QC Low	0.515	48.209	0.502	97.60%	96.40% (0.01 / 1.43%)
		48.205	0.502	97.59%	
	0.513	46.804	0.488	95.11%	
		46.896	0.489	95.30%	
QC High	1.030	97.509	1.011	98.08%	98.32% (0.00 / 0.34%)
		97.413	1.010	97.98%	
	1.033	98.277	1.019	98.62%	
		98.267	1.019	98.61%	
y = 96.98x - 0.51					

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TABLE 3 (cont.): METHOD VALIDATION RESULTS

Specificity (Analyzed on 03/15/2017)

	Peak Area	Specificity
LIN 1-A	11.996	NA
	12.259	
HO 1 AM-1	ND	
HO 1 AM-2	ND	
HO 1 AM-3	ND	

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TABLE 4: NEAT TEST SUBSTANCE CONCENTRATION VERIFICATION ANALYSIS

Analyzed on 03/15/2017

Day[1]	Sample Name	Sample Weight (g)	Final Conc. (mg/g)	Peak Area	Calculated Conc. (mg/g)	% Recovery	Avg. % Recovery	SD / %RSD
0	NT 1 A	0.1037	0.515	50.477	0.526	102.02%	102.67%	0.02 / 2.09%
		0.1026	0.512	51.630	0.538	105.07%		
		0.1022	0.509	49.361	0.514	100.93%		
y = 96.98x + 0.51								

[1] Days relative to the initial diet preparation.

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TABLE 5: DIETARY MIXTURE SAMPLE ANALYSIS

Study Day 0 Analyzed on 03/15/2017 – 03/16/2017

Sample ID	Sample Wt. (g)	Lysis Reagent Wt. (g)	Dose Level (ppm)	Peak Area	Calc. Conc. (ng/g)	Dose Conc. (ppm)	Average (ppm)	%RSD	% Target	% Target Average	%Target between the strata (Avg. / RSD)
HO1 A M-1	0.5029	4.9678	0	ND	NA	NA	NA	NA	NA	NA	NA
HO1 A M-2	0.5096	4.9801		ND	NA	NA	NA	NA	NA		
HO1 A M-3	0.5211	4.9605		ND	NA	NA	NA	NA	NA		
HO2 A T-1	0.5036	5.0567	5863	50.961	0.531	5329	5513	1.88%	90.89%	94.07%	93.80% / 1.21%
HO2 A T-2	0.5080	5.0331		55.043	0.579	5759			98.23%		
HO2 A T-3	0.5141	5.0687		54.041	0.562	5546			94.59%		
HO3 A M-1	0.5185	5.0303		37.475	0.398	5031	5649	2.58%	90.12%	96.36%	
HO3 A M-2	0.5110	5.0372		54.663	0.569	5608			95.65%		
HO3 A M-3	0.5041	5.0540		52.965	0.551	5528			94.26%		
HO4 A B-1	0.5183	5.0239		55.770	0.580	5626	5304	3.44%	95.96%	90.16%	
HO4 A B-2	0.5178	5.0121		50.272	0.524	5069			86.45%		
HO4 A B-3	0.5346	5.0654		53.362	0.555	5217			88.99%		
HO5 A T-1	0.5032	10.0232	11594	49.646	0.517	10352	10933	1.00%	91.18%	91.66%	93.86% / 2.27%
HO5 A T-2	0.5033	10.1017		52.900	0.548	10901			96.82%		
HO5 A T-3	0.5082	10.0495		51.249	0.534	10554			92.95%		
HO6 A M-1	0.5126	10.0720		50.691	0.528	10374	10433	2.29%	91.37%	91.89%	
HO6 A M-2	0.5059	10.0924		49.216	0.513	10229			90.09%		
HO6 A M-3	0.5262	10.0811		53.653	0.558	10996			94.20%		
HO7 A B-1	0.5012	10.0436		50.254	0.523	10488	10915	3.86%	92.38%	96.13%	
HO7 A B-2	0.5171	10.0951		55.291	0.575	11332			99.81%		
HO7 A B-3	0.5225	10.0710		54.185	0.566	11022			96.20%		
y = 95.08x + 0.51											

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TABLE 5 (cont.) INDIVIDUAL SAMPLE ANALYSES

Study Day 0 (cont.) Analyzed on 03/13/2017 - 03/16/2017

Sample ID	Sample Wt. (g)	Lysis Reagent Wt. (g)	Dose Level (ppm)	Peak Area	Calc. Conc. (mg/g)	Dose Conc. (ppm)	Average (ppm)	%RSD	% Target	% Target Average	%Target between the strata (Avg. / RSD)
HOBAT-1	0.5146	15.1256	16936	51.456	0.536	15753	16160	2.30%	93.02%	91.52%	93.91% 0.68%
HOBAT-2	0.5131	15.1869		51.386	0.535	15439			91.22%		
HOBAT-3	0.5019	15.1315		52.343	0.545	16439			97.12%		
HO9AM3-1	0.5044	15.1564		50.123	0.522	15938	15882	1.95%	92.63%	93.78%	
HO9AM3-2	0.5261	15.1204		53.285	0.555	15936			94.16%		
HO9AM3-3	0.5163	15.1318		52.555	0.547	15021			94.68%		
HO10AB-1	0.5271	15.1673		52.692	0.549	15788	15822	4.67%	93.22%	93.42%	
HO10AB-2	0.5176	15.1514		54.314	0.566	16578			97.89%		
HO10AB-3	0.5086	15.0832		48.871	0.509	15101			89.17%		
) = 96.98% (0.1)											

NA = Not Applicable; ND = Not Detected

APPENDIX D: INDIVIDUAL ANIMAL IN-LIFE OBSERVATIONS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal In-Life Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrous cycle Determination

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	f	7001	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7002	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7003	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7004	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7005	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7006	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	.	.
			Alopecia	Abdomen	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	M	M	M	M	M
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	S	M	M	M	M	M
		7007	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7008	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.	.	.	.
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	S	S	S	S
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	S	M	M	M	M	M
		7009	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7010	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7011	No Abnormalities Detected		X	X	X	X	X	X	.	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Back	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.
		7012	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7013	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7014	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7015	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Severity Codes: X = Present; S = Slight; M = Moderate

Group 1 - 0 mg/kg/day Group 2 - 512 mg/kg/day
Group 3 - 1024 mg/kg/day Group 4 - 1536 mg/kg/day

Individual Animal In-Life Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrous cycle Determination

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
1	f	7001	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7002	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7003	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7004	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7005	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7006	No Abnormalities Detected		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Abdomen	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Left Forepaw	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
			Alopecia	Right Forepaw	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M	M
		7007	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7008	No Abnormalities Detected		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Left Forepaw	S	S	S	S	S	S	S	S	S	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Forepaw	M	M	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
		7009	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7010	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7011	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Back	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7012	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7013	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7014	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7015	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	S	S	S	S	S	S	S	S	S	S

Severity Codes: X = Present; S = Slight; M = Moderate

Group 1 - 0 mg/kg/day Group 2 - 512 mg/kg/day
Group 3 - 1024 mg/kg/day Group 4 - 1536 mg/kg/day

Individual Animal In-Life Clinical Observations

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in Rats with a 14-Day Pre-Dosing Estrous cycle Determination

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	38	39	40	41	42	43
1	f	7001	No Abnormalities Detected		X	X	X	X	X	X
		7002	No Abnormalities Detected		X	X	X	X	X	X
		7003	No Abnormalities Detected		X	X	X	X	X	X
		7004	No Abnormalities Detected		X	X	X	X	X	X
		7005	No Abnormalities Detected		X	X	X	X	X	X
		7006	No Abnormalities Detected		.	.	.	.	.	.
			Alopecia	Abdomen	.	.	.	.	S	S
			Alopecia	Left Forepaw	M	M	M	M	M	M
			Alopecia	Right Forepaw	M	M	M	M	M	M
		7007	No Abnormalities Detected		X	X	X	X	X	X
		7008	No Abnormalities Detected		.	.	X	X	X	X
			Alopecia	Left Forepaw	.	.	.	.	.	.
			Alopecia	Right Forepaw	S	S	.	.	.	.
		7009	No Abnormalities Detected		X	X	X	X	X	X
		7010	No Abnormalities Detected		X	X	X	X	X	X
		7011	No Abnormalities Detected		X	X	X	X	X	X
			Alopecia	Back	.	.	.	.	.	.
		7012	No Abnormalities Detected		X	X	X	X	X	X
		7013	No Abnormalities Detected		X	X	X	X	X	X
		7014	No Abnormalities Detected		X	X	X	X	X	X
		7015	No Abnormalities Detected		.	.	.	.	.	.
			Alopecia	Left Forepaw	S	S	S	S	S	S

Severity Codes: X = Present; S = Slight; M = Moderate

Group 1 - 0 mg/kg/day Group 2 - 512 mg/kg/day
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Individual Animal In-Life Clinical Observations

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Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
2	f	7016	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7017	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7018	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7019	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7020	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7021	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7022	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7023	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Broken Tooth	Upper Right Incisor	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7024	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7025	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7026	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7027	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7028	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7029	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7030	No Abnormalities Detected		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Flank	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Severity Codes: X = Present; S = Slight; M = Moderate

Group 1 - 0 mg/kg/day Group 2 - 512 mg/kg/day
Group 3 - 1024 mg/kg/day Group 4 - 1536 mg/kg/day

Individual Animal In-Life Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrous cycle Determination

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
2	f	7016	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7017	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7018	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7019	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7020	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7021	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7022	No Abnormalities Detected		X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Forepaw	.	.	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
		7023	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.
			Broken Tooth	Upper Right Incisor	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	X	X
		7024	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	.	.	.	.	.	.	.	.	.	.
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	S
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	S	S	S	S	S	S	S	S	S	S
		7025	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7026	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7027	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7028	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7029	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7030	No Abnormalities Detected		.	.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Right Flank	S	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

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PSL Study Number 44856
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in Rats with a 14-Day Pre-Dosing Estrous cycle Determination

Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	38	39	40	41	42	43
2	f	7016	No Abnormalities Detected		X	X	X	X	X	X
		7017	No Abnormalities Detected		X	X	X	X	X	X
		7018	No Abnormalities Detected		X	X	X	X	X	X
		7019	No Abnormalities Detected		X	X	X	X	X	X
		7020	No Abnormalities Detected		X	X	X	X	X	X
		7021	No Abnormalities Detected		X	X	X	X	X	X
		7022	No Abnormalities Detected		.	.	X	X	X	X
			Alopecia	Right Forepaw	S	S	.	.	.	.
		7023	No Abnormalities Detected		.	.	.	.	.	X
			Broken Tooth	Upper Right Incisor	X	X	X	X	X	.
		7024	No Abnormalities Detected		.	.	.	.	.	.
			Alopecia	Left Forepaw	S	S	S	S	S	S
			Alopecia	Right Forepaw	S	S	S	S	S	S
		7025	No Abnormalities Detected		X	X	X	X	X	X
		7026	No Abnormalities Detected		X	X	X	X	X	X
		7027	No Abnormalities Detected		X	X	X	X	X	X
		7028	No Abnormalities Detected		X	X	X	X	X	X
		7029	No Abnormalities Detected		X	X	X	X	X	X
		7030	No Abnormalities Detected		X	X	X	X	X	X
			Alopecia	Right Flank	.	.	.	.	.	.

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Group	Sex	Animal	Clinical Sign	Site	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
3	f	7031	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7032	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7033	No Abnormalities Detected		X	X	X	X	X	.	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Back	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Flank	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	.	.	.	.
		7034	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7035	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7036	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7037	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7038	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7039	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7040	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7041	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7042	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7043	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7044	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7045	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

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Group	Sex	Animal	Clinical Sign	Site	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
3	f	7031	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7032	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7033	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Back	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Flank	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7034	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7035	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7036	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	S
		7037	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7038	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7039	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7040	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	.	.	.
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	S	S
		7041	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7042	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7043	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7044	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7045	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

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Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	38	39	40	41	42	43
3	f	7031	No Abnormalities Detected		X	X	X	X	X	X
		7032	No Abnormalities Detected		X	X	X	X	X	X
		7033	No Abnormalities Detected		X	X	X	X	X	X
			Alopecia	Back	.	.	.	.	.	.
			Alopecia	Right Flank	.	.	.	.	.	.
		7034	No Abnormalities Detected		X	X	X	X	X	X
		7035	No Abnormalities Detected		X	X	X	X	X	X
		7036	No Abnormalities Detected		.	.	X	X	X	X
			Alopecia	Left Forepaw	S	S	.	.	.	.
		7037	No Abnormalities Detected		X	X	X	X	X	X
		7038	No Abnormalities Detected		X	X	X	X	X	X
		7039	No Abnormalities Detected		X	X	X	X	X	X
		7040	No Abnormalities Detected		.	.	.	.	.	.
			Alopecia	Left Forepaw	.	.	.	.	S	S
			Alopecia	Right Forepaw	S	S	S	S	S	S
		7041	No Abnormalities Detected		X	X	X	X	X	X
		7042	No Abnormalities Detected		X	X	X	X	X	X
		7043	No Abnormalities Detected		X	X	X	X	X	X
		7044	No Abnormalities Detected		X	X	X	X	X	X
		7045	No Abnormalities Detected		X	X	X	X	X	X

Severity Codes: X = Present; S = Slight; M = Moderate

Group 1 - 0 mg/kg/day Group 2 - 512 mg/kg/day
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Day numbers relative to Start Date

Group	Sex	Animal	Clinical Sign	Site	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
4	f	7046	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7047	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
			Alopecia	Left Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Right Forepaw	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		7048	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7049	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7050	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7051	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7052	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7053	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7054	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7055	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7056	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7057	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7058	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7059	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7060	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

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Group	Sex	Animal	Clinical Sign	Site	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
4	f	7046	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7047	No Abnormalities Detected		X	X	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
			Alopecia	Left Forepaw	.	.	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
			Alopecia	Right Forepaw	.	.	S	S	S	S	S	S	S	S	S	S	S	S	S	M	M	M	M
		7048	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7049	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7050	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7051	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7052	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7053	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7054	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7055	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7056	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7057	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7058	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7059	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
		7060	No Abnormalities Detected		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

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Group	Sex	Animal	Clinical Sign	Site	38	39	40	41	42	43
4	f	7046	No Abnormalities Detected		X	X	X	X	X	X
		7047	No Abnormalities Detected		.	.	X	X	X	X
			Alopecia	Left Forepaw	S	S	.	.	.	.
			Alopecia	Right Forepaw	M	M	.	.	.	.
		7048	No Abnormalities Detected		X	X	X	X	X	X
		7049	No Abnormalities Detected		X	X	X	X	X	X
		7050	No Abnormalities Detected		X	X	X	X	X	X
		7051	No Abnormalities Detected		X	X	X	X	X	X
		7052	No Abnormalities Detected		X	X	X	X	X	X
		7053	No Abnormalities Detected		X	X	X	X	X	X
		7054	No Abnormalities Detected		X	X	X	X	X	X
		7055	No Abnormalities Detected		X	X	X	X	X	X
		7056	No Abnormalities Detected		X	X	X	X	X	X
		7057	No Abnormalities Detected		X	X	X	X	X	X
		7058	No Abnormalities Detected		X	X	X	X	X	X
		7059	No Abnormalities Detected		X	X	X	X	X	X
		7060	No Abnormalities Detected		X	X	X	X	X	X

Severity Codes: X = Present; S = Slight; M = Moderate

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APPENDIX E: DETAILED CLINICAL OBSERVATIONS ASSESSMENT METHODS SCORING KEY

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Removal from Cage and Open Field Observations	
Activity/Arousal	0. Alternating behaviors - animal goes through normal repertoire of behaviors during observation period. These consist of exploring, sniffing, grooming, rearing, etc. 1. Inactive/Alert - animal sits in one place during the observation period but appears to be aware of its surroundings. It may go through its normal repertoire of activities but the majority of the observation period is spent not moving. 2. Hypoactive/Not alert - animal sits in one place during the observation period. Animal appears to be unaware of its surroundings or in a stupor. 3. Hyperactive/Hyperalert - animal appears excited. Animal may dart and freeze during the observation period or animal may sit in one place and jump at any sound or movement.
Convulsions	0. None 1. Clonic – alternating periods of contraction and relaxation of muscles 2. Tonic – prolonged period of muscle contractions
Defecation	0. None/Normal 1. Soft (partially formed) 2. Diarrhea (watery feces)
Ease of Removal/Handling	0. Slight/moderate resistance - animal is easy to handle, may squirm or vocalize occasionally. 1. No resistance - animal is limp/flaccid when being handled. 2. High resistance - animal is difficult to handle, and/or squirms continuously. 3. Aggressive - biting or lunging behavior specifically directed at handler.
Emaciation	0. Absent 1. Present (confirmed using body weights)
Eyes	0. Normal 1. Exophthalmos - abnormal protrusion of eyeball 2. Enophthalmos – sunken eyeball 3. Eye damaged – mechanical damage (e.g. orbital bleeding, etc.)
Fur/Skin Appearance	0. Normal 1. Unkempt - coat rough or ungroomed, may be slightly stained 2. Urine stained/wetness (ano-genital staining) 3. Hair loss
Gait	0. Normal 1. Abnormal – limbs exaggerated/splayed, hind limbs and/or forelimbs show exaggerated placement or movement 2. Non weight bearing (Limping)
Lacrimation	0. Absent 1. Present - lacrimation noticeable. 2. Excessive - animal has excessive amount of tearing. Note: Descriptors (i.e. color of ocular discharge will be noted on daily observation sheet).

Locomotion	0. Normal 1. Somewhat impaired 2. Totally impaired
Mucous Membranes	0. Normal 1. Present – mucous noticeable 2. Excessive – animal has an excessive amount of mucous present
Muscle Tone	0. Normal - muscles are resilient and firm and the hind legs go through their full range of motion. 1. Increased - muscles are rigid, hind limbs will not go through their full range of motion. 2. Decreased - muscles are flaccid, hind limbs have little or no resistance to movement
Palpebral Closure	0. Eyes wide open 1. Eyes halfway shut 2. Eyes completely shut
Piloerection	0. Absent 1. Present
Posture	0. Normal (awake) – alert, sitting, standing, or rearing 1. Normal (sleeping) – curled up, usually with head down 2. Hunched – abnormal posture 3. Flattened (prone) – limbs spread out lying flat or on one side
Respiratory Pattern	0. Normal 1. Slow 2. Rapid 3. Rales (Moist or Dry) 4. Gasping 5. Labored - Dyspnea
Salivation	0. None 1. Present - salivation is noticeable around the edge of the mouth 2. Excessive - salivation extends to the fur around the jaw
Tremors	0. None 1. Slight – localized to one area, or a twitch/spasm of a localized area 2. Severe – more than one area or involving whole body 3. Fasciculation – wave-like ripples of a muscle or group of muscles
Unusual Behaviors	0. Absent 1. Present – Be specific in describing all unusual behaviors on data sheet.
Urination	0. None/Normal 1. Excessive
Vocalization, removal from cage	0. Absent 1. Present - animal vocalizes unprovoked or continuously vocalizes when being handled.
Vocalizations, open field observations	0. Absent 1. Present
Manipulative Tests	
Pupillary reflex	0. Normal 1. Slow or absent- pupil reaction is slow or absent.

APPENDIX F: INDIVIDUAL ANIMAL DETAILED CLINICAL OBSERVATIONS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Removal from Cage)													
	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Palpebral Closure	Palpebral Closure	Palpebral Closure	Palpebral Closure
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

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Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Removal from Cage)													
	Palpebral Closure	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Eye	Eye	Eye	Eye	Eye	Mucous Membranes	Mucous Membranes	Mucous Membranes
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
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Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Removal from Cage)													
	Mucous Membranes	Mucous Membranes	Salivation	Salivation	Salivation	Salivation	Salivation	Emaciation	Emaciation	Emaciation	Emaciation	Emaciation	Piloerection	Piloerection
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Removal from Cage)													
	Piloerection	Piloerection	Piloerection	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Respiratory Pattern
	28	35	42	14	21	28	35	42	14	21	28	35	42	14
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	3	3	3	3	3	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	3	3	3	3	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	3	3	3	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Removal from Cage)									DetClinObs (Open Field Obs)				
	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal
	21	28	35	42	14	21	28	35	42	14	21	28	35	42
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Open Field Obs)													
	Convulsions	Convulsions	Convulsions	Convulsions	Convulsions	Tremors	Tremors	Tremors	Tremors	Tremors	Posture	Posture	Posture	Posture
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Open Field Obs)													
	Posture	Gait	Gait	Gait	Gait	Gait	Locomotion	Locomotion	Locomotion	Locomotion	Locomotion	Defecation	Defecation	Defecation
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Open Field Obs)													
	Defecation	Defecation	Urination	Urination	Urination	Urination	Urination	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Vocalization (OF)	Vocalization (OF)
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7001	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
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Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	DetClinObs (Open Field Obs)		
	Vocalization (OF)	Vocalization (OF)	Vocalization (OF)
	28	35	42
7001	0	0	0
7002	0	0	0
7003	0	0	0
7004	0	0	0
7005	0	0	0
7006	0	0	0
7007	0	0	0
7008	0	0	0
7009	0	0	0
7010	0	0	0
7011	0	0	0
7012	0	0	0
7013	0	0	0
7014	0	0	0
7015	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Removal from Cage)													
	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Palpebral Closure	Palpebral Closure	Palpebral Closure	Palpebral Closure
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

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Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Removal from Cage)													
	Palpebral Closure	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Eye	Eye	Eye	Eye	Eye	Mucous Membranes	Mucous Membranes	Mucous Membranes
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

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Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Removal from Cage)													
	Mucous Membranes	Mucous Membranes	Salivation	Salivation	Salivation	Salivation	Salivation	Emaciation	Emaciation	Emaciation	Emaciation	Emaciation	Piloerection	Piloerection
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Removal from Cage)													
	Piloerection	Piloerection	Piloerection	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Respiratory Pattern
	28	35	42	14	21	28	35	42	14	21	28	35	42	14
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	3	3	3	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	3	3	3	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	3	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Removal from Cage)									DetClinObs (Open Field Obs)				
	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal
	21	28	35	42	14	21	28	35	42	14	21	28	35	42
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Open Field Obs)													
	Convulsions	Convulsions	Convulsions	Convulsions	Convulsions	Tremors	Tremors	Tremors	Tremors	Tremors	Posture	Posture	Posture	Posture
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Open Field Obs)													
	Posture	Gait	Gait	Gait	Gait	Gait	Locomotion	Locomotion	Locomotion	Locomotion	Locomotion	Defecation	Defecation	Defecation
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Open Field Obs)													
	Defecation	Defecation	Urination	Urination	Urination	Urination	Urination	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Vocalization (OF)	Vocalization (OF)
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7016	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7017	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7018	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7019	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7020	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7021	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7022	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7023	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7024	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7025	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7026	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7027	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7028	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7029	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7030	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	DetClinObs (Open Field Obs)		
	Vocalization (OF)	Vocalization (OF)	Vocalization (OF)
	28	35	42
7016	0	0	0
7017	0	0	0
7018	0	0	0
7019	0	0	0
7020	0	0	0
7021	0	0	0
7022	0	0	0
7023	0	0	0
7024	0	0	0
7025	0	0	0
7026	0	0	0
7027	0	0	0
7028	0	0	0
7029	0	0	0
7030	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Removal from Cage)													
	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Palpebral Closure	Palpebral Closure	Palpebral Closure	Palpebral Closure
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Removal from Cage)													
	Palpebral Closure	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Eye	Eye	Eye	Eye	Eye	Mucous Membranes	Mucous Membranes	Mucous Membranes
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Removal from Cage)													
	Mucous Membranes	Mucous Membranes	Salivation	Salivation	Salivation	Salivation	Salivation	Emaciation	Emaciation	Emaciation	Emaciation	Emaciation	Piloerection	Piloerection
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Removal from Cage)													
	Piloerection	Piloerection	Piloerection	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Respiratory Pattern
	28	35	42	14	21	28	35	42	14	21	28	35	42	14
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	3	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	3	3	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Removal from Cage)									DetClinObs (Open Field Obs)				
	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal
	21	28	35	42	14	21	28	35	42	14	21	28	35	42
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Open Field Obs)													
	Convulsions	Convulsions	Convulsions	Convulsions	Convulsions	Tremors	Tremors	Tremors	Tremors	Tremors	Posture	Posture	Posture	Posture
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Open Field Obs)													
	Posture	Gait	Gait	Gait	Gait	Gait	Locomotion	Locomotion	Locomotion	Locomotion	Locomotion	Defecation	Defecation	Defecation
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Open Field Obs)													
	Defecation	Defecation	Urination	Urination	Urination	Urination	Urination	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Vocalization (OF)	Vocalization (OF)
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7031	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7032	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7033	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7034	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7035	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7036	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7037	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7038	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7039	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7040	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7041	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7042	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7043	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7044	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7045	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	DetClinObs (Open Field Obs)		
	Vocalization (OF)	Vocalization (OF)	Vocalization (OF)
	28	35	42
7031	0	0	0
7032	0	0	0
7033	0	0	0
7034	0	0	0
7035	0	0	0
7036	0	0	0
7037	0	0	0
7038	0	0	0
7039	0	0	0
7040	0	0	0
7041	0	0	0
7042	0	0	0
7043	0	0	0
7044	0	0	0
7045	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Removal from Cage)													
	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Handling Reactivity	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Vocalization (RC)	Palpebral Closure	Palpebral Closure	Palpebral Closure	Palpebral Closure
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Removal from Cage)													
	Palpebral Closure	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Lacrimation	Eye	Eye	Eye	Eye	Eye	Mucous Membranes	Mucous Membranes	Mucous Membranes
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Removal from Cage)													
	Mucous Membranes	Mucous Membranes	Salivation	Salivation	Salivation	Salivation	Salivation	Emaciation	Emaciation	Emaciation	Emaciation	Emaciation	Piloerection	Piloerection
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Removal from Cage)													
	Piloerection	Piloerection	Piloerection	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Fur/Skin	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Muscle Tone	Respiratory Pattern
	28	35	42	14	21	28	35	42	14	21	28	35	42	14
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	3	3	3	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Removal from Cage)									DetClinObs (Open Field Obs)				
	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Respiratory Pattern	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Pupillary Reflex	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal	Activity/ Arousal
	21	28	35	42	14	21	28	35	42	14	21	28	35	42
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Open Field Obs)													
	Convulsions	Convulsions	Convulsions	Convulsions	Convulsions	Tremors	Tremors	Tremors	Tremors	Tremors	Posture	Posture	Posture	Posture
	14	21	28	35	42	14	21	28	35	42	14	21	28	35
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Open Field Obs)													
	Posture	Gait	Gait	Gait	Gait	Gait	Locomotion	Locomotion	Locomotion	Locomotion	Locomotion	Defecation	Defecation	Defecation
	42	14	21	28	35	42	14	21	28	35	42	14	21	28
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Open Field Obs)													
	Defecation	Defecation	Urination	Urination	Urination	Urination	Urination	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Unusual Behaviors	Vocalization (OF)	Vocalization (OF)
	35	42	14	21	28	35	42	14	21	28	35	42	14	21
7046	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7047	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7048	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7049	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7050	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7051	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7052	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7053	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7054	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7055	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7056	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7057	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7058	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7059	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7060	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Individual Animal Detailed Clinical Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	DetClinObs (Open Field Obs)		
	Vocalization (OF)	Vocalization (OF)	Vocalization (OF)
	28	35	42
7046	0	0	0
7047	0	0	0
7048	0	0	0
7049	0	0	0
7050	0	0	0
7051	0	0	0
7052	0	0	0
7053	0	0	0
7054	0	0	0
7055	0	0	0
7056	0	0	0
7057	0	0	0
7058	0	0	0
7059	0	0	0
7060	0	0	0

APPENDIX G: INDIVIDUAL ANIMAL ESTROUS CYCLES

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

0 mg/kg/day Group 1	Day(s) Relative to Start Date									
	0	1	2	3	4	5	6	7	8	9
7001	E	D	D	P	E	D	D	P	E	D
7002	D	D	P	E	D	D	P	E	D	D
7003	E	D	D	P	E	D	D	P	E	D
7004	D	D	E	D	D	D	E	D	D	P
7005	P	E	D	D	P	E	D	D	P	E
7006	D	D	E	D	D	P	E	D	D	P
7007	D	P	E	D	D	D	E	D	D	P
7008	D	D	E	D	P	E	E	D	D	P
7009	D	P	E	D	D	P	E	D	D	P
7010	D	P	E	D	P	P	E	D	D	P
7011	D	D	P	D	P	E	D	D	P	E
7012	D	P	E	E	D	P	E	D	D	P
7013	D	D	P	E	D	D	P	E	D	D
7014	D	P	E	D	D	P	E	D	D	P
7015	D	D	E	E	D	D	P	E	D	D

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

0 mg/kg/day Group 1	Day(s) Relative to Start Date									
	10	11	12	13	29	30	31	32	33	34
7001	D	D	E	D	E	E	D	D	P	E
7002	P	E	D	D	D	P	E	D	D	P
7003	D	P	E	D	D	D	E	E	D	D
7004	E	D	D	P	P	E	D	D	P	E
7005	D	D	P	E	E	D	D	D	E	D
7006	E	D	D	P	P	E	D	D	P	E
7007	P	D	D	P	E	D	D	D	E	D
7008	E	D	D	P	D	E	P	D	D	P
7009	E	D	D	P	P	E	D	D	P	E
7010	P	D	D	P	E	D	D	D	E	D
7011	D	D	P	E	E	D	D	D	E	D
7012	E	D	D	P	D	E	D	D	P	E
7013	P	E	D	D	D	P	E	D	D	P
7014	E	D	D	P	P	E	D	D	P	E
7015	P	E	D	D	D	P	E	D	D	P

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

0 mg/kg/day Group 1	Day(s) Relative to Start Date								
	35	36	37	38	39	40	41	42	43
7001	E	D	D	D	P	E	D	D	P
7002	E	D	D	P	E	D	P	E	E
7003	P	E	D	D	P	E	D	D	D
7004	D	D	P	E	D	D	P	E	D
7005	D	P	E	E	D	D	E	D	D
7006	D	D	P	E	D	D	E	E	D
7007	D	P	E	D	D	P	P	E	D
7008	E	D	D	E	D	D	P	E	D
7009	D	P	P	E	D	P	P	D	D
7010	D	P	E	E	D	D	D	E	D
7011	D	P	E	D	D	P	E	D	D
7012	D	D	P	E	D	D	D	E	D
7013	E	D	D	P	E	E	D	D	D
7014	D	D	P	E	D	D	P	E	D
7015	E	D	D	P	E	D	D	P	E

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

512 mg/kg/day Group 2	Day(s) Relative to Start Date									
	0	1	2	3	4	5	6	7	8	9
7016	D	P	E	D	P	E	D	D	P	E
7017	E	E	D	D	P	E	D	D	P	E
7018	P	E	D	D	P	E	D	D	P	E
7019	D	D	E	E	D	P	E	D	D	P
7020	D	E	D	D	P	E	E	D	D	P
7021	P	D	D	P	D	D	E	E	D	D
7022	P	E	D	D	E	E	D	D	P	E
7023	E	E	D	D	P	E	D	D	P	E
7024	P	E	D	D	P	E	D	D	P	E
7025	P	E	D	D	P	E	D	D	P	E
7026	D	P	E	D	D	D	P	E	D	D
7027	E	D	D	P	E	D	D	P	E	D
7028	D	D	P	E	D	D	D	E	D	D
7029	P	E	D	D	P	E	D	D	P	E
7030	D	E	D	D	P	E	D	D	P	E

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

512 mg/kg/day Group 2	Day(s) Relative to Start Date									
	10	11	12	13	29	30	31	32	33	34
7016	D	D	P	E	D	D	D	D	E	D
7017	E	D	P	E	E	D	D	D	D	E
7018	D	D	P	E	E	D	D	P	E	D
7019	E	D	D	P	P	E	E	D	D	E
7020	E	D	D	P	D	P	E	E	D	D
7021	P	E	E	D	P	E	D	D	P	E
7022	D	D	P	E	E	D	D	P	E	D
7023	D	D	D	E	E	D	D	E	E	D
7024	D	D	P	E	E	D	D	P	E	D
7025	D	D	P	E	E	D	D	P	E	D
7026	P	E	E	D	D	P	D	D	D	P
7027	D	D	E	D	D	D	P	E	D	D
7028	P	E	D	D	D	P	E	P	D	D
7029	D	D	P	E	E	D	D	D	E	D
7030	D	D	P	E	E	D	D	E	E	D

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

512 mg/kg/day Group 2	Day(s) Relative to Start Date								
	35	36	37	38	39	40	41	42	43
7016	D	P	E	D	D	P	E	D	D
7017	E	D	D	P	E	D	D	P	D
7018	D	P	E	D	D	P	E	D	D
7019	D	D	P	E	D	D	P	E	D
7020	P	E	E	D	D	P	E	D	D
7021	M	D	D	P	P	E	E	D	D
7022	D	P	E	D	D	P	E	D	D
7023	D	P	E	D	D	P	E	D	D
7024	D	P	E	D	D	P	E	D	D
7025	D	P	E	D	D	P	E	D	D
7026	E	D	D	P	E	E	D	D	D
7027	P	E	D	D	D	E	D	P	E
7028	E	D	D	P	E	D	D	P	E
7029	D	P	E	D	D	P	E	D	D
7030	D	P	E	E	D	D	P	E	D

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

1024 mg/kg/day Group 3	Day(s) Relative to Start Date									
	0	1	2	3	4	5	6	7	8	9
7031	D	D	D	E	E	D	D	P	E	E
7032	E	E	D	D	P	E	D	D	P	E
7033	P	E	D	D	P	E	D	D	P	E
7034	P	E	D	D	P	E	D	D	P	E
7035	P	E	D	D	P	E	D	D	P	E
7036	P	E	D	D	P	E	D	D	P	E
7037	E	E	D	P	E	E	D	D	P	E
7038	P	E	D	D	P	E	D	D	P	E
7039	D	D	P	E	D	D	P	E	D	D
7040	D	D	D	P	D	D	D	P	E	D
7041	D	P	E	E	E	D	D	P	E	D
7042	D	P	E	D	D	P	E	D	D	P
7043	P	E	D	D	P	E	D	D	P	E
7044	D	P	E	D	D	P	E	D	D	P
7045	D	D	E	E	D	D	P	E	D	D

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

1024 mg/kg/day Group 3	Day(s) Relative to Start Date									
	10	11	12	13	29	30	31	32	33	34
7031	D	D	P	E	D	D	D	D	P	E
7032	D	D	E	D	E	D	D	D	E	D
7033	D	D	P	E	E	D	D	P	E	D
7034	D	D	P	E	E	D	D	D	E	D
7035	D	D	P	E	E	D	D	P	E	D
7036	D	D	P	E	E	D	D	E	E	D
7037	D	D	P	E	E	D	D	D	E	D
7038	D	D	D	E	E	D	D	D	E	D
7039	P	E	D	D	D	P	E	D	D	P
7040	D	P	E	D	D	D	P	E	D	D
7041	D	D	P	E	D	D	P	E	E	D
7042	E	E	D	D	D	P	E	D	D	P
7043	E	D	D	P	E	D	D	P	E	D
7044	E	D	D	P	P	E	D	D	D	E
7045	E	D	D	P	D	D	E	D	D	P

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

1024 mg/kg/day Group 3	Day(s) Relative to Start Date								
	35	36	37	38	39	40	41	42	43
7031	D	D	P	P	E	D	E	D	D
7032	D	P	E	D	P	E	E	D	D
7033	D	P	E	D	D	P	E	D	D
7034	D	P	E	D	D	P	E	D	D
7035	D	P	E	D	D	P	E	D	D
7036	D	P	E	D	D	P	E	D	D
7037	D	P	E	D	D	P	E	D	D
7038	D	P	E	D	D	P	E	D	D
7039	E	D	D	P	E	D	D	P	E
7040	P	E	D	D	P	E	D	D	D
7041	D	P	E	E	D	D	P	E	E
7042	E	D	D	P	E	D	D	P	E
7043	D	P	E	D	P	P	E	D	D
7044	D	D	P	E	D	D	P	E	D
7045	E	E	D	D	D	E	D	D	D

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

1536 mg/kg/day Group 4	Day(s) Relative to Start Date									
	0	1	2	3	4	5	6	7	8	9
7046	P	E	D	D	P	P	E	D	D	P
7047	D	D	E	D	D	P	E	D	D	P
7048	E	E	D	D	P	E	D	D	P	E
7049	D	E	D	D	P	E	D	D	P	E
7050	E	E	D	D	P	E	D	D	P	E
7051	D	P	E	E	D	P	E	D	D	P
7052	D	D	D	P	E	D	D	P	E	E
7053	E	D	D	P	E	D	D	P	E	D
7054	D	P	E	D	D	P	E	D	D	P
7055	E	D	D	P	E	D	D	P	E	D
7056	P	E	D	D	P	E	D	D	P	E
7057	D	P	E	D	D	D	E	D	D	P
7058	P	E	D	D	P	E	D	D	P	E
7059	E	D	D	P	E	D	D	P	E	D
7060	E	D	D	P	E	D	D	P	E	D

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

1536 mg/kg/day Group 4	Day(s) Relative to Start Date									
	10	11	12	13	29	30	31	32	33	34
7046	E	E	D	D	P	E	D	D	P	E
7047	E	D	D	P	P	E	D	D	P	E
7048	D	D	D	E	D	D	D	D	D	E
7049	D	D	P	E	E	D	D	P	E	D
7050	D	D	D	E	E	D	D	P	E	D
7051	E	D	D	P	P	E	D	D	P	E
7052	D	D	D	D	D	D	P	P	E	D
7053	D	D	E	D	D	D	P	E	D	D
7054	E	D	D	P	D	P	E	D	D	P
7055	D	P	E	D	D	D	D	E	D	D
7056	D	D	D	P	P	E	D	D	E	D
7057	E	D	D	E	E	E	D	D	P	E
7058	D	D	P	E	E	D	D	D	E	D
7059	D	P	E	D	D	D	E	E	D	D
7060	D	P	E	D	D	D	P	E	D	D

Individual Animal Estrus Cycles

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Stage of Estrus

1536 mg/kg/day Group 4	Day(s) Relative to Start Date								
	35	36	37	38	39	40	41	42	43
7046	D	D	P	E	E	D	D	P	E
7047	D	D	P	E	D	D	D	E	D
7048	D	D	P	E	D	D	P	E	D
7049	D	P	E	D	D	P	E	D	D
7050	D	P	E	D	D	P	E	D	D
7051	E	D	D	D	P	E	D	D	D
7052	D	P	E	D	P	E	D	P	E
7053	P	E	D	D	P	E	D	D	D
7054	E	D	D	P	E	D	D	P	E
7055	P	E	D	D	P	E	D	D	D
7056	D	P	E	D	D	P	E	D	D
7057	D	D	P	E	D	D	P	E	D
7058	D	P	E	D	D	P	E	D	D
7059	P	E	D	D	P	E	E	D	D
7060	P	E	D	D	P	E	D	D	P

APPENDIX H: INDIVIDUAL ANIMAL WEEKLY BODY WEIGHTS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Mean Weekly Body Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Bodyweight (g)

0 mg/kg/day Group 1	Day(s) Relative to Start Date						
	0	7	14	21	28	35	42
7001	160	186	200	209	214	218	229
7002	180	196	211	220	235	244	263
7003	182	209	231	246	249	258	270
7004	159	174	182	196	202	208	209
7005	200	229	238	248	263	273	287
7006	170	197	214	233	243	243	250
7007	195	204	220	256	264	273	275
7008	191	216	232	246	253	261	266
7009	191	205	208	225	232	235	247
7010	174	200	218	226	237	242	250
7011	174	189	212	225	234	246	254
7012	179	191	203	222	224	224	231
7013	176	199	218	238	246	249	264
7014	164	181	192	198	199	203	212
7015	200	208	226	237	240	234	242
Mean	179.7	198.9	213.7	228.3	235.7	240.7	249.9
SD	13.5	14.0	15.3	17.8	19.5	21.2	22.4
N	15	15	15	15	15	15	15

Individual Animal Mean Weekly Body Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Bodyweight (g)

512 mg/kg/day Group 2	Day(s) Relative to Start Date						
	0	7	14	21	28	35	42
7016	200	215	227	231	252	255	265
7017	163	179	191	204	211	215	223
7018	185	198	201	210	222	229	237
7019	195	217	229	258	276	279	288
7020	176	192	209	224	235	240	247
7021	187	202	219	235	247	252	267
7022	180	200	212	222	237	244	252
7023	174	189	202	201	215	222	224
7024	175	194	207	226	235	247	251
7025	201	223	239	256	268	279	289
7026	171	191	215	230	243	243	265
7027	167	195	214	221	232	242	237
7028	159	177	194	207	213	214	226
7029	193	216	233	242	258	267	277
7030	176	190	199	200	213	221	228
Mean	180.1	198.5	212.7	224.5	237.1	243.3	251.7
SD	13.0	13.8	14.5	18.3	20.4	21.0	22.7
N	15	15	15	15	15	15	15

Individual Animal Mean Weekly Body Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Bodyweight (g)

1024 mg/kg/day Group 3	Day(s) Relative to Start Date						
	0	7	14	21	28	35	42
7031	174	190	192	200	200	202	220
7032	172	198	213	231	239	251	262
7033	204	221	233	240	264	270	269
7034	199	224	237	238	248	262	273
7035	180	204	219	221	233	246	253
7036	157	173	182	188	202	213	214
7037	190	200	199	204	213	224	237
7038	183	198	212	221	242	251	260
7039	176	196	214	229	234	241	259
7040	162	195	210	221	223	233	242
7041	194	217	237	249	255	272	282
7042	176	181	198	211	214	217	221
7043	164	183	202	205	226	234	252
7044	192	217	236	260	275	282	290
7045	169	203	209	230	237	240	253
Mean	179.5	200.2	212.9	223.2	233.7	242.5	252.5
SD	14.0	14.7	17.1	19.5	21.6	23.0	22.4
N	15	15	15	15	15	15	15

Individual Animal Mean Weekly Body Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Bodyweight (g)

1536 mg/kg/day Group 4	Day(s) Relative to Start Date						
	0	7	14	21	28	35	42
7046	198	220	239	253	272	271	285
7047	190	210	233	257	281	278	279
7048	193	213	217	240	248	262	275
7049	182	199	215	228	249	263	276
7050	175	191	200	207	210	223	232
7051	204	220	233	258	272	272	288
7052	159	184	196	214	217	228	241
7053	186	220	246	262	267	283	292
7054	198	205	217	232	235	244	261
7055	160	173	184	193	190	195	203
7056	169	188	198	207	215	217	225
7057	173	189	197	205	212	215	218
7058	176	208	224	235	246	256	263
7059	177	208	230	245	253	279	282
7060	171	187	205	216	218	229	240
Mean	180.7	201.0	215.6	230.1	239.0	247.7	257.3
SD	13.8	14.9	18.4	22.2	27.6	27.9	28.6
N	15	15	15	15	15	15	15

APPENDIX I: INDIVIDUAL ANIMAL MEAN DAILY BODY WEIGHT GAIN

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Mean Daily Body Weight Gain

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Body Weight Gain (g/day)

0 mg/kg/day Group 1	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7001	3.7	2.0	1.3	0.7	0.6	1.6	1.6
7002	2.3	2.1	1.3	2.1	1.3	2.7	2.0
7003	3.9	3.1	2.1	0.4	1.3	1.7	2.1
7004	2.1	1.1	2.0	0.9	0.9	0.1	1.2
7005	4.1	1.3	1.4	2.1	1.4	2.0	2.1
7006	3.9	2.4	2.7	1.4	0.0	1.0	1.9
7007	1.3	2.3	5.1	1.1	1.3	0.3	1.9
7008	3.6	2.3	2.0	1.0	1.1	0.7	1.8
7009	2.0	0.4	2.4	1.0	0.4	1.7	1.3
7010	3.7	2.6	1.1	1.6	0.7	1.1	1.8
7011	2.1	3.3	1.9	1.3	1.7	1.1	1.9
7012	1.7	1.7	2.7	0.3	0.0	1.0	1.2
7013	3.3	2.7	2.9	1.1	0.4	2.1	2.1
7014	2.4	1.6	0.9	0.1	0.6	1.3	1.1
7015	1.1	2.6	1.6	0.4	-0.9	1.1	1.0
Mean	2.75	2.10	2.10	1.05	0.72	1.31	1.67
SD	1.02	0.77	1.05	0.61	0.68	0.69	0.38
N	15	15	15	15	15	15	15

Individual Animal Mean Daily Body Weight Gain

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Body Weight Gain (g/day)

512 mg/kg/day Group 2	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7016	2.1	1.7	0.6	3.0	0.4	1.4	1.5
7017	2.3	1.7	1.9	1.0	0.6	1.1	1.4
7018	1.9	0.4	1.3	1.7	1.0	1.1	1.2
7019	3.1	1.7	4.1	2.6	0.4	1.3	2.2
7020	2.3	2.4	2.1	1.6	0.7	1.0	1.7
7021	2.1	2.4	2.3	1.7	0.7	2.1	1.9
7022	2.9	1.7	1.4	2.1	1.0	1.1	1.7
7023	2.1	1.9	-0.1	2.0	1.0	0.3	1.2
7024	2.7	1.9	2.7	1.3	1.7	0.6	1.8
7025	3.1	2.3	2.4	1.7	1.6	1.4	2.1
7026	2.9	3.4	2.1	1.9	0.0	3.1	2.2
7027	4.0	2.7	1.0	1.6	1.4	-0.7	1.7
7028	2.6	2.4	1.9	0.9	0.1	1.7	1.6
7029	3.3	2.4	1.3	2.3	1.3	1.4	2.0
7030	2.0	1.3	0.1	1.9	1.1	1.0	1.2
Mean	2.63	2.03	1.68	1.81	0.88	1.21	1.70
SD	0.59	0.69	1.08	0.56	0.51	0.85	0.34
N	15	15	15	15	15	15	15

Individual Animal Mean Daily Body Weight Gain

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Body Weight Gain (g/day)

1024 mg/kg/day Group 3	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7031	2.3	0.3	1.1	0.0	0.3	2.6	1.1
7032	3.7	2.1	2.6	1.1	1.7	1.6	2.1
7033	2.4	1.7	1.0	3.4	0.9	-0.1	1.5
7034	3.6	1.9	0.1	1.4	2.0	1.6	1.8
7035	3.4	2.1	0.3	1.7	1.9	1.0	1.7
7036	2.3	1.3	0.9	2.0	1.6	0.1	1.4
7037	1.4	-0.1	0.7	1.3	1.6	1.9	1.1
7038	2.1	2.0	1.3	3.0	1.3	1.3	1.8
7039	2.9	2.6	2.1	0.7	1.0	2.6	2.0
7040	4.7	2.1	1.6	0.3	1.4	1.3	1.9
7041	3.3	2.9	1.7	0.9	2.4	1.4	2.1
7042	1.1	2.0	1.9	0.4	0.4	0.6	1.1
7043	2.7	2.7	0.4	3.0	1.1	2.6	2.1
7044	3.6	2.7	3.4	2.1	1.0	1.1	2.3
7045	4.9	0.9	3.0	1.0	0.4	1.9	2.0
Mean	2.96	1.81	1.48	1.50	1.27	1.42	1.74
SD	1.07	0.89	0.99	1.04	0.62	0.82	0.41
N	15	15	15	15	15	15	15

Individual Animal Mean Daily Body Weight Gain

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Body Weight Gain (g/day)

1536 mg/kg/day Group 4	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7046	3.1	2.7	2.0	2.7	-0.1	2.0	2.1
7047	2.9	3.3	3.4	3.4	-0.4	0.1	2.1
7048	2.9	0.6	3.3	1.1	2.0	1.9	2.0
7049	2.4	2.3	1.9	3.0	2.0	1.9	2.2
7050	2.3	1.3	1.0	0.4	1.9	1.3	1.4
7051	2.3	1.9	3.6	2.0	0.0	2.3	2.0
7052	3.6	1.7	2.6	0.4	1.6	1.9	2.0
7053	4.9	3.7	2.3	0.7	2.3	1.3	2.5
7054	1.0	1.7	2.1	0.4	1.3	2.4	1.5
7055	1.9	1.6	1.3	-0.4	0.7	1.1	1.0
7056	2.7	1.4	1.3	1.1	0.3	1.1	1.3
7057	2.3	1.1	1.1	1.0	0.4	0.4	1.1
7058	4.6	2.3	1.6	1.6	1.4	1.0	2.1
7059	4.4	3.1	2.1	1.1	3.7	0.4	2.5
7060	2.3	2.6	1.6	0.3	1.6	1.6	1.6
Mean	2.90	2.09	2.08	1.27	1.24	1.38	1.82
SD	1.07	0.88	0.83	1.09	1.10	0.69	0.48
N	15	15	15	15	15	15	15

APPENDIX J: INDIVIDUAL ANIMAL MEAN DAILY FOOD CONSUMPTION

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Mean Daily Food Consumption

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Food Consumption (g/day)

0 mg/kg/day Group 1	Day(s) Relative to Start Date										
	3 → 7	7 → 10	10 → 14	14 → 17	17 → 21	21 → 24	24 → 28	28 → 31	31 → 35	35 → 38	3 → 38
7001	13.3	18.0	19.8	20.0	19.5	17.7	18.3	18.0	16.3	18.7	17.9
7002	26.0	21.0	22.8	19.0	20.0	17.3	21.5	20.3	21.0	23.0	21.3
7003	9.3	19.0	19.0	19.3	20.5	19.7	18.8	19.0	18.3	18.3	18.0
7004	23.0	15.3	17.0	17.0	18.8	16.3	16.5	16.3	16.8	17.0	17.5
7005	16.0	30.0	21.8	19.7	22.5	24.0	22.0	22.3	21.3	22.3	22.0
7006	18.8	19.3	20.3	22.7	20.8	19.7	21.0	17.3	19.8	20.0	20.0
7007	19.3	17.7	13.8	25.7	22.3	20.3	25.3	22.3	21.0	21.0	20.8
7008	19.8	18.0	21.8	21.7	21.0	20.0	19.0	19.3	19.3	21.0	20.1
7009	21.5	20.7	20.3	24.0	22.0	20.7	20.0	23.0	30.0	22.7	22.5
7010	18.0	18.0	19.0	18.0	19.3	20.0	18.5	18.0	18.3	19.0	18.6
7011	16.8	19.0	20.8	19.0	20.3	20.0	19.5	19.7	19.0	19.3	19.3
7012	17.8	17.7	17.8	18.7	19.5	17.3	17.0	16.7	16.3	19.7	17.8
7013	20.0	21.7	19.5	23.3	23.0	19.3	22.8	21.7	21.5	24.7	21.7
7014	16.5	16.0	17.5	18.0	18.0	17.3	23.0	16.0	16.3	16.7	17.6
7015	17.8	19.3	18.5	18.7	20.5	17.0	22.0	16.7	17.3	19.0	18.7
Mean	18.23	19.38	19.28	20.31	20.52	19.11	20.33	19.11	19.47	20.16	19.59
SD	3.93	3.40	2.25	2.55	1.44	1.97	2.44	2.38	3.48	2.27	1.73
N	15	15	15	15	15	15	15	15	15	15	15

Individual Animal Mean Daily Food Consumption

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Food Consumption (g/day)

512 mg/kg/day Group 2	Day(s) Relative to Start Date										
	3 — 7	7 — 10	10 — 14	14 — 17	17 — 21	21 — 24	24 — 28	28 — 31	31 — 35	35 — 38	3 — 38
7016	18.0	16.0	17.8	17.3	19.3	19.7	18.5	18.7	16.8	18.0	18.0
7017	16.5	16.7	17.8	20.0	20.3	18.7	19.3	18.3	18.8	21.3	18.7
7018	17.0	15.0	16.5	17.0	18.0	17.3	16.8	17.3	16.8	17.7	16.9
7019	23.8	21.0	22.0	24.7	25.5	22.7	25.0	22.7	23.3	25.0	23.6
7020	17.5	19.3	20.0	18.7	19.5	18.3	21.0	20.0	20.0	19.0	19.4
7021	20.8	19.7	19.8	18.3	23.0	21.3	20.5	20.7	19.5	22.0	20.6
7022	18.5	15.7	18.8	18.7	19.3	20.0	19.3	18.3	18.8	18.0	18.6
7023	17.0	16.0	18.5	25.0	18.3	20.0	18.0	17.7	16.8	20.3	18.6
7024	17.3	15.7	18.3	21.7	21.5	20.3	20.0	19.3	19.8	19.3	19.3
7025	21.0	18.7	20.5	19.3	22.0	21.7	21.8	21.3	21.0	21.3	20.9
7026	19.8	21.0	20.8	19.0	25.8	21.0	23.0	21.7	21.3	24.7	21.8
7027	19.0	18.3	19.0	18.0	19.3	18.3	19.8	21.0	18.0	16.7	18.8
7028	17.0	18.3	18.3	17.3	18.5	16.3	20.0	17.3	17.0	19.3	18.0
7029	20.5	19.3	21.3	19.7	21.8	21.7	21.3	21.0	20.8	21.7	20.9
7030	17.8	16.7	18.8	15.7	19.0	19.7	17.0	18.3	17.5	20.0	18.0
Mean	18.75	17.82	19.18	19.36	20.72	19.80	20.07	19.58	19.05	20.29	19.47
SD	2.03	2.00	1.50	2.64	2.48	1.75	2.18	1.73	1.97	2.43	1.76
N	15	15	15	15	15	15	15	15	15	15	15

Individual Animal Mean Daily Food Consumption

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Food Consumption (g/day)

1024 mg/kg/day Group 3	Day(s) Relative to Start Date										
	3 → 7	7 → 10	10 → 14	14 → 17	17 → 21	21 → 24	24 → 28	28 → 31	31 → 35	35 → 38	3 → 38
7031	16.8	15.0	16.5	15.7	16.3	13.3	15.5	17.7	14.3	19.3	16.0
7032	20.3	20.0	21.8	24.0	23.0	22.3	21.3	22.0	21.0	21.0	21.6
7033	19.5	19.0	20.0	19.0	20.5	21.7	24.3	20.3	20.8	19.7	20.5
7034	24.0	23.0	27.0	21.3	22.0	20.7	23.5	23.0	23.8	25.7	23.5
7035	19.5	18.0	18.8	18.0	18.8	20.0	19.5	19.7	18.8	20.3	19.1
7036	16.8	15.7	16.3	15.7	17.0	17.3	17.5	19.0	16.8	17.0	16.9
7037	16.5	15.0	16.0	15.3	15.8	15.7	16.5	16.7	17.5	18.7	16.4
7038	18.5	19.0	19.5	18.0	22.0	19.3	21.5	21.7	22.5	23.7	20.6
7039	17.8	19.0	19.5	18.3	19.5	16.3	18.0	18.0	18.3	20.0	18.5
7040	19.8	18.7	18.5	23.7	19.3	17.7	23.3	21.0	17.8	17.7	19.7
7041	20.3	19.7	23.0	22.0	22.5	18.7	23.3	23.0	20.3	20.7	21.4
7042	16.3	16.7	17.5	17.3	18.3	17.7	17.3	17.3	17.3	17.3	17.3
7043	20.8	17.3	20.8	18.0	20.0	20.0	21.3	20.3	19.8	21.7	20.1
7044	17.8	19.0	26.0	20.7	21.8	21.0	21.3	23.0	21.0	22.0	21.4
7045	19.8	18.0	18.5	21.0	20.5	17.3	18.8	19.3	19.0	19.3	19.2
Mean	18.93	18.20	19.97	19.20	19.80	18.60	20.17	20.13	19.23	20.27	19.47
SD	2.07	2.09	3.30	2.79	2.28	2.45	2.80	2.14	2.42	2.35	2.16
N	15	15	15	15	15	15	15	15	15	15	15

Individual Animal Mean Daily Food Consumption

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Mean Daily Food Consumption (g/day)

1536 mg/kg/day Group 4	Day(s) Relative to Start Date										
	3 → 7	7 → 10	10 → 14	14 → 17	17 → 21	21 → 24	24 → 28	28 → 31	31 → 35	35 → 38	3 → 38
7046	19.0	20.3	20.8	20.0	24.5	21.0	20.3	20.0	19.8	22.7	20.8
7047	18.3	19.0	22.3	22.3	21.5	20.3	22.5	19.3	21.0	21.7	20.9
7048	19.5	18.3	19.5	21.0	21.3	19.7	20.3	21.0	21.5	24.3	20.6
7049	17.5	19.0	19.8	18.3	22.0	22.7	21.3	22.3	22.0	22.3	20.7
7050	16.5	15.7	16.3	14.7	17.0	14.7	15.5	15.7	16.3	18.0	16.1
7051	20.8	23.7	26.3	21.0	22.8	18.7	22.3	20.7	18.8	23.0	21.8
7052	17.0	17.0	18.0	19.0	19.0	17.0	22.3	19.7	17.8	18.7	18.6
7053	20.3	20.3	21.8	20.3	22.8	19.7	24.5	22.0	20.5	20.3	21.3
7054	17.0	17.0	18.0	18.7	18.8	15.0	17.3	17.7	18.8	20.3	17.9
7055	15.5	16.3	16.5	16.0	17.3	14.3	15.8	17.3	15.3	17.0	16.1
7056	17.5	17.0	17.5	17.3	17.0	15.7	16.3	16.3	15.5	15.7	16.6
7057	19.5	21.7	22.5	20.7	17.8	15.7	16.3	17.3	16.5	17.7	18.5
7058	19.8	19.3	21.0	20.7	22.3	20.7	23.5	20.0	19.8	19.3	20.7
7059	23.5	23.0	21.0	20.0	23.5	22.0	22.5	25.3	24.5	23.0	22.9
7060	16.8	19.3	18.0	16.7	18.3	16.0	21.8	18.7	17.5	18.0	18.1
Mean	18.55	19.13	19.93	19.11	20.37	18.20	20.13	19.56	19.02	20.13	19.44
SD	2.06	2.38	2.68	2.15	2.60	2.86	3.09	2.54	2.63	2.61	2.17
N	15	15	15	15	15	15	15	15	15	15	15

APPENDIX K: INDIVIDUAL ANIMAL FOOD EFFICIENCY[1]

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

[1] Food efficiency = Mean Daily Body Weight Gain / Mean Daily Food Consumption

Individual Animal Mean Food Efficiency

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Food Efficiency

0 mg/kg/day Group 1	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7001	0.20	0.11	0.07	0.04	0.03	0.09	0.09
7002	0.11	0.10	0.07	0.11	0.06	0.12	0.10
7003	0.21	0.17	0.11	0.02	0.07	0.09	0.11
7004	0.13	0.07	0.11	0.05	0.05	0.01	0.07
7005	0.20	0.05	0.07	0.09	0.07	0.09	0.09
7006	0.20	0.12	0.13	0.07	0.00	0.05	0.10
7007	0.07	0.15	0.22	0.05	0.06	0.01	0.09
7008	0.19	0.11	0.09	0.05	0.06	0.03	0.09
7009	0.09	0.02	0.11	0.05	0.02	0.08	0.06
7010	0.20	0.14	0.06	0.08	0.04	0.06	0.10
7011	0.13	0.16	0.09	0.07	0.09	0.06	0.10
7012	0.10	0.10	0.14	0.02	0.00	0.05	0.07
7013	0.17	0.13	0.12	0.05	0.02	0.09	0.10
7014	0.15	0.09	0.05	0.01	0.04	0.07	0.07
7015	0.06	0.14	0.08	0.02	-0.05	0.06	0.05
Mean	0.149	0.110	0.100	0.052	0.037	0.065	0.085
SD	0.053	0.041	0.042	0.029	0.035	0.031	0.017
N	15	15	15	15	15	15	15

Individual Animal Mean Food Efficiency

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Food Efficiency

512 mg/kg/day Group 2	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7016	0.12	0.10	0.03	0.16	0.02	0.08	0.09
7017	0.14	0.10	0.09	0.05	0.03	0.06	0.08
7018	0.11	0.03	0.07	0.10	0.06	0.06	0.07
7019	0.14	0.08	0.16	0.11	0.02	0.05	0.09
7020	0.13	0.12	0.11	0.08	0.04	0.05	0.09
7021	0.10	0.12	0.11	0.08	0.04	0.10	0.09
7022	0.15	0.10	0.08	0.11	0.05	0.06	0.09
7023	0.13	0.11	-0.01	0.11	0.06	0.01	0.06
7024	0.16	0.11	0.13	0.06	0.09	0.03	0.09
7025	0.16	0.12	0.12	0.08	0.07	0.07	0.10
7026	0.15	0.16	0.09	0.08	0.00	0.14	0.10
7027	0.21	0.15	0.05	0.08	0.07	-0.04	0.09
7028	0.15	0.13	0.10	0.05	0.01	0.09	0.09
7029	0.16	0.12	0.06	0.11	0.06	0.07	0.10
7030	0.12	0.07	0.01	0.10	0.06	0.05	0.07
Mean	0.142	0.108	0.081	0.091	0.046	0.059	0.087
SD	0.026	0.032	0.046	0.027	0.026	0.040	0.012
N	15	15	15	15	15	15	15

Individual Animal Mean Food Efficiency

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Food Efficiency

1024 mg/kg/day Group 3	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7031	0.13	0.02	0.07	0.00	0.02	0.14	0.07
7032	0.19	0.10	0.11	0.05	0.08	0.07	0.10
7033	0.12	0.09	0.05	0.15	0.04	-0.01	0.08
7034	0.15	0.07	0.01	0.06	0.09	0.06	0.08
7035	0.18	0.12	0.02	0.09	0.10	0.05	0.09
7036	0.14	0.08	0.05	0.11	0.09	0.01	0.08
7037	0.08	-0.01	0.05	0.08	0.09	0.10	0.07
7038	0.12	0.10	0.06	0.15	0.06	0.06	0.09
7039	0.16	0.13	0.11	0.04	0.06	0.13	0.11
7040	0.24	0.12	0.07	0.01	0.07	0.07	0.10
7041	0.17	0.13	0.08	0.04	0.11	0.06	0.10
7042	0.07	0.12	0.10	0.02	0.02	0.03	0.06
7043	0.14	0.14	0.02	0.14	0.06	0.12	0.10
7044	0.20	0.12	0.16	0.10	0.05	0.05	0.11
7045	0.25	0.05	0.14	0.06	0.02	0.09	0.10
Mean	0.157	0.092	0.074	0.074	0.064	0.070	0.089
SD	0.052	0.044	0.045	0.049	0.029	0.042	0.016
N	15	15	15	15	15	15	15

Individual Animal Mean Food Efficiency

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Food Efficiency

1536 mg/kg/day Group 4	Day(s) Relative to Start Date						
	0 → 7	7 → 14	14 → 21	21 → 28	28 → 35	35 → 42	0 → 42
7046	0.16	0.13	0.09	0.13	-0.01	0.09	0.10
7047	0.16	0.16	0.16	0.16	-0.02	0.01	0.10
7048	0.15	0.03	0.16	0.06	0.09	0.08	0.09
7049	0.14	0.12	0.09	0.14	0.09	0.08	0.11
7050	0.14	0.08	0.06	0.03	0.12	0.07	0.08
7051	0.11	0.07	0.16	0.10	0.00	0.11	0.09
7052	0.21	0.10	0.14	0.02	0.08	0.10	0.11
7053	0.24	0.18	0.11	0.03	0.11	0.06	0.12
7054	0.06	0.10	0.11	0.03	0.07	0.12	0.08
7055	0.12	0.10	0.08	-0.03	0.04	0.07	0.06
7056	0.16	0.08	0.08	0.07	0.02	0.07	0.08
7057	0.12	0.05	0.06	0.06	0.03	0.02	0.06
7058	0.24	0.11	0.07	0.07	0.07	0.05	0.10
7059	0.21	0.14	0.10	0.05	0.15	0.02	0.11
7060	0.13	0.14	0.09	0.01	0.09	0.08	0.09
Mean	0.157	0.106	0.103	0.062	0.062	0.069	0.093
SD	0.050	0.040	0.035	0.051	0.050	0.032	0.017
N	15	15	15	15	15	15	15

APPENDIX L: INDIVIDUAL ANIMAL MEAN DAILY DIETARY INTAKE OF SOY LEGHEMOGLOBIN PREPARATION

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Mean Daily Dietary Intake

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Dietary Intake Variable (mg/kg/day)

0 mg/kg/day Group 1	Day(s) Relative to Start Date								
	10 → 14	14 → 17	17 → 21	21 → 24	24 → 28	28 → 31	31 → 35	35 → 38	3 → 38
7001	0	0	0	0	0	0	0	0	0
7002	0	0	0	0	0	0	0	0	0
7003	0	0	0	0	0	0	0	0	0
7004	0	0	0	0	0	0	0	0	0
7005	0	0	0	0	0	0	0	0	0
7006	0	0	0	0	0	0	0	0	0
7007	0	0	0	0	0	0	0	0	0
7008	0	0	0	0	0	0	0	0	0
7009	0	0	0	0	0	0	0	0	0
7010	0	0	0	0	0	0	0	0	0
7011	0	0	0	0	0	0	0	0	0
7012	0	0	0	0	0	0	0	0	0
7013	0	0	0	0	0	0	0	0	0
7014	0	0	0	0	0	0	0	0	0
7015	0	0	0	0	0	0	0	0	0
Mean	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SD	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
N	15	15	15	15	15	15	15	15	15

Individual Animal Mean Daily Dietary Intake

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Dietary Intake Variable (mg/kg/day)

512 mg/kg/day Group 2	Day(s) Relative to Start Date								
	10 → 14	14 → 17	17 → 21	21 → 24	24 → 28	28 → 31	31 → 35	35 → 38	3 → 38
7016	458	448	477	487	446	451	424	456	460
7017	545	614	568	524	555	528	563	640	564
7018	481	496	491	472	459	475	472	498	482
7019	563	632	566	503	551	499	538	578	565
7020	561	524	498	468	543	518	538	511	531
7021	529	491	560	520	505	509	499	563	537
7022	519	516	496	516	494	470	496	476	502
7023	537	726	520	569	509	500	487	591	545
7024	517	614	544	515	518	500	516	505	525
7025	503	474	492	484	494	484	486	493	498
7026	566	518	641	523	576	542	564	655	587
7027	521	493	498	475	518	551	480	444	513
7028	552	524	511	452	571	495	513	583	539
7029	535	495	514	512	501	495	501	524	519
7030	552	462	544	563	485	523	511	584	531
Mean	529.2	535.0	528.0	505.5	515.0	502.7	505.8	540.1	526.6
SD	30.7	76.8	43.0	33.3	38.2	27.0	36.2	64.6	33.0
N	15	15	15	15	15	15	15	15	15

Individual Animal Mean Daily Dietary Intake

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Dietary Intake Variable (mg/kg/day)

1024 mg/kg/day Group 3	Day(s) Relative to Start Date								
	10 → 14	14 → 17	17 → 21	21 → 24	24 → 28	28 → 31	31 → 35	35 → 38	3 → 38
7031	976	926	949	779	952	1085	895	1214	976
7032	1159	1279	1164	1130	1093	1131	1061	1061	1147
7033	975	926	998	1055	1129	946	975	924	1004
7034	1293	1022	1080	1015	1164	1140	1150	1242	1166
7035	972	933	991	1058	1028	1037	967	1048	1021
7036	1014	977	1057	1077	1065	1156	997	1012	1058
7037	913	875	902	897	952	962	991	1057	945
7038	1044	964	1163	1022	1092	1100	1137	1196	1100
7039	1035	973	995	833	945	945	960	1053	996
7040	1000	1280	1018	934	1281	1157	966	962	1093
7041	1102	1054	1056	876	1120	1108	944	964	1044
7042	1004	994	1011	978	991	995	1008	1013	1013
7043	1166	1012	1140	1140	1155	1106	1070	1174	1146
7044	1251	994	978	944	950	1028	944	989	1010
7045	1005	1141	1042	881	972	1002	1004	1022	1028
Mean	1060.6	1023.3	1036.3	974.7	1059.3	1059.9	1004.7	1062.1	1049.8
SD	110.3	121.0	76.0	108.4	100.6	76.3	71.4	99.0	66.8
N	15	15	15	15	15	15	15	15	15

Individual Animal Mean Daily Dietary Intake

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Dietary Intake Variable (mg/kg/day)

1536 mg/kg/day Group 4	Day(s) Relative to Start Date								
	10 → 14	14 → 17	17 → 21	21 → 24	24 → 28	28 → 31	31 → 35	35 → 38	3 → 38
7046	1470	1417	1725	1479	1416	1399	1442	1655	1526
7047	1617	1623	1490	1409	1523	1309	1495	1542	1523
7048	1522	1639	1577	1460	1553	1611	1624	1838	1605
7049	1556	1444	1719	1771	1623	1706	1655	1680	1648
7050	1376	1242	1463	1262	1404	1419	1442	1597	1432
7051	1908	1526	1571	1289	1556	1445	1364	1673	1599
7052	1555	1642	1582	1415	1950	1724	1541	1620	1644
7053	1497	1400	1547	1337	1745	1567	1433	1422	1536
7054	1405	1457	1440	1152	1396	1430	1521	1649	1449
7055	1519	1473	1592	1323	1577	1735	1548	1725	1585
7056	1497	1483	1463	1348	1438	1445	1413	1429	1489
7057	1934	1777	1543	1361	1458	1555	1519	1626	1675
7058	1588	1563	1687	1567	1817	1546	1527	1494	1629
7059	1546	1473	1709	1600	1692	1905	1738	1631	1730
7060	1487	1377	1505	1320	1898	1629	1512	1555	1581
Mean	1565.2	1502.3	1574.2	1406.2	1603.1	1561.6	1518.2	1609.2	1576.8
SD	157.5	131.2	96.8	153.1	181.1	159.8	97.7	109.5	84.0
N	15	15	15	15	15	15	15	15	15

APPENDIX M: ANIMAL NUMBERS, DOSE GROUPS, AND FATES

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Fates

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrous cycle Determination

Group	Dose Level	Sex	Animal	Cage	Removal Day	Removal Week	Removal Date	Removal Time	Time Slot	Removal Symptom	Pathology Reason
1	0 mg/kg/day	Female	7001	7001	43	6	23/03/17	9:08	.	Term	Term
			7002	7002	43	6	23/03/17	9:08	.	Term	Term
			7003	7003	43	6	23/03/17	9:08	.	Term	Term
			7004	7004	43	6	23/03/17	9:08	.	Term	Term
			7005	7005	43	6	23/03/17	9:08	.	Term	Term
			7006	7006	43	6	23/03/17	9:08	.	Term	Term
			7007	7007	43	6	23/03/17	9:08	.	Term	Term
			7008	7008	43	6	23/03/17	9:08	.	Term	Term
			7009	7009	43	6	23/03/17	9:08	.	Term	Term
			7010	7010	43	6	23/03/17	9:08	.	Term	Term
			7011	7011	43	6	23/03/17	9:08	.	Term	Term
			7012	7012	43	6	23/03/17	9:08	.	Term	Term
			7013	7013	43	6	23/03/17	9:08	.	Term	Term
			7014	7014	43	6	23/03/17	9:09	.	Term	Term
			7015	7015	43	6	23/03/17	9:09	.	Term	Term
2	512 mg/kg/day	Female	7016	7016	43	6	23/03/17	9:09	.	Term	Term
			7017	7017	43	6	23/03/17	9:09	.	Term	Term
			7018	7018	43	6	23/03/17	9:09	.	Term	Term
			7019	7019	43	6	23/03/17	9:09	.	Term	Term
			7020	7020	43	6	23/03/17	9:09	.	Term	Term
			7021	7021	43	6	23/03/17	9:09	.	Term	Term
			7022	7022	43	6	23/03/17	9:09	.	Term	Term
			7023	7023	43	6	23/03/17	9:09	.	Term	Term
			7024	7024	43	6	23/03/17	9:09	.	Term	Term
			7025	7025	43	6	23/03/17	9:09	.	Term	Term
			7026	7026	43	6	23/03/17	9:09	.	Term	Term
			7027	7027	43	6	23/03/17	9:09	.	Term	Term
			7028	7028	43	6	23/03/17	9:09	.	Term	Term
			7029	7029	43	6	23/03/17	9:09	.	Term	Term
			7030	7030	43	6	23/03/17	9:09	.	Term	Term

Individual Animal Fates

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrous cycle Determination

Group	Dose Level	Sex	Animal	Cage	Removal Day	Removal Week	Removal Date	Removal Time	Time Slot	Removal Symptom	Pathology Reason
3	1024 mg/kg/day	Female	7031	7031	43	6	23/03/17	9:09	.	Term	Term
			7032	7032	43	6	23/03/17	9:09	.	Term	Term
			7033	7033	43	6	23/03/17	9:09	.	Term	Term
			7034	7034	43	6	23/03/17	9:09	.	Term	Term
			7035	7035	43	6	23/03/17	9:09	.	Term	Term
			7036	7036	43	6	23/03/17	9:09	.	Term	Term
			7037	7037	43	6	23/03/17	9:09	.	Term	Term
			7038	7038	43	6	23/03/17	9:09	.	Term	Term
			7039	7039	43	6	23/03/17	9:10	.	Term	Term
			7040	7040	43	6	23/03/17	9:10	.	Term	Term
			7041	7041	43	6	23/03/17	9:10	.	Term	Term
			7042	7042	43	6	23/03/17	9:10	.	Term	Term
			7043	7043	43	6	23/03/17	9:10	.	Term	Term
			7044	7044	43	6	23/03/17	9:10	.	Term	Term
			7045	7045	43	6	23/03/17	9:10	.	Term	Term
4	1536 mg/kg/day	Female	7046	7046	43	6	23/03/17	9:10	.	Term	Term
			7047	7047	43	6	23/03/17	9:10	.	Term	Term
			7048	7048	43	6	23/03/17	9:10	.	Term	Term
			7049	7049	43	6	23/03/17	9:10	.	Term	Term
			7050	7050	43	6	23/03/17	9:10	.	Term	Term
			7051	7051	43	6	23/03/17	9:10	.	Term	Term
			7052	7052	43	6	23/03/17	9:10	.	Term	Term
			7053	7053	43	6	23/03/17	9:10	.	Term	Term
			7054	7054	43	6	23/03/17	9:10	.	Term	Term
			7055	7055	43	6	23/03/17	9:10	.	Term	Term
			7056	7056	43	6	23/03/17	9:10	.	Term	Term
			7057	7057	43	6	23/03/17	9:10	.	Term	Term
			7058	7058	43	6	23/03/17	9:10	.	Term	Term
			7059	7059	43	6	23/03/17	9:10	.	Term	Term
			7060	7060	43	6	23/03/17	9:11	.	Term	Term

APPENDIX N: INDIVIDUAL ANIMAL NECROPSY OBSERVATIONS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Animal:	7001	Group:	1	Sex:	Female
		Dose:	0		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7002	Group:	1	Sex:	Female
		Dose:	0		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7003	Group:	1	Sex:	Female
		Dose:	0		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

uterus : fluid filled

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7004	Group:	1	Sex:	Female
		Dose:	0		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7005	Group:	1	Sex:	Female
		Dose:	0		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7006	Group:	1	Sex:	Female
		Dose:	0		

Necropsy Date: 3/23/2017

Last Clinical Observations:

Alopecia, Abdomen, Slight
Alopecia, Left Forepaw, Moderate

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Last Clinical Observations (Continued):

Alopecia, Right Forepaw, Moderate

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7007 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7008 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7009 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7010 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7011 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7012 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7013 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

uterus : fluid filled

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7014 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7015 Group: 1 Sex: Female
Dose: 0
Necropsy Date: 3/23/2017

Last Clinical Observations:

Alopecia, Left Forepaw, Slight

Gross Pathology Observations [Correlation]:

non correlated finding : no correlated finding [Alopecia, Left Forepaw, Slight (C)]

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7016 Group: 2 Sex: Female
Dose: 512
Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7017 Group: 2 Sex: Female
Dose: 512

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7018 Group: 2 Sex: Female
Dose: 512

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7019 Group: 2 Sex: Female
Dose: 512

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7020 Group: 2 Sex: Female
Dose: 512

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7021 Group: 2 Sex: Female
Dose: 512

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7022 Group: 2 Sex: Female
Dose: 512

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Animal:	7023	Group:	2	Sex:	Female
		Dose:	512		
Necropsy Date:	3/23/2017				

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7024	Group:	2	Sex:	Female
		Dose:	512		
Necropsy Date:	3/23/2017				

Last Clinical Observations:

Alopecia, Left Forepaw, Slight
Alopecia, Right Forepaw, Slight

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7025	Group:	2	Sex:	Female
		Dose:	512		
Necropsy Date:	3/23/2017				

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7026	Group:	2	Sex:	Female
		Dose:	512		
Necropsy Date:	3/23/2017				

Gross Pathology Observations [Correlation]:

uterus : fluid filled

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7027	Group:	2	Sex:	Female
		Dose:	512		
Necropsy Date:	3/23/2017				

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7028	Group:	2	Sex:	Female
		Dose:	512		
Necropsy Date:	3/23/2017				

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7029 Group: 2 Sex: Female
Dose: 512

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7030 Group: 2 Sex: Female
Dose: 512

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7031 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7032 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7033 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7034 Group: 3 Sex: Female
Dose: 1024

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7035 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7036 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7037 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7038 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7039 Group: 3 Sex: Female
Dose: 1024

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Animal:	7040	Group:	3	Sex:	Female
		Dose:	1024		

Necropsy Date: 3/23/2017

Last Clinical Observations:

Alopecia, Left Forepaw, Slight
Alopecia, Right Forepaw, Slight

Gross Pathology Observations [Correlation]:

uterus : fluid filled

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7041	Group:	3	Sex:	Female
		Dose:	1024		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7042	Group:	3	Sex:	Female
		Dose:	1024		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7043	Group:	3	Sex:	Female
		Dose:	1024		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7044	Group:	3	Sex:	Female
		Dose:	1024		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7045	Group:	3	Sex:	Female
		Dose:	1024		

Necropsy Date: 3/23/2017

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7046 Group: 4 Sex: Female
Dose: 1536

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7047 Group: 4 Sex: Female
Dose: 1536

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

urinary bladder : 13x5x8 mm
urinary bladder : thick
urinary bladder : urolith

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7048 Group: 4 Sex: Female
Dose: 1536

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7049 Group: 4 Sex: Female
Dose: 1536

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal: 7050 Group: 4 Sex: Female
Dose: 1536

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Animal:	7051	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations (Correlation):

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7052	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations (Correlation):

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7053	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations (Correlation):

uterus : fluid filled

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7054	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations (Correlation):

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7055	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations (Correlation):

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7056	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations (Correlation):

No observations found

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7057	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7058	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7059	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

No observations found

Any remaining protocol required tissues, which have been examined, have no visible lesions

Animal:	7060	Group:	4	Sex:	Female
		Dose:	1536		

Necropsy Date: 3/23/2017

Gross Pathology Observations [Correlation]:

uterus : fluid filled

Any remaining protocol required tissues, which have been examined, have no visible lesions

Individual Animal Necropsy Observations

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Key Page

Codes

(TGL) = Trackable Gross Lesion, (MPF) = Major Pathological Finding, (?) = Questionable, (E) = Excluded
(C) = Clinical Observation, (M) = Mass, (G) = Gross Pathology, (H) = Histo Pathology

Group Information

Short Name	Long Name
1	1
2	2
3	3
4	4

APPENDIX O: INDIVIDUAL ANIMAL TERMINAL BODY AND ORGAN WEIGHTS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Terminal Body and Organ Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	Terminal BW (g)	Ovaries with Oviducts Wt (g)	Uterus Wt (g)
7001	229	0.120	0.83
7002	258	0.172	0.72
7003	269	0.137	0.97
7004	217	0.130	0.35
7005	291	0.125	0.54
7006	249	0.134	0.49
7007	271	0.128	0.58
7008	271	0.109	0.40
7009	247	0.158	0.69
7010	252	0.139	0.47
7011	255	0.129	0.57
7012	234	0.109	0.37
7013	258	0.129	1.10
7014	210	0.108	0.42
7015	237	0.139	0.56
Mean	249.9	0.1311	0.604
SD	21.8	0.0174	0.221
N	15	15	15

Individual Animal Terminal Body and Organ Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	Terminal BW (g)	Ovaries with Oviducts Wt (g)	Uterus Wt (g)
7016	267	0.174	0.54
7017	224	0.106	0.48
7018	236	0.124	0.49
7019	290	0.144	0.54
7020	251	0.153	0.55
7021	269	0.171	0.57
7022	256	0.123	0.49
7023	230	0.132	0.44
7024	253	0.154	0.65
7025	290	0.106	0.47
7026	260	0.123	0.84
7027	235	0.128	0.51
7028	223	0.117	0.58
7029	282	0.130	0.62
7030	231	0.130	0.43
Mean	253.1	0.1343	0.547
SD	23.1	0.0209	0.102
N	15	15	15

Individual Animal Terminal Body and Organ Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	Terminal BW (g)	Ovaries with Oviducts Wt (g)	Uterus Wt (g)
7031	225	0.124	0.51
7032	261	0.128	0.55
7033	273	0.130	0.55
7034	276	0.122	0.55
7035	254	0.152	0.53
7036	218	0.106	0.48
7037	238	0.118	0.47
7038	259	0.123	0.55
7039	259	0.137	0.53
7040	239	0.122	1.14
7041	279	0.120	0.53
7042	218	0.104	0.60
7043	251	0.124	0.53
7044	297	0.136	0.47
7045	253	0.105	0.56
Mean	253.3	0.1234	0.570
SD	22.9	0.0128	0.162
N	15	15	15

Individual Animal Terminal Body and Organ Weights

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	Terminal BW (g)	Ovaries with Oviducts Wt (g)	Uterus Wt (g)
7046	287	0.147	0.84
7047	288	0.140	0.60
7048	282	0.157	0.52
7049	274	0.143	0.60
7050	235	0.149	0.54
7051	288	0.160	0.71
7052	239	0.143	0.48
7053	289	0.129	1.08
7054	258	0.146	0.62
7055	202	0.111	0.87
7056	229	0.129	0.55
7057	221	0.118	0.47
7058	267	0.127	0.69
7059	290	0.110	0.74
7060	236	0.146	1.24
Mean	259.0	0.1370	0.703
SD	29.6	0.0156	0.223
N	15	15	15

APPENDIX P: INDIVIDUAL ANIMAL ORGAN-TO-BODY WEIGHT RATIOS

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Individual Animal Organ-to-Body Weight Ratios

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

0 mg/kg/day Group 1	Ovaries with oviducts/TBW (Ratio)	Uterus /TBW (Ratio)
7001	0.524	3.62
7002	0.667	2.79
7003	0.509	3.61
7004	0.599	1.61
7005	0.430	1.86
7006	0.538	1.97
7007	0.472	2.14
7008	0.402	1.48
7009	0.640	2.79
7010	0.552	1.87
7011	0.506	2.24
7012	0.466	1.58
7013	0.500	4.26
7014	0.514	2.00
7015	0.586	2.36
Mean	0.5270	2.412
SD	0.0733	0.841
N	15	15

Individual Animal Organ-to-Body Weight Ratios

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

512 mg/kg/day Group 2	Ovaries with oviducts/TBW (Ratio)	Uterus /TBW (Ratio)
7016	0.652	2.02
7017	0.473	2.14
7018	0.525	2.08
7019	0.497	1.86
7020	0.610	2.19
7021	0.636	2.12
7022	0.480	1.91
7023	0.574	1.91
7024	0.609	2.57
7025	0.366	1.62
7026	0.473	3.23
7027	0.545	2.17
7028	0.525	2.60
7029	0.461	2.20
7030	0.563	1.86
Mean	0.5325	2.166
SD	0.0772	0.390
N	15	15

Individual Animal Organ-to-Body Weight Ratios

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1024 mg/kg/day Group 3	Ovaries with oviducts/TBW (Ratio)	Uterus /TBW (Ratio)
7031	0.551	2.27
7032	0.490	2.11
7033	0.476	2.01
7034	0.442	1.99
7035	0.598	2.09
7036	0.486	2.20
7037	0.496	1.97
7038	0.475	2.12
7039	0.529	2.05
7040	0.510	4.77
7041	0.430	1.90
7042	0.477	2.75
7043	0.494	2.11
7044	0.458	1.58
7045	0.415	2.21
Mean	0.4886	2.276
SD	0.0467	0.731
N	15	15

Individual Animal Organ-to-Body Weight Ratios

PSL Study Number 44856
Soy Leghemoglobin Preparation: An Investigative 28-Day Dietary Study
in Rats with a 14-Day Pre-Dosing Estrus Cycle Determination

Sex: Female Day(s) Relative to Start Date

1536 mg/kg/day Group 4	Ovaries with oviducts/TBW (Ratio)	Uterus /TBW (Ratio)
7046	0.512	2.93
7047	0.486	2.08
7048	0.557	1.84
7049	0.522	2.19
7050	0.634	2.30
7051	0.556	2.47
7052	0.598	2.01
7053	0.446	3.74
7054	0.566	2.40
7055	0.550	4.31
7056	0.563	2.40
7057	0.534	2.13
7058	0.476	2.58
7059	0.379	2.55
7060	0.619	5.25
Mean	0.5332	2.745
SD	0.0667	0.957
N	15	15

APPENDIX Q: INDIVIDUAL ANIMAL BLIND HISTOPATHOLOGAL ESTROUS CYCLE EVALUATION

Submitted By:

Regan Path/Tox Services
1457 Township Rd. 853
Ashland, OH 44805

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

INDIVIDUAL ANIMAL ESTROUS CYCLES

Blind Histopathological Determination* (Day 43)

Group	Animal ID	Stage of Estrous Cycle
1 0 mg/kg/day	7001	P
	7002	E
	7003	P
	7004	M
	7005	D
	7006	M
	7007	E
	7008	M
	7009	D
	7010	M
	7011	D[1]
	7012	M
	7013	P
	7014	M
	7015	E
2 512 mg/kg/day	7016	D[1]
	7017	D[1]
	7018	D
	7019	M
	7020	D
	7021	D
	7022	D[1]
	7023	D
	7024	D[1]
	7025	D[1]
	7026	P
	7027	PE[2]
	7028	E
	7029	D
	7030	M

P – Proestrus, E – Estrus, M – Metestrus, D – Diestrus

* Estrous cycle determination was based on the blind evaluation of the anterior portion of the vagina, the cervix, the uterine bifurcation and horns, the oviducgts and the ovary by light microscopy with the knowledge of the vaginal estrous cycle determined by cytology evlauation collected immediately prior to scheduled sacrifice.

[1] Diestrus with evidence of early proestrus

[2] Prolonged Estrus

INDIVIDUAL ANIMAL ESTROUS CYCLES

Blind Histopathological Determination* (Day 43)

Group	Animal ID	Stage of Estrous Cycle
3 1024 mg/kg/day	7031	D
	7032	D
	7033	D
	7034	D[1]
	7035	D
	7036	D
	7037	D
	7038	D
	7039	E
	7040	P
	7041	E
	7042	E
	7043	D[1]
	7044	M
	7045	P
4 1536 mg/kg/day	7046	P
	7047	E
	7048	M
	7049	D*
	7050	D
	7051	P
	7052	E
	7053	P
	7054	E
	7055	P
	7056	D[1]
	7057	M
	7058	D[1]
	7059	D
	7060	P

P – Proestrus, E – Estrus, M – Metestrus, D – Diestrus

* Estrous cycle determination was based on the blind evaluation of the anterior portion of the vagina, the cervix, the uterine bifurcation and horns, the oviducgts and the ovary by light microscopy with the knowledge of the vaginal estrous cycle determined by cytology evlauation collected immediately prior to scheduled sacrifice.

[1] Diestrus with evidence of early proestrus

APPENDIX R: HISTOPATHOLOGY

Submitted By:

Regan Path/Tox Services
1457 Township Rd. 853
Ashland, OH 44805

PRODUCT IDENTIFICATION

Soy Leghemoglobin Preparation

Regan Path/Tox Services, Inc

ANATOMIC PATHOLOGY REPORT
Final Report July 25, 2017

SOY LEGHEMOGLOBIN PREPARATION; AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY PRE-DOSING ESTRUS CYCLE DETERMINATION

STUDY NUMBER 44856

Prepared by:

Regan Path/Tox Services, Inc
1457 Township Rd. 853
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Submitted to:

Product Safety Labs
2394 US Highway 130
Dayton, New Jersey 08810

TABLE OF CONTENTS

INTRODUCTION

The objective of this study was to evaluate the potential reproductive toxicity (estrous cycle and reproductive organ histopathology) of Soy Leghemoglobin Preparation in female rats continuously exposed to the test substance in the diet for at least 28 days.

METHODS

The study design is presented in Text Table 1. The complete study design is described in the study protocol and amendment 1. Briefly, 60 female CRL: Sprague-Dawley CD® IGS rats, approximately 7-8 weeks old, were assigned to the study. The test substance was administered in the diet to three groups of 15 female rats at target dietary dose levels of 512, 1024 and 1536 mg/kg/day that corresponded to target dose levels of 250, 500 and 750 mg/kg/day of the active ingredient, Soy Leghemoglobin. Concentrations in the test diets were calculated based on the most recent group body weight and food consumption data. For 14 days prior to the start of dosing (study days 0-13), vaginal lavage was performed on all rats to evaluate for regular estrous cyclicity. Vaginal lavage was also performed on study days 29-42, and at study termination on day 43 to determine the stage of estrus at the scheduled necropsy. Necropsies and organ weight determinations were performed by Product Safety Labs. Tissue processing and slide preparation were performed by Histoserv, Inc Germantown, MD. Slides containing formalin-fixed, paraffin-embedded, H&E-stained sections of ovaries, oviducts, uterus, cervix and the anterior-most portion of the vagina for all animals were transferred to Regan Pathology/Toxicology Services, Inc, Ashland, OH, for microscopic examination by Karen S Regan DVM, Dipl ACVP, DABT. The initial examination was conducted with the pathologist blinded to treatment group and involved only estrous cycle staging for all animals (the cycle as determined from the final vaginal lavage on the day of scheduled necropsy was provided to the pathologist for this examination). Once this initial examination was completed, the slides were unblinded and the reproductive tissues (ovaries, oviducts, uterus, cervix and vagina) were examined microscopically for all animals in Groups 1 and 4. Gradable microscopic findings were given a severity score based upon a scale of minimal (grade 1), mild (grade 2), moderate (grade 3), marked (grade 4) and severe (grade 5). Clinical and necropsy observations and organ weight data were provided to the study pathologist for refinement of interpretation of results.

Text Table 1. Study Design

Group	Number of Females	Group Name	Target Exposure of Active Ingredient (mg/kg/day)	Target Dietary Dose Level of Test Substance (mg/kg/day)*
1	15	Basal Diet Control	0	0
2	15	Low Dose	250	512
3	15	Intermediate Dose	500	1024
4	15	High Dose	750	1536

*Based on 48.82% active ingredient (AI, Soy Leghemoglobin) of Soy Leghemoglobin Preparation (Lot # PP-PGM2-16-088-301)

RESULTS AND DISCUSSION

Mortality
There were no early deaths. All animals survived to the scheduled necropsy on Study Day 43.

Macroscopic Observations
There were no test substance-related macroscopic observations. The only macroscopic observations were urinary bladder thickened (size recorded as 13 x 5 x 8 mm) and urolith present for 4F 7047, and uterus fluid-filled for animals 1F 7003, 1F 7013, 2F 7026, 3F 7040, 4F 7053, and 4F 7060. Per protocol, the urinary bladder was not saved for microscopic examination. Fluid-filled uterus correlated with the proestrus stage of the estrous cycle as determined from the microscopic examination; dilated/fluid-filled uterus is a normal physiologic change at the proestrus stage of the estrous cycle. See Microscopic Observations section, below, for discussion on estrous cyclicity.

Organ Weights
There were no test article-related changes in the mean values of absolute weights of the ovaries with oviducts or the uterus.

Microscopic Observations
Individual animal estrous cycle stage determinations conducted without knowledge of treatment group are provided in Appendix Table 1. Summary microscopic observations are presented in Appendix Table 2, and Individual Animal Microscopic Data are presented in Appendix Table 3.

There were no test substance-related microscopic observations in the reproductive tissues examined. Determination of the stage of the estrous cycle was initially conducted with the pathologist blinded to treatment group, but the estrous cycle stage as determined from vaginal lavage smears obtained on the day of necropsy was provided for each animal. From these data alone, all but one animal (2F 7027) were considered to be cycling normally. Animal 2F 7027 appeared to have a prolonged estrus based on morphology of the ovaries (large atretic follicles, multiple CLs at a similar age/stage of atresia) and the presence of squamous epithelial metaplasia in the uterus. After treatment groups were revealed, this finding was considered spontaneous and incidental because of the lack of similar findings in animals at the higher dose levels. There were some differences between the assessment of cycle stage obtained from the vaginal lavage on the day of study termination and the cycle stage as determined from the microscopic examination. These differences may have arisen from timing (even a few hours between obtaining the vaginal lavage and euthanasia may cause differences) and/or the fact that only the anterior-most vagina was present for microscopic assessment. Generally, the section used for cycle assessment comes from the mid vagina. In some animals, very little vagina was present for microscopic exam.

Importantly, regarding estrous cycle data, determination of stage alone without morphologic examination and recording of the reproductive tissues for other changes is an incomplete assessment and can be potentially misleading without consideration of morphology of the reproductive tissues. In the current study, however, microscopic examination of the reproductive tissues for other changes was conducted on the control and Group 4 animals. There was no

evidence of a test substance-related effect in the tissues examined from these two groups. All animals in these two groups had evidence of old and recent corpora lutea (CL) and follicles at various stages of development in the ovaries, and had reproductive tissue morphology consistent with the stage of the cycle they were in. One control animal (7013) had large atretic follicles observed in both ovaries, and one Group 4 animal (7048) had luteinized follicles (follicles with evidence of luteinization in the wall but have not ovulated) in both ovaries. Both of these observations are reported as background findings observed in rats of the strain and age used in this study (Dixon et al, 2014) and were considered incidental because of their singular occurrences.

SUMMARY

The test substance, Soy Leghemoglobin Preparation, was administered in the diet to three groups of 15 female rats at target dietary dose levels of 512, 1024 and 1536 mg/kg/day that corresponded to target dose levels of 250, 500 and 750 mg/kg/day of the active ingredient, Soy Leghemoglobin. For 14 days prior to the start of dosing (study days 0-13), vaginal lavage was performed on all rats to evaluate for regular estrous cyclicity. Vaginal lavage was also performed on study days 29-42, and at study termination (study day 43) to determine the stage of estrus at the scheduled necropsy. The ovaries, oviducts, uterus, cervix and anterior-most portion of the vagina were collected from all animals for potential microscopic examination. The tissues for all animals were initially evaluated for stage of estrous cycle only; this was performed without knowledge of treatment group. Following this assessment, the tissues were evaluated microscopically from animals in Groups 1 and 4. There were no test substance-related changes in macroscopic or microscopic observations, organ weights or estrous cyclicity.

(b) (6)

Karen S Regan, DVM, DABT, DACVP
Study Pathologist

July 25 2017
Date

REFERENCE

Dixon, D; Alison, R; Bach, U; Colman, K; Foley, GL; Harleman, JH; Haworth, R; Herbert, R; Heuser, A; Long, G; Mirsky, M; Regan, K; van Esch, E; Westwood, FR; Vidal, J; Yoshida, Y. 2014. Nonproliferative and proliferative lesions of the rat and mouse female reproductive system, *J Toxicol Pathol* 27(3&4 Suppl):1S-107S

**SOY LEGHEMOGLOBIN PREPARATION:
AN INVESTIGATIVE 28-DAY DIETARY STUDY IN RATS WITH A 14-DAY
PRE-DOSING ESTRUS CYCLE DETERMINATION**

Summary and Individual Pathology Data Tables

Product Safety Labs
Study 44856

Appendix Table 1
Individual Animal Estrous Cycle Data

#	Slide ID	Day 43 Cytology Estrus	Blind estrus determination*	Animal ID	Group	Pertinent Macroscopic Observations
35	1078	P	P	**7001**	**Group 1**	none
34	1074	E	E	**7002**	**Group 1**	none
3	1003	D	P	**7003**	**Group 1**	uterus: fluid-filled
38	1081	D	M	**7004**	**Group 1**	none
36	1079	D	D	**7005**	**Group 1**	none
27	1061	D	M	**7006**	**Group 1**	none
11	1023	D	E	**7007**	**Group 1**	none
33	1072	D	M	**7008**	**Group 1**	none
5	1006	D	D	**7009**	**Group 1**	none
42	1090	D	M	**7010**	**Group 1**	none
7	1013	D	D*	**7011**	**Group 1**	none
10	1017	D	M	**7012**	**Group 1**	none
4	1005	D	P	**7013**	**Group 1**	uterus: fluid-filled
31	1066	D	M	**7014**	**Group 1**	none
59	1181	E	E	**7015**	**Group 1**	none
25	1056	D	D*	**7016**	**Group 2**	none
19	1042	D	D*	**7017**	**Group 2**	none
17	1040	D	D	**7018**	**Group 2**	none
13	1028	D	M	**7019**	**Group 2**	none
50	1105	D	D	**7020**	**Group 2**	none
49	1103	D	D	**7021**	**Group 2**	none
30	1064	D	D*	**7022**	**Group 2**	none
53	1117	D	D	**7023**	**Group 2**	none
22	1050	D	D*	**7024**	**Group 2**	none
28	1062	D	D*	**7025**	**Group 2**	none
45	1096	D	P	**7026**	**Group 2**	uterus: fluid-filled
32	1068	E	PE	**7027**	**Group 2**	none
15	1033	E	E	**7028**	**Group 2**	none
54	1123	D	D	**7029**	**Group 2**	none
48	1100	D	M	**7030**	**Group 2**	none
51	1113	D	D	**7031**	**Group 3**	none
6	1009	D	D	**7032**	**Group 3**	none
39	1084	D	D	**7033**	**Group 3**	none
29	1063	D	D*	**7034**	**Group 3**	none
1	1001	D	D	**7035**	**Group 3**	none
56	1163	D	D	**7036**	**Group 3**	none
2	1002	D	D	**7037**	**Group 3**	none
57	1164	D	D	**7038**	**Group 3**	none
41	1088	E	E	**7039**	**Group 3**	none
23	1051	D	P	**7040**	**Group 3**	uterus: fluid-filled
12	1024	E	E	**7041**	**Group 3**	none

*Late diestrus/early proestrus

Product Safety Labs
Study 44856

Appendix Table 1
Individual Animal Estrous Cycle Data

#	Slide ID	Day 43 Cytology Estrus	Blind estrus determination*	Animal ID	Group	Pertinent Macroscopic Observations
37	1080	E	E	**7042**	**Group 3**	none
58	1166	D	D*	**7043**	**Group 3**	none
43	1092	D	M	**7044**	**Group 3**	none
40	1087	D	P	**7045**	**Group 3**	none
18	1041	E	P	**7046**	**Group 4**	none
9	1016	D	E	**7047**	**Group 4**	none
46	1097	D	M	**7048**	**Group 4**	none
60	1264	D	D*	**7049**	**Group 4**	none
44	1093	D	D	**7050**	**Group 4**	none
24	1055	D	P	**7051**	**Group 4**	none
26	1058	E	E	**7052**	**Group 4**	none
20	1043	D	P	**7053**	**Group 4**	uterus: fluid-filled
8	1015	E	E	**7054**	**Group 4**	none
14	1031	D	P	**7055**	**Group 4**	none
55	1133	D	D*	**7056**	**Group 4**	none
52	1115	D	M	**7057**	**Group 4**	none
21	1049	D	D*	**7058**	**Group 4**	none
16	1038	D	D	**7059**	**Group 4**	none
47	1099	P	P	**7060**	**Group 4**	uterus: fluid-filled

*Late diestrus/early proestrus

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Appendix Table 2
Summary Animal Data - Groups 1 and 4

Group	1	4
	Incidence	Incidence
Tissue/Diagnosis		
Ovary (No. Examined)	**(15)**	**(15)**
Within normal limits	14	14
Large atretic follicles	1	0
Luteinized follicles	0	1
Uterus (No. Examined)	**(15)**	**(15)**
Within normal limits	15	15
Cervix (No. Examined)	**(15)**	**(15)**
Within normal limits	15	15
Vagina (No. Examined)	**(14)**	**(15)**
Not examined	1	0
Within normal limits	14	15
Oviducts (No. Examined)	**(15)**	**(15)**
Within normal limits	15	15

Product Safety Labs
Study No: 44856

Appendix Table 3
Individual Animal Data
Group 1

Animal Number (Group 1)	7001	7002	7003	7004	7005	7005	7007	7008	7009	7010	7011	7012	7013	7014	7015
Blind slide ID	1078	1074	1003	1081	1079	1061	1023	1072	1006	1090	1013	1017	1005	1066	1181
Tissue/Diagnosis															
Ovary															
Within normal limits	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y
Large atretic follicles													B,1		
Uterus															
Within normal limits	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Vagina'															
Not examined												Y			
Within normal limits	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y		Y	Y	Y
Cervix															
Within normal limits	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Estrous Cycle															
Stage	P	E	P	M	D	M	E	M	D	M	D*	M	P	M	E
Normal	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Oviduct															
Within normal limits	Y	Y	Y	Y	Y"	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y

" - One of pair present
Y - Present
B - Bilateral

*Late diestrus/early proestrus
' - Anterior vagina only

E - Estrus
D - Diestrus
M - Metestrus
P - Proestrus

Product Safety Labs
Study No: 44856

Appendix Table 3
Individual Animal Data
Group 4

Animal Number (Group 4)	7046	7047	7048	7049	7050	7051	7052	7053	7054	7055	7056	7057	7058	7059	7060
Blind slide ID	1041	1016	1097	1264	1093	1055	1058	1043	1015	1031	1133	1115	1049	1038	1099
Tissue/Diagnosis															
Ovary															
Within normal limits	Y	Y		Y	Y	Y	Y	Y^	Y	Y	Y	Y	Y	Y[4]	Y
Luteinized follicles			B,2												
Uterus															
Within normal limits	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Vagina'															
Within normal limits	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Cervix															
Within normal limits	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Estrous Cycle															
Stage	P	E	M	D*	D	P	E	P	E	P	D*	M	D*	D	P
Normal	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Oviduct															
Within normal limits	Y	Y"	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y"

" - One of pair present
Y - Present
' - Anterior vagina only

*Late diestrus/early proestrus
^Minimal tissue unilateral
[4] Minimal tissue bilateral

E - Estrus
D - Diestrus
M - Metestrus
P - Proestrus

Annex 4

STUDY TITLE

Soy Leghemoglobin Preparation :
Bacterial Reverse Mutation Test (Ames Test)

DATA REQUIREMENT

US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 1. a. (2007)

OECD Guidelines for Testing of Chemicals, Section 4 (Test No. 471): "Bacterial Reverse Mutation Test" (1997)

Commission regulation (EC) No 440/2008 B.13/14
"Mutagenicity-Reverse Mutation Test using Bacteria"

AUTHOR

Mithila Shitut, BVSc & AH, MS

STUDY COMPLETED ON

December 7, 2016

PERFORMING LABORATORY

Product Safety Labs

LABORATORY STUDY NUMBER

42759

SPONSOR

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No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA sec. 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA 10(g).

Submitter: (b) (6) Date: 12/7/16

Name of Signer: Rachel Fraser, PhD

Name of Company: Impossible Foods, Inc.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Soy Leghemoglobin Preparation

This study meets the requirements of Good Laboratory Practices as stated in U.S. FDA GLP: 21 CFR Part 58, 1987; which is compatible with OECD Principles of GLP (as revised in 1997): ENV/MC/CHEM(98)17, OECD, Paris, 1998; and EC Directive 2004/10/EC, Official Journal of the European Union, L50/44, Feb. 20, 2004 with the following exception:

Characterization of the positive control substances and verification of concentration of the positive control substances in their carriers during this study were not determined analytically; however, the purity of the materials used were certified by a reputable supplier and all preparations were thoroughly documented.

Specific information related to the characterization of the test substance as received and tested is the responsibility of the study Sponsor (see Test Substance section).

Study Director: (b) (6) Date: 12/07/16

Name of Signer: Mithila Shitut, BVSc & AH, MS

Name of Company: Product Safety Labs

Sponsor: (b) (6) Date: 12/7/16

Name of Signer: Rachel Fraser, PhD

Name of Company: Impossible Foods, Inc.

Submitter: (b) (6) Date: 12/7/16

Name of Signer: Rachel Fraser, PhD

Name of Company: Impossible Foods, Inc.

QUALITY ASSURANCE STATEMENT

The Product Safety Labs' Quality Assurance Unit has reviewed this final study report to assure the report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study.

QA activities for this study:

QA Activity	Date Conducted	Date Findings Reported To Study Director And Management
Protocol review	Aug 21, 2015[1]; May 17, 2016	Aug 21, 2015; May 17, 2016
In-process inspection: *Test substance preparation of main and confirmatory tests*	Apr 12, 2016	Apr 12, 2016
In-process inspection: *Main test colony counting of TA1535*	Apr 29, 2016	Apr 29, 2016
Raw data audit	May 17, 2016	May 17, 2016
Draft report review	May 17, 2016	May 17, 2016

Final report reviewed by:

(b) (6)

Maryann Zakrzewski
Quality Assurance Auditor
Product Safety Labs

Dec. 7, 2016
Date

[1] PSL's "generic" protocol used for this study was reviewed by the Quality Assurance group on this date.

TABLE OF CONTENTS

SOY LEGHEMOGLOBIN PREPARATION : BACTERIAL REVERSE MUTATION TEST (AMES TEST)

PROTOCOL NO.:	P600.AMES
AGENCY:	EPA (FIFRA), OECD, EC Directive
STUDY NUMBER:	42759
SPONSOR:	Impossible Foods, Inc. 525 Chesapeake Dr. Redwood City, CA 94063
TEST SUBSTANCE IDENTIFICATION:	Soy Leghemoglobin Preparation Lot #: PP-PGM2-16-015-101
DATE RECEIVED:	January 27, 2016
PSL REFERENCE NO:	160127-7D
STUDY INITIATION DATE:	March 29, 2016
DATES OF TEST:	April 12 - April 29, 2016
NOTEBOOK NO.:	16-42759: pages 1-96

1. PURPOSE

To evaluate the potential for Soy Leghemoglobin Preparation to induce gene mutations in bacteria using the Ames assay. Point mutations which involve substitution, addition or deletion of one or a few DNA base pairs are detected in amino acid-requiring strains of *Salmonella typhimurium* (*S. typhimurium*, ST) and *Escherichia coli* (*E. coli*, EC) by their ability to functionally reverse mutations. These reverse mutations result in revertant colonies of bacteria with restored capability to synthesize the essential amino acid.

2. SUMMARY

The Ames test was conducted with Soy Leghemoglobin Preparation at levels of 23.384, 74, 233.84, 740, 2338.4, 7400, 23,384, and 74,000 µg/plate corresponding to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate with the high level being the standard limit for this test. The main test was conducted using the plate incorporation method in both the absence and presence of metabolic activation (chemically-induced rat liver S9 mix). The results of the test were confirmed using a similar study design but employing the pre-incubation modification of the Ames test.

No signs of precipitation or contamination were noted in any of the strains. No signs of toxicity were noticed in any strains in either plate incorporation or pre-incubation method in presence or absence of S9.

Eight dose levels without precipitation, toxicity or plate contamination were evaluated for all strains, therefore bacterial mutagenicity was adequately assessed.

In conclusion, based on these findings and on the evaluation system used, Soy Leghemoglobin Preparation did not elicit evidence of bacterial mutagenicity in the Ames assay.

3. TEST SUBSTANCE

The test substance, identified as Soy Leghemoglobin Preparation , Lot #: PP-PGM2-16-015-101, was received on January 27, 2016, and was further identified with PSL Reference Number 160127-7D. The test substance was stored frozen: -20°C to -80°C. Documentation of the methods of synthesis, fabrication, or derivation of the test substance is retained at 600 Galveston Dr., Redwood City, CA, 94063.

The following information related to the characterization of the test substance was provided by the Sponsor (see also Appendix B):

Composition: Soy Leghemoglobin - 6.74%[1]
Other Ingredients - 8.11%

Physical Description: Red frozen liquid

Stability: Test substance was expected to be stable for the duration of testing.

Expiration Date: Not applicable

In the preparation of formulations a correction for purity was used based on soy leghemoglobin concentration, and all dose levels are expressed in terms of material as supplied.

4. POSITIVE CONTROL SUBSTANCES

The positive control substances (known mutagens) were received on the dates listed below and were further identified using Product Safety Labs' identification numbers. The substances were stored refrigerated. Documentation of the methods of synthesis, fabrication, or derivation of the positive controls is retained by the vendor (Molecular Toxicology, Inc.).

Positive Control Substance Identification	Lot No.	CAS No.	Date of Receipt	PSL ID No.	Expiration Date
Sodium Azide (NaN_3)	6350SA 6350SA	26628-22-8	Mar 8, 2016 Apr 13, 2016	160308-5H 160413-5H	Aug 5, 2017 Aug 5, 2017
ICR 191 Acridine	6260ICR	17070-45-0	Mar 8, 2016	160308-3H	Feb 23, 2018
Daunomycin	2175DU	20830-81-3	Mar 8, 2016	160308-2H	Jun 3, 2017
Methyl methanesulfonate (MMS)	8201MS	66-27-3	Mar 8, 2016	160308-4H	Nov 6, 2017
2-Aminoanthracene (2-AA)	6421AA 6425AA	613-13-8	Mar 8, 2016 Apr 13, 2016	160308-1H 160413-1H	Dec 2, 2017 Mar 31, 2018

5. VEHICLE CONTROL SUBSTANCE

Sterile water was used as the vehicle control.

[1] A GLP Certificate of Analysis was prepared by PSL (from PSL study numbers 43970 for the active ingredient) and gave a Percent Leghemoglobin of 6.68%. This is within an acceptable margin of error of the analytical measurement. The value original value provided by Impossible Foods was used to calculate study doses.

6. GENERAL TEST SYSTEM PARAMETERS

A. Test System Identification

Each of the *S. typhimurium* and *E. coli* strains received for use on this study was accompanied by documentation that includes lot number, preparation and expiration dates, and confirmation of phenotype and response to specific mutagens. The following bacterial strains were purchased from Molecular Toxicology, Inc.:

Strain	Characteristics	Mutations Detected	Lot Number	Expiration Date
ST TA1535	his; rfa; uvrB	Base-pair substitution	5107D	Mar 17, 2018
ST TA1537	his; rfa; uvrB	Frameshift	5100D	Feb 04, 2018
ST TA98	his; rfa; uvrB; R-factor	Frameshift	5047D	Aug 20, 2017
ST TA100	his; rfa; uvrB; R-factor	Base-pair substitution	5029D	Jun 19, 2017
EC WP2 uvrA	trp; uvrA	Base-pair substitution	5087D	Jan 07, 2018

Legend:

his	histidine required as a growth factor
rfa	deep rough mutation involves loss of a major component of the cell coat increasing permeability to larger molecules; this deletion also involves the gene coding for biotin synthesis
uvrA/B	deletion of DNA nucleotide excision repair system
R-factor	contains the pKM101 plasmid which increases sensitivity by enhancing error-prone DNA repair systems
trp	tryptophan required as a growth factor

B. Justification for the Selection of the Test System

The referenced guidelines (Section 10) accept the combination of *S. typhimurium* (TA1535, TA1537, TA98, TA100) and *E. coli* (WP2 uvrA) strains selected for use in this study.

7. ASSAY MATERIALS

A. Growth Media and Plates

Overlay agar (supplemented with biotin and limited amounts of histidine and tryptophan) and minimal glucose agar plates were purchased from Molecular Toxicology, Inc.

B. Metabolic Activation System (S9 Mix) and Substitution Buffer

S9 mix (cofactor supplemented post-mitochondrial fraction) was included in the Ames test to simulate mammalian metabolism since some test substances only become mutagenic following metabolic activation. S9 liver fraction was purchased from Molecular Toxicology, Inc., and sourced from male Sprague-Dawley rats induced with phenobarbital and benzoflavone.

The S9 mix, freshly prepared on the day of use, was maintained on ice prior to and during use and contained 5% v/v S9 fraction. The prepared S9 mix contained the following sterile cofactors

(Maron & Ames, 1983): 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate and 4 mM NADP.

Sodium phosphate buffer was used as the substitution buffer for plates treated in the absence of S9.

C. Bacteria (Test Systems)

Fresh bacterial suspension cultures in nutrient broth were prepared so that they were in the late exponential phase of growth at the time of use (roughly 1 x 10^9 bacteria/mL).

D. Test Substance Preparation

The test substance was formulated as a solution in Sterile water (0.2338, 0.74, 2.3384, 7.4, 23.384, 74, 233.84 and 740mg/mL) to provide corresponding dose levels of up to 74,000 µg/plate. These levels correspond to active component soy leghemoglobin concentrations of 0.0158, 0.05, 0.158, 0.50, 1.58, 5.0, 15.80, 50.00mg/mL. The solutions were vortexed prior to use.

E. Positive Control Substances

The performance of this test was evaluated with positive controls for each tester strain used, with and without metabolic activation (S9). Appropriate dilutions were prepared using the solvents listed below prior to testing.

Positive Control Substance (Concentration)	Solvent	Tester Strain	Metabolic Activation (S9)
Sodium Azide (15 µg/mL)	Sterile water	*S. typhimurium* TA100, TA1535	Absent
ICR 191 Acridine (10 µg/mL)	Sterile water	*S. typhimurium* TA1537	Absent
Daunomycin (60 µg/mL)	Sterile water	*S. typhimurium* TA98	Absent
Methyl methanesulfonate (25 µL/mL)	Sterile water	*E. coli* WP2 uvrA	Absent
2-Aminoanthracene (100 µg/mL)	DMSO	All	Present

8. EXPERIMENTAL DESIGN

A. Main Test

The initial test followed the plate incorporation method, in which the following materials were mixed and poured over the surface of a minimal agar plate:

- 100 µL of the prepared test substance solutions, negative (vehicle) control, or prepared positive control substance
- 500 µL S9 mix or substitution buffer
- 100 µL bacteria suspension (ST or EC)
- 2000 µL overlay agar maintained at approximately 45°C

Plates were prepared in triplicate and uniquely identified.

For each of the bacterial strains, plates were prepared at each experimental point as follows:

Treatment	Dose No.	Final Dose (µg/plate)	Number of Replicates		Number of Strains
			-S9	+S9	
Vehicle control	0	0	3	3	5
Test substance	1	23.384	3	3	5
	2	74	3	3	5
	3	233.84	3	3	5
	4	740	3	3	5
	5	2338.4	3	3	5
	6	7400	3	3	5
	7	23,384	3	3	5
	8	74,000[A]	3	3	5
Positive control	*[B]	*[B]	3	3	5

[A] The OECD standard limit dose
[B] Dose depends on the test organism and the positive control

Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate.

In addition, an untreated (negative control group) was included. Appropriate sterility control check plates (treated with critical components in the absence of bacteria) were included as a standard procedural check. After pouring, plates were placed on a level surface until the agar was gelled then incubated at approximately 37°C until growth was adequate for enumeration (approximately 65 hours).

B. Confirmatory Test

The confirmatory test employed the pre-incubation modification of the plate incorporation test. The test or control substances, bacteria suspension, and S9/substitution buffer were incubated under agitation for approximately 30 minutes at approximately 37°C prior to mixing with the overlay agar and pouring onto the minimal agar plates before proceeding as described for the initial test. The study design for the confirmatory test, including strains, dose levels etc. was as described above for the initial (main) test.

C. Control of Bias

General procedures associated with the balanced design and conduct of this study were employed to control bias.

D. Results

After incubation, the number of colonies per plate was counted manually and/or with the aid of a plate counter (Colony Plate Reader: Model Colony-Doc-It™). The mean and standard deviation were calculated for each set of triplicate plates.

E. Criteria for Validity

The background lawn for vehicle control plates should appear normal (i.e., slightly hazy with abundant microscopic non-revertant bacterial colonies). The mean revertant colony counts for each strain treated with the vehicle should lie close to or within the expected range taking into

account the laboratory historical control range and/or published values (Mortelmans & Zeiger, 2000; Gatehouse, 2012). The positive controls (with S9 where required) should produce substantial increases in revertant colony numbers with the appropriate bacterial strain as specified in the Evaluation of Mutagenicity Section below.

In the case where part of the study is invalid based on these criteria (e.g., the positive control does not induce an appropriate response with an individual strain or generally poor growth of the background lawn with that strain), detailed results for that part of the study will not be reported and the affected part of the study would normally be subjected to an automatic repeat as described in an amendment, if appropriate.

F. Evaluation of Toxicity

Toxic effects of the test substance are indicated by the partial or complete absence of a background lawn of non-revertant bacteria (colony counts, if any, should not be reported) or a substantial dose-related reduction in revertant colony counts compared with lower dose levels and concurrent vehicle control taking into account the laboratory historical control range. Where precipitation obscures observations on the condition of the background lawn, the lawn can be considered normal and intact if the revertant colony counts are within the expected range based on results for lower dose levels and historical control counts for that strain.

G. Evaluation of Mutagenicity

For each experimental point, the Mutation Factor (MF) was calculated by dividing the mean revertant colony count by the mean revertant colony count for the corresponding concurrent vehicle control group. The mutagenic activity of the test item was assessed by applying the following criteria:

The results were considered positive (i.e., indicative of mutagenic potential) if:

- The results for the test item showed a substantial increase in revertant colony counts, i.e., response MF $\geq$ 2 for strains TA98, TA100, and WP2 uvrA or MF $\geq$ 3 for strains TA1535 and TA1537, with mean value(s) outside the laboratory historical control range. Otherwise, results were considered negative.

- The above increase must be dose related and/or reproducible, i.e., increases must be obtained at more than one experimental point (at least one strain, more than one dose level, more than one occasion or with different methodologies).

If the second criterion is not met, the results may be classified as equivocal, and further testing may be appropriate.

A test substance that produces neither a concentration related increase in the number of revertant colonies nor a reproducible substantial increase in revertant colonies is considered to be non-mutagenic in this test system.

9. STATISTICAL ANALYSIS

Product Safety Labs calculated means and standard deviations for all quantitative data collected.

10. STUDY CONDUCT

This study was conducted at Product Safety Labs' (PSL) test facility at 2394 US Highway 130, Dayton, New Jersey 08810. The Study Director for this study was Mithila Shitut, BVSc & AH,

MS. The primary scientist for this study was Anupama Dubey, BS, with contributions from Kathleen Quinn, BS. This study was conducted to comply with the Good Laboratory Practice (GLP) regulations as defined in:

- U.S. FDA GLP: 21 CFR 58, 1987

Which is compatible with:

- OECD Principles of GLP (as revised in 1997): ENV/MC/CHEM(98)17, OECD, Paris, 1998
- EC Directive 2004/10/EC, Official Journal of the European Union, L50/44, Feb. 20, 2004

The procedures as described in this protocol are based on the most recent version of the following testing guidelines:

- US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 1. a. (2007)
- OECD Guidelines for Testing of Chemicals, Section 4 (Test No. 471):"Bacterial Reverse Mutation Test" (1997)
- Commission regulation (EC) No 440/2008 B.13/14

11. QUALITY ASSURANCE

The final report was audited for agreement with the raw data records and for compliance with the protocol, Product Safety Labs Standard Operating Procedures and appropriate Good Laboratory Practice Standards. Dates of inspections and audits performed during the study and the dates of reporting of the inspection and audit findings to the Study Director and Facility Management are presented in the Quality Assurance Statement.

12. AMENDMENTS TO THE PROTOCOL

1) Per sponsor request, to calculate the active ingredient in the test substance, Section 7A. Main Test was updated to:

   Plates were prepared in triplicate and uniquely identified. For each of the bacterial strains, plates were prepared at each experimental point as follows:

Treatment	Dose No.	Final Dose (µg/plate)	Number of Replicates		Number of Strains
			-S9	+S9	
Vehicle control	0	0	3	3	5
Test substance	1	23.384	3	3	5
	2	74	3	3	5
	3	233.84	3	3	5
	4	740	3	3	5
	5	2338.4	3	3	5
	6	7400	3	3	5
	7	23,384	3	3	5
	8	74,000[A]	3	3	5
Positive control	*[B]	*[B]	3	3	5

[A] The OECD standard limit dose
[B] Dose depends on the test organism and the positive control

In addition, an untreated (negative control group) was included when considered appropriate. Appropriate sterility control check plates (treated with critical components in the absence of bacteria) were included as a standard procedural check. After pouring, plates were placed on a level surface until the agar was gelled then incubated at approximately 37°C until growth was adequate for enumeration (approximately 65 hours). Note that the loss of an individual plate (e.g., due to microbial contamination) does not affect the validity of the study.

2) The test substance name, active ingredient and lot number will be changed to:

Test article name - Soy Leghemoglobin Preparation
Active ingredient - Soy Leghemoglobin
Lot #: PP-PGM2-PP-PGM2-16-015-101

throughout the protocol in all applicable places as per the GLP-Certificate of Analysis.

13. DEVIATIONS FROM THE PROTOCOL

None.

14. FINAL REPORT AND RECORDS TO BE MAINTAINED

Information on equipment maintenance and calibration, storage, usage, and disposition of the test substance, and all other records that would demonstrate adherence to the protocol will be maintained. Facility records which are not specific to the subject study will be maintained by the testing facility and archived according to PSL SOP.

The original, final report will be sent to the Sponsor. A copy of the signed report, together with the protocol, associated amendments and/or deviations if applicable, and all raw data generated at PSL will be maintained in the PSL Archives. PSL will maintain these records for a period of at least five years. After this time, the Sponsor of the study will be offered the opportunity to take possession of the records or request continued archiving by PSL.

15. RESULTS

Revertant colony counts for each strain are presented in Tables 1-5. Historical Control Data is presented in Appendix A. The PSL Certificate of Analysis is presented is Appendix B.

The mean revertant colony counts for each strain treated with the vehicle were close to or within the expected range, considering the laboratory historical control range and/or published values (Mortelmans & Zeiger, 2000; Gatehouse, 2012). The positive control substances caused 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. Therefore, each phase of the test is considered valid.

No signs of precipitation or contamination were noted in any of the strains. No signs of toxicity were noticed in any strains in either plate incorporation or pre-incubation method in presence or absence of S9.

Eight dose levels without precipitation, toxicity or plate contamination were evaluated for all strains, therefore bacterial mutagenicity was adequately assessed.

There was no concentration-related or substantial test substance related increases in the number of revertant colonies observed with strains TA1535, TA1537, TA98 , TA100 or E. Coli WP2 uvrA in both the absence and presence of S9 using either the plate incorporation or the pre-incubation method.

16. CONCLUSION

Based on these findings and on the evaluation system used, Soy Leghemoglobin Preparation did not elicit evidence of bacterial mutagenicity in the Ames assay.

17. REFERENCES

Ames, B. N., Durston, W. E., Lee, F. D., & Yamasaki, E. (1973). Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proceedings of the National Academy of Sciences, Vol. 70* (No. 8), 2281-2285.

Gatehouse D (2012). Bacterial mutagenicity assays: test methods. *Methods in Molecular Biology, Vol. 817*: 21-34.

Maron, D. M., & Ames, B. N. (1983). Revised methods for the Salmonella mutagenicity test. *Mutation Research, 113*, 173-215.

Mortelmans, K., & Zeiger, E. (2000). The Ames *Salmonella*/microsome mutagenicity assay. *Mutation Research, 455*, 29–60.

U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention & National Institutes of Health. (2009). Biosafety in Microbiological and Biomedical Laboratories (5th ed.). HHS Publication No. (CDC) 21-1112.

SIGNATURE

Soy Leghemoglobin Preparation

I, the undersigned, declare that the methods, results and data contained in this report faithfully reflect the procedures used and raw data collected during the study.

(b) (6)

Mithila Shitut, BVSc & AH, MS
Study Director
Product Safety Labs

12/07/16
Date

TABLE 1A: REVERTANT COLONY COUNTS – TA 1535

Plate Incorporation Method - Main Test									
TA1535		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose* (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	20 16 19	18	2.1	13 10 15	13	2.5	1.00	1.00
Test Substance	23.384	11 10 7	9	2.1	14 11 7	11	3.5	0.50	0.85
Test Substance	74	7 18 12	12	5.5	14 15 14	14	0.6	0.67	1.08
Test Substance	233.84	9 9 12	10	1.7	16 10 12	13	3.1	0.56	1.00
Test Substance	740	16 11 8	12	4.0	10 9 8	9	1.0	0.67	0.69
Test Substance	2338.4	11 14 11	12	1.7	14 10 14	13	2.3	0.67	1.00
Test Substance	7400	4 11 18	11	7.0	12 10 11	11	1.0	0.61	0.85
Test Substance	23384	18 18 9	15	5.2	14 8 7	10	3.8	0.83	0.77
Test Substance	74000	11 11 13	12	1.2	12 16 10	13	3.1	0.67	1.00
Sodium Azide	1.5	557 564 580	567	11.8	-			31.50	-
2-AA	10	-			328 331 339	333	5.7	-	25.62

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate.

TABLE 1B: REVERTANT COLONY COUNTS – TA 1535

Pre-Incubation Method - Confirmatory Test									
TA1535		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	15 12 11	13	2.1	9 12 11	11	1.5	1.00	1.00
Test Substance	74	12 16 17	15	2.6	8 15 10	11	3.6	1.15	1.00
Test Substance	7.4	12 9 13	11	2.1	9 7 14	10	3.6	0.85	0.91
Test Substance	233.84	19 11 17	16	4.2	9 13 8	10	2.6	1.23	0.91
Test Substance	740	12 11 11	11	0.6	9 11 11	10	1.2	0.85	0.91
Test Substance	2338.4	17 8 15	13	4.7	11 4 9	8	3.6	1.00	0.73
Test Substance	7400	15 7 11	11	4.0	13 9 14	12	2.6	0.85	1.09
Test Substance	23384	6 5 9	7	2.1	9 8 12	10	2.1	0.54	0.91
Test Substance	74000	6 14 16	12	5.3	14 11 9	11	2.5	0.92	1.00
Sodium Azide	1.5	547 607 600	585	32.8	-			45.00	-
2-AA	10	-			275 279 272	275	3.5	-	25.00

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate.

TABLE 2A: REVERTANT COLONY COUNTS – TA 1537

Plate Incorporation Method - Main Test									
TA1537		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	12 13 13	13	0.6	9 10 18	12	4.9	1.00	1.00
Test Substance	23.384	9 9 13	10	2.3	13 13 15	14	1.2	0.77	1.17
Test Substance	74	9 15 9	11	3.5	10 16 15	14	3.2	0.85	1.17
Test Substance	233.84	11 12 11	11	0.6	10 17 14	14	3.5	0.85	1.17
Test Substance	740	11 18 11	13	4.0	8 8 13	10	2.9	1.00	0.83
Test Substance	2338.4	10 11 11	11	0.6	14 14 15	14	0.6	0.85	1.17
Test Substance	7400	9 9 8	9	0.6	7 6 12	8	3.2	0.69	0.67
Test Substance	23384	6 7 7	7	0.6	8 11 11	10	1.7	0.54	0.83
Test Substance	74000	8 7 10	8	1.5	12 10 17	13	3.6	0.62	1.08
ICR 191 Acridine	1	520 450 549	506	50.9	-			38.92	-
2-AA	10	-			410 370 399	393	20.7	-	32.75

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate.

TABLE 2B: REVERTANT COLONY COUNTS – TA 1537

Pre-Incubation Method - Confirmatory Test									
TA1537		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	7 9 8	8	1.0	8 13 14	12	3.2	1.00	1.00
Test Substance	23.384	9 13 18	13	4.5	15 20 17	17	2.5	1.63	1.42
Test Substance	74	24 13 13	17	6.4	19 13 13	15	3.5	2.13	1.25
Test Substance	233.84	6 10 7	8	2.1	10 11 15	12	2.6	1.00	1.00
Test Substance	740	9 6 18	11	6.2	5 6 13	8	4.4	1.38	0.67
Test Substance	2338.4	10 9 5	8	2.6	9 11 12	11	1.5	1.00	0.92
Test Substance	7400	9 7 14	10	3.6	11 10 9	10	1.0	1.25	0.83
Test Substance	23384	10 10 15	12	2.9	14 20 7	14	6.5	1.50	1.17
Test Substance	74000	14 13 15	14	1.0	18 13 16	16	2.5	1.75	1.33
ICR 191 Acridine	1	5486 5640 5465	5530	95.6	-			691.25	-
2-AA	10	-			351 389 404	381	27.3	-	31.75

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate

TABLE 3A: REVERTANT COLONY COUNTS – TA 98

Plate Incorporation Method - Main Test									
TA98		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	25 27 24	25	1.5	23 32 25	27	4.7	1.00	1.00
Test Substance	23.384	26 23 23	24	1.7	24 28 25	26	2.1	0.96	0.96
Test Substance	74	21 20 20	20	0.6	21 23 25	23	2.0	0.80	0.85
Test Substance	233.84	21 28 21	23	4.0	29 25 30	28	2.6	0.92	1.04
Test Substance	740	20 24 23	22	2.1	27 25 29	27	2.0	0.88	1.00
Test Substance	2338.4	20 23 18	20	2.5	27 26 26	26	0.6	0.80	0.96
Test Substance	7400	20 25 22	22	2.5	30 24 20	25	5.0	0.88	0.93
Test Substance	23384	25 26 26	26	0.6	18 17 21	19	2.1	1.04	0.70
Test Substance	74000	23 20 27	23	3.5	30 23 32	28	4.7	0.92	1.04
Daunomycin	6	809 815 778	801	19.9	-			32.04	-
2-AA	10	-			2786 2471 2644	2634	157.8	-	97.56

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate

TABLE 3B: REVERTANT COLONY COUNTS – TA 98

Pre-Incubation Method - Confirmatory Test									
TA98		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	21 23 20	21	1.5	22 28 29	26	3.8	1.00	1.00
Test Substance	23.384	24 21 22	22	1.5	24 24 26	25	1.2	1.05	0.96
Test Substance	74	27 22 14	21	6.6	26 25 36	29	6.1	1.00	1.12
Test Substance	233.84	18 22 17	19	2.6	21 23 25	23	2.0	0.90	0.88
Test Substance	740	24 28 31	28	3.5	22 23 22	22	0.6	1.33	0.85
Test Substance	2338.4	23 23 18	21	2.9	21 29 25	25	4.0	1.00	0.96
Test Substance	7400	19 23 21	21	2.0	29 25 29	28	2.3	1.00	1.08
Test Substance	23384	26 22 18	22	4.0	31 19 27	26	6.1	1.05	1.00
Test Substance	74000	25 19 28	24	4.6	25 35 29	30	5.0	1.14	1.15
Daunomycin	6	305 320 302	309	9.6	-			14.71	-
2-AA	10	-			2344 2637 2356	2446	165.8	-	94.08

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate

TABLE 4A: REVERTANT COLONY COUNTS – TA 100

Plate Incorporation Method - Main Test									
TA100		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	98 99 112	103	7.8	128 109 121	119	9.6	1.00	1.00
Test Substance	23.384	91 95 109	98	9.5	110 97 112	106	8.1	0.95	0.89
Test Substance	74	93 87 92	91	3.2	114 108 105	109	4.6	0.88	0.92
Test Substance	233.84	81 108 103	97	14.4	97 119 90	102	15.1	0.94	0.86
Test Substance	740	83 93 90	89	5.1	104 118 102	108	8.7	0.86	0.91
Test Substance	2338.4	94 95 106	98	6.7	91 95 97	94	3.1	0.95	0.79
Test Substance	7400	102 92 93	96	5.5	96 103 99	99	3.5	0.93	0.83
Test Substance	23384	103 94 93	97	5.5	103 109 108	107	3.2	0.94	0.90
Test Substance	74000	97 95 108	100	7.0	90 106 107	101	9.5	0.97	0.85
Sodium Azide	1.5	509 508 499	505	5.5	-			4.90	-
2-AA	10	-			2372 3010 3109	2830	400.0	-	23.78

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate

TABLE 4B: REVERTANT COLONY COUNTS – TA 100

Pre-Incubation Method - Confirmatory Test									
TA100		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	106 112 92	103	10.3	120 124 115	120	4.5	1.00	1.00
Test Substance	23.384	94 85 87	89	4.7	91 104 98	98	6.5	0.86	0.82
Test Substance	74	80 84 94	86	7.2	105 102 110	106	4.0	0.83	0.88
Test Substance	233.84	113 90 86	96	14.6	88 114 110	104	14.0	0.93	0.87
Test Substance	740	108 101 94	101	7.0	117 105 109	110	6.1	0.98	0.92
Test Substance	2338.4	87 100 95	94	6.6	93 80 102	92	11.1	0.91	0.77
Test Substance	7400	94 72 78	81	11.4	90 81 96	89	7.5	0.79	0.74
Test Substance	23384	107 91 88	95	10.2	90 105 99	98	7.5	0.92	0.82
Test Substance	74000	97 106 86	96	10.0	113 116 104	111	6.2	0.93	0.93
Sodium Azide	1.5	517 535 521	524	9.5	-			5.09	-
2-AA	10	-			2426 2607 2468	2500	94.7	-	20.83

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate

TABLE 5A: REVERTANT COLONY COUNTS – EC WP2 uvrA

Plate Incorporation Method - Main Test									
E. Coli WP2 uvrA		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	37 36 46	40	5.5	60 40 55	52	10.4	1.00	1.00
Test Substance	23.384	52 42 36	43	8.1	44 37 40	40	3.5	1.08	0.77
Test Substance	74	29 42 30	34	7.2	34 42 39	38	4.0	0.85	0.73
Test Substance	233.84	45 33 47	42	7.6	54 55 50	53	2.6	1.05	1.02
Test Substance	740	47 46 44	46	1.5	52 32 44	43	10.1	1.15	0.83
Test Substance	2338.4	43 42 50	45	4.4	54 52 30	45	13.3	1.13	0.87
Test Substance	7400	52 43 29	41	11.6	50 54 66	57	8.3	1.03	1.10
Test Substance	23384	47 42 49	46	3.6	50 39 51	47	6.7	1.15	0.90
Test Substance	74000	47 31 40	39	8.0	59 49 50	53	5.5	0.98	1.02
MMS	2.5	806 810 821	812	7.8	-			20.30	-
2-AA	10	-			119 99 121	113	12.2	-	2.17

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate

TABLE 5B: REVERTANT COLONY COUNTS – EC WP2 uvrA

Pre-Incubation Method - Confirmatory Test									
E. Coli WP2 uvrA		Revertant Colonies per Plate						Mutation Factor	
		Without Activation (-S9)			With Activation (+S9)				
Treatment	Dose * (µg/plate)	Counts	Mean	SD	Counts	Mean	SD	-S9	+S9
Sterile Water	N/A	30 37 36	34	3.8	46 42 35	41	5.6	1.00	1.00
Test Substance	23.384	45 32 43	40	7.0	55 42 43	47	7.2	1.18	1.15
Test Substance	74	20 34 30	28	7.2	49 48 47	48	1.0	0.82	1.17
Test Substance	233.84	34 48 42	41	7.0	35 40 36	37	2.6	1.21	0.90
Test Substance	740	31 31 35	32	2.3	53 39 41	44	7.6	0.94	1.07
Test Substance	2338.4	33 28 33	31	2.9	40 41 41	41	0.6	0.91	1.00
Test Substance	7400	27 37 45	36	9.0	56 52 48	52	4.0	1.06	1.27
Test Substance	23384	31 28 38	32	5.1	47 50 43	47	3.5	0.94	1.15
Test Substance	74000	43 46 42	44	2.1	43 49 26	39	11.9	1.29	0.95
MMS	2.5	360 357 402	373	25.2	-			10.97	-
2-AA	10	-			130 120 108	119	11.0	-	2.90

N/A = Not applicable

* Test substance levels correspond to active component soy leghemoglobin concentrations of 1.58, 5.0, 15.8, 50, 158, 500, 1580, 5000 µg/plate

APPENDIX A: HISTORICAL CONTROL DATA[1]

Plate Incorporation Method - Revertants Per Plate							
Strain	**Treatment**	**Dose (µg/plate)**	**S9**	**Mean**	**SD**	**Min**	**Max**
TA1535	Sodium Azide	1.5	-	618	91	359	1192
TA1537	ICR 191 Acridine	1	-	1136	1437	119	6388
TA98	Daunomycin	6	-	938	343	350	1500
TA100	Sodium Azide	1.5	-	600	126	394	1003
E. Coli	MMS	2.5	-	634	101	386	846
TA1535	2-AA	10	+	267	86	85	636
TA1537	2-AA	10	+	280	99	42	542
TA98	2-AA	10	+	2321	971	83	3915
TA100	2-AA	10	+	2377	806	976	4169
E. Coli	2-AA	10	+	125	30	63	196
TA1535	Sterile Water	N/A	-	13	2	7	21
TA1537	Sterile Water	N/A	-	12	4	6	25
TA98	Sterile Water	N/A	-	28	8	16	49
TA100	Sterile Water	N/A	-	130	16	104	155
E. Coli	Sterile Water	N/A	-	45	7	29	57
TA1535	Sterile Water	N/A	+	13	1	9	20
TA1537	Sterile Water	N/A	+	15	3	8	28
TA98	Sterile Water	N/A	+	29	5	18	40
TA100	Sterile Water	N/A	+	145	14	116	170
E. Coli	Sterile Water	N/A	+	59	13	31	81

[1] Historical Data maintained by PSL from 2015.

APPENDIX A (cont.): HISTORICAL CONTROL DATA[1]

Pre-Incubation Method - Revertants Per Plate							
Strain	Treatment	Dose (µg/plate)	S9	Mean	SD	Min	Max
TA1535	Sodium Azide	1.5	-	622	71	478	831
TA1537	ICR 191 Acridine	1	-	3227	1227	875	5700
TA98	Daunomycin	6	-	602	345	146	1227
TA100	Sodium Azide	1.5	-	539	166	138	904
E. Coli	MMS	2.5	-	509	143	313	808
TA1535	2-AA	10	+	293	64	64	391
TA1537	2-AA	10	+	260	107	112	541
TA98	2-AA	10	+	2384	938	506	3530
TA100	2-AA	10	+	2388	583	1308	3620
E. Coli	2-AA	10	+	128	29	60	188
TA1535	Sterile Water	N/A	-	16	3	8	23
TA1537	Sterile Water	N/A	-	14	5	5	23
TA98	Sterile Water	N/A	-	29	7	14	46
TA100	Sterile Water	N/A	-	118	18	83	143
E. Coli	Sterile Water	N/A	-	46	10	30	67
TA1535	Sterile Water	N/A	+	12	2	8	19
TA1537	Sterile Water	N/A	+	14	5	6	26
TA98	Sterile Water	N/A	+	35	7	23	50
TA100	Sterile Water	N/A	+	125	16	88	147
E. Coli	Sterile Water	N/A	+	53	9	36	76

[1] Historical Data maintained by PSL from 2015.

APPENDIX B: CERTIFICATE OF ANALYSIS

Product Safety Labs

CERTIFICATE OF ANALYSIS

Product: Soy Leghemoglobin Preparation

Lot #: PP-PGM2-16-015-101

PSL Reference No.: 160809-3D

Date of Analysis: September 1, 2016

Result:

Soy Leghemoglobin – 6.68%

Approval: (b) (6) _____ 9/7/16

David Sinning
Analytical Services
Product Safety Labs

Date

QA Release: (b) (6) _____ Sep 14, 2016

Rhonda Krick, B.S.
Quality Assurance
Product Safety Labs

Date

This material was analyzed in compliance with Good Laboratory Practice (40 CFR 160) standards. Data are reported in PSL GLP Study No. 43970

PRODUCT SAFETY LABS
2394 US Highway 130
Dayton, NJ 08810
USA

732-438-5100
psl@productsafetylabs.com
www.productsafetylabs.com

Annex 5

eurofins | BioPharma Product Testing

In vitro Mammalian Chromosome Aberration Test in Human Lymphocytes with Soy Leghemoglobin Preparation

Report

Version: Final

Study Completion Date: 27 FEB 2017

Eurofins Munich Study No.: 160931

Sponsor:
Impossible Foods Incorporated
525 Chesapeake Drive
Redwood City
California 94063
USA

Eurofins BioPharma Product Testing Munich GmbH
Behringstr. 6/8
D-82152 Planegg/Munich
Germany

Tel +49 (0)89 899 650-0
Fax +49 (0)89 899 650-11

GLP/GMP FDA Approved

1. Copy of the GLP Certificate

Bayerisches Landesamt für
Gesundheit und Lebensmittelsicherheit

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

EUROFINS BIOPHARMA PRODUCT TESTING MUNICH GMBH
BEHRINGSTRAßE 6-8
82152 PLANEGG

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

Kategorie 2/ Category 2
Kategorie 3/ Category 3
Kategorie 8/ Category 8
Kategorie 9*/ Category 9*

Sonstige Prüfungen:
biologische und mikrobiologische Sicherheitsprüfungen an Medizinprodukten und Arzneimitteln; Auftragsarchivierung

other tests:
biological an microbiological safety evaluation on medical devices and pharmaceuticals; contract archiving

Datum der Inspektion/Date of Inspection
(Tag.Monat.Jahr/day.month.year)

18. bis 19.03.2015

Die/Der genannte Prüfeinrichtung/Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/ diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility/test site is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Schwabach, 05.06.2015

(b) (6)

Dr. Peter Franke
Leiter der GLP-Landesleitstelle Bayern

GLP- Landesleitstelle Bayern
Bayerisches Landesamt für Gesundheit
und Lebensmittelsicherheit
Rathausgasse 4
91126 Schwabach

2. Contents

3. List of Tables

4. Preface

4.1. Abbreviations

Art.	Artikel *(article)*
ATCC	American Type Culture Collection
BGBl.	Bundesgesetzblatt *(Federal Law Gazette)*
BrdU	5-bromo-2-deoxyuridine
bw	body weight
CA	chromosome aberration
CPA	cyclophosphamide
DNA	desoxyribonucleic acid
e.g.	exempli gratia *(for example)*
EC	European Commission
EMS	ethylmethanesulfonate
EPA	Environmental Protection Agency
FBS	fetal bovine serum
GLP	Good Laboratory Practice
GmbH	Gesellschaft mit beschränkter Haftung *(company with limited liability)*
i.e.	id est *(that is)*
KCl	potassium chloride
NADP	nicotinamide adenine di-phosphate
No.	number
OECD	Organisation for Economic Cooperation and Development
PBS	phosphate buffered saline
PHA-L	phytohemagglutinin-L
QA	Quality Assurance
QAU	Quality Assurance Unit
RPMI	Roswell Park Memorial Institute medium
S9	microsomal fraction of rat liver homogenate
SOPs	Standard Operating Procedures
v/v	volume per volume

The following abbreviations are used in the tables with structural chromosomal aberrations:

g / ig	gap/ iso-gap; gaps are achromatic lesions of chromatid or chromosome type where no dislocation of chromosomal material is visible (independent of the size of the achromatic region).
b / ib	break / iso-break
f / if	fragment / iso-fragment
d / id	deletion / iso-deletion
ma	multiple aberration is defined as a metaphase containing more than 4 events [excluding gaps]; only exchanges are recorded additionally in these cells
ex	chromatid type exchange
cx	chromosome type exchange
cd	chromosomal disintegration (pulverisation)

4.2. General

Sponsor:	Impossible Foods Incorporated 525 Chesapeake Drive Redwood City California 94063 USA
Study Monitor:	Dr. Rachel Fraser
Test Facility:	Eurofins BioPharma Product Testing Munich GmbH Behringstraße 6/8 82152 Planegg Germany
Eurofins Munich Study No.:	160931
Test Item:	Soy Leghemoglobin Preparation
Title:	*In vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes with Soy Leghemoglobin Preparation

4.3. Project Staff

Study Director:	Christine Tiessen
Management:	Dr. Angela Lutterbach Dr. Katrin Witschital Jure Kapetan
Head of GLP Quality Assurance Unit:	Dipl.-Biol. Carolin Schmidt

4.4. Schedule

Arrival of the Test Item:	11 February 2016
Study Initiation Date:	08 March 2016
Date of 1st Amendment to Study Plan:	21 March 2016
Date of 2nd Amendment to Study Plan:	04 May 2016
Experimental Starting Date:	02 March 2016
Experimental Completion Date:	19 May 2016

5. Quality Assurance

5.1. GLP Compliance

This study was conducted to comply with:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998 [3].

The OECD Principles of Good Laboratory Practice are accepted by regulatory authorities throughout the European Community, USA and Japan.

This study was assessed for compliance with the study plan and the Standard Operating Procedures of Eurofins Munich. The study and/or the test facility are inspected periodically by the Quality Assurance Unit according to the corresponding SOPs. These inspections and audits are carried out by the Quality Assurance Unit, personnel independent of staff involved in the study. A signed quality assurance statement, listing all performed audits, is included in the report.

5.2. Guidelines

This study followed the procedures indicated by internal Eurofins Munich SOPs and the following internationally accepted guidelines and recommendations:

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473, „*In vitro* Mammalian Chromosome Aberration Test", adopted 26 September, 2014 [4].

Commission Regulation (EC) No. 440/2008 B.10: "Mutagenicity – *In vitro* Mammalian Chromosome Aberration Test", dated May 30, 2008 [5].

5.3. Archiving

For a period of 15 years (or shorter if in compliance with the GLP regulations) Eurofins Munich will store the records, materials and specimens in their scientific archives according to the GLP regulations.

The following records have to be stored according to the GLP regulations:

A copy of the final report, the study plan and documentation of all raw data generated during the conduct of the study (documentation forms as well as any other notes of raw data, printouts of instruments and computers) and the correspondence with the sponsor concerning the study. Any document relating to the study will be discarded only with the prior consent of the sponsor.

The following materials and samples have to be stored according to the period of time specified in the GLP regulations:

A retained sample of the test item will be archived according to the GLP regulations, if possible, and will be discarded without the sponsor's prior consent.

Other materials and specimens have to be stored according to the GLP regulations and disposed of after the respective archiving period with the sponsor's prior consent.

Unless otherwise agreed in writing, the remaining test item will be discarded three months after the release of the report.

6. Statement of Compliance

Eurofins Munich Study No.:	160931
Test Item:	Soy Leghemoglobin Preparation
Title:	*In vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes with Soy Leghemoglobin Preparation
Study Director:	Christine Tiessen

This study performed in the test facility Eurofins Munich was conducted in compliance with Good Laboratory Practice Regulations:

Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498) [1].

Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998 [2].

"OECD Principles of Good Laboratory Practice (as revised in 1997)", Paris 1998 [3].

There were no circumstances that may have affected the quality or integrity of the study.

Study Director: Christine Tiessen

(b) (6)

Date: 27 February 2017

This statement does not include the solubility test.

7. Statement of the Quality Assurance Unit

Eurofins Munich Study No.:	160931
Test Item:	Soy Leghemoglobin Preparation
Title:	*In vitro* Mammalian Chromosome Aberration Test in Human Lymphocytes with Soy Leghemoglobin Preparation
Study Director:	Christine Tiessen

This report and the conduct of this study were inspected by the Quality Assurance Unit on the following dates:

Phase of QAU Inspection	Date of QAU Inspection	Date of Reporting to the Study Director and Management
Audit Final Study Plan:	08 March 2016	08 March 2016
Audit 1st Amendment to Study Plan:	21 March 2016	
Audit 2nd Amendment to Study Plan:	04 May 2016	
Audit Experimental Phase (process-based):	30 August 2016	30 August 2016
Audit Final Report:	24 FEB 2017	24 FEB 2017

This report reflects the raw data.

Member of the
Quality Assurance Unit: (b) (6)

Print Name: Katrin Seidel

Date: 01 Mar 2017

This statement does not include the solubility test.

8. Summary

8.1. Summary Results

A chromosome aberration assay was carried out in order to investigate a possible potential of Soy Leghemoglobin Preparation to induce structural chromosome aberrations in human lymphocytes.

The metaphases were prepared 24 h after start of treatment with the test item. The treatment interval was 4 h **without** and **with** metabolic activation (experiment I) and 24 h **without** metabolic activation (experiment II). Duplicate cultures were set up. Per culture 150 metaphases were scored for structural chromosomal aberrations (for exceptions see Tables).

The following concentrations of Soy Leghemoglobin (active ingredient) were evaluated:

Experiment I

Without and **with** metabolic activation, 4 h treatment, 24 h preparation interval:

500, 1000, 2500 and 5000 µg/mL

Experiment II

Without metabolic activation, 24 h treatment, 24 h preparation interval:

100, 200, 500 and 1000 µg/mL

In experiment I and II, precipitation was not observed after treatment of the lymphocytes with the different test item concentrations. However, the test item itself showed a dark-red colouring

In Experiment II, precipitation occurred in the concentrations 500 µg/mL and higher during the fixation of the cells. In contrast to experiment I, in the experiment with long-term treatment the test item is not removed by repeated washing steps, as the treatment period is stopped by the fixation step directly. When the cells were spread on the object slides the precipitation appeared as a greenish lacquer coat, visible by eye and with the aid of an inverted microscope. The evaluation of aberration rates was not affected.

In experiment I **without** metabolic activation, toxic effects (decrease below 70% rel. mitotic index) were seen at concentrations of 1000 µg/mL (69%), 2500 µg/mL (56%) and 5000 µg/mL (54%) (Table 5). In experiment I **with** metabolic activation no toxic effects (decrease below 70% rel. mitotic index) were observed.

In experiment II **without** metabolic activation, toxic effects regarding the mitotic index were noted in the concentrations 500 µg/mL (69%), 1000 µg/mL (53%), 2000 µg/mL (26%), 3000 µg/mL (13%), 4000 µg/mL (38%) and 5000 µg/mL (42%) (Table 7). However, only concentrations up to 1000 µg/ml were evaluated for chromosomal aberrations, as precipitation was noted from concentrations of 500 µg/mL and higher.

In experiment I, no biologically relevant decreases of the proliferation index was observed. In experiment II, a concentration-dependent cell cycle delay was detected. As cytotoxicity was determined at the evaluated concentrations without any increase in chromosome aberrations, the cell cycle delay seemed to be cytotoxicity related.

In experiment I **without** metabolic activation, aberration rates of 500 µg/mL (4.0%), 1000 µg/mL (4.3%) and 2500 µg/mL (4.0%) evaluated are slightly increased above the historical control range of 0-3% (Table 15). However, as the negative control with 3.3% is slightly above the control data, which indicates a raise of the basic level of chromosome aberrations, these effects are regarded as not biologically relevant. Moreover, this statement is in line with the result of the highest tested concentration of 5000 µg/mL (1.7%), which is within the historical control data. Reduced clastogenic effects due to cytotoxicity in the highest test item concentration of 5000 µg/mL (rel. mitotic index 54%) can be excluded as for the lower concentration of 2500 µg/mL a similar mitotic index was determined (rel. mitotic index 56%). The variation observed in the two highest concentrations (3.7% aberrant cells at 2500 µg/mL and 1.7% aberrant cells at 5000 µg/mL) is most likely due to the variability observed regularly in a biological test system. Additionally, neither a concentration

dependent increase towards higher aberration rates nor a statistically significance was observed. In conclusion, in experiment I **without** metabolic activation, the test item Soy Leghemoglobin Preparation did not induce structural chromosomal aberrations in human lymphocytes.

In experiment I **with** metabolic activation, the negative control is with 3.7% chromosomal aberrations within the range of the historical negative control data (0-3.7%) (Table 15). The lowest concentration evaluated (1000 µg/mL) is slightly above the control range with 4.3% of chromosomal aberrations (Table 6). However, as the negative control is at the upper limit of the historical control data and test item concentrations 2500 µg/mL (2.0%) and 5000 µg/mL (2.7%) are within the control data and no dose dependent increase or a trend towards higher aberration rates is obvious, this effect is regarded as not biologically relevant.

In experiment II, no biologically relevant increase of the aberration rates was noted after treatment with the test item **without** metabolic activation (Table 8). The aberration rates of all dose groups treated with the test item were within the historical control data of the negative control (Table 16).

In the experiments I and II **without** and **with** metabolic activation, no biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item as compared to the controls.

The χ^2 Test for trend was performed to test whether there is a concentration-related increase in chromosomal aberrations. No statistically significant increase was observed in all experimental conditions.

EMS (400 and 900 µg/mL) and CPA (7.5 µg/mL) were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations, thus proving the efficiency of the test system to indicate potential clastogenic effects.

Table 1: Summary: Experiment I, without and with metabolic activation

	Dose Group	Concentration [µg/mL]	Relative Mitotic Index [%]	Proliferation Index	Mean % Aberrant Cells		Historical Laboratory Negative Control Range	Precipitation[a]	Statistical Significance[b]
					incl. Gaps	excl. Gaps			
without 4 h treatment, 24 h preparation interval	C	0	100	1.16	**5.7**	**3.3**	0.0% - 3.0% aberrant cells	-	-
	2	500	86	/	**6.0**	**4.0**		-	-
	3	1000	69	/	**7.3**	**4.3**		-	-
	4	2500	56	/	**6.0**	**4.0**		-	-
	5	5000	54	1.09	**6.7**	**1.7**		-	-
	EMS	900	63	/	**18.5**	**16.0**		-	+
with 4 h treatment, 24 h preparation interval	C	0	100	1.12	**7.7**	**3.7**	0.0% - 3.7% aberrant cells	-	-
	3	1000	111	/	**7.3**	**4.3**		-	-
	4	2500	83	/	**5.0**	**2.0**		-	-
	5	5000	105	1.07	**5.0**	**2.7**		-	-
	CPA	7.5	88	/	**18.5**	**15.5**		-	+

Less than 300 cells were evaluated for chromosome aberration of the positive controls EMS (200 cells) and CPA (200 cells)
The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the negative controls.

C: Negative Control (Culture Medium)
EMS: Ethylmethanesulfonate
a: - without precipitation. + with precipitation
b: statistical significant increase compared to negative controls (Fisher's exact test, $p < 0.05$), +: significant; -not significant

Table 2: Summary: Experiment II, without metabolic activation

	Dose Group	Concentration [µg/mL]	Relative Mitotic Index [%]	Proliferation Index	Mean % Aberrant Cells		Historical Laboratory Negative Control Range	Precipitation[a]	Statistical Significance[b]
					incl. Gaps	excl. Gaps			
without 24 h treatment, 24 h preparation interval	C	0	100	1.56	**4.3**	**2.3**	0.0% - 4.2% aberrant cells	-	-
	1	100	87	/	**6.0**	**2.7**		-	-
	2	200	95	/	**6.7**	**3.3**		-	-
	3	500	69	1.23	**3.3**	**2.0**		+	-
	4	1000	53	1.12	**3.3**	**2.0**		+	-
	EMS	400	48	/	**57.3**	**54.7**		-	+

Less than 300 cells were evaluated for chromosome aberration of the positive control EMS (75 cells)
The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the negative controls.

C: Negative Control (Culture Medium)
CPA: Cyclophosphamide
a: - without precipitation. + with precipitation during the preparation of the cells
b: statistical significant increase compared to negative controls (Fishers exact test, $p < 0.05$), +: significant; -not significant

8.2. Conclusion

In conclusion, it can be stated that during the described *in vitro* chromosomal aberration test and under the experimental conditions reported, the test item Soy Leghemoglobin Preparation did not induce structural chromosomal aberrations in human lymphocyte cells.

Therefore, Soy Leghemoglobin Preparation is considered to be non-clastogenic in this chromosome aberration test.

9. Introduction

9.1. Aim of the Study

The purpose of the *in vitro* chromosome aberration (CA) test is to identify agents that cause structural chromosome aberrations in stimulated cultured human lymphocytes.

Chromosome aberration assays aim to detect the induction of chromosome breakage (clastogenesis). Although substances produce structural chromosome aberrations by a variety of mechanisms, the endpoint is a discontinuity in the chromosomal DNA which is left unrejoined, or rejoined inaccurately to produce a mutated chromosome. Many of these changes will be lethal to the cell during the first few cell cycles after their induction but are used as indicators of the presence of non-lethal changes such as reciprocal translocations, inversions and small deletions. These more subtle changes may have important consequences in both germ and somatic cells. Chromosomal mutations and related events are the cause of many human genetic diseases and there is substantial evidence that these changes including oncogenes and tumor suppressor genes are involved in cancer in humans and experimental systems. CAs are generally evaluated in first post treatment mitosis.

Short-term cultures of peripheral blood lymphocytes are stimulated to divide by the addition of a mitogen (e.g. phytohemagglutinin: PHA) to the culture medium. Mitotic activity begins at about 40 h after PHA stimulation and reaches a maximum at around 3 days. The chromosome constitution remains diploid during short-term culture.

Treatments should commence at around 48 h after culture initiation when the cells are actively proliferating and should be sampled first at about 24 h later (1 - 1.5 fold of the normal cell cycle time), i.e. at 72 h after culture initiation (the cycle time of lymphocytes, except first cycle averages about 11 - 17 h). The cell cycle of the actual lymphocyte cultures is monitored using a BrdU-labeling technique. If toxicity occurs or cell cycle delay is indicated an additional sampling time should be used at about 24 h after the first fixation (e.g. 48 h after beginning of treatment or 96 h after culture initiation).

For soluble, non-toxic test items the highest concentration should correspond to 2 mg/mL, 2 µL/mL or 10 mM, whichever is the lowest. When the test chemical is not of defined composition, e.g. substance of unknown or variable composition, the top concentration may need to be higher (e.g. 5 mg/mL) in the absence of sufficient cytotoxicity. If the highest concentration is based on cytotoxicity the highest concentration chosen for evaluation should show a reduction of the mitotic index to 45 ± 5%. The lowest concentration should be in the range of the negative control.

At least three concentrations of the test item with concentration intervals of approximately 2 to 3 fold should be used at fixation time of 24 h.

Though the purpose of the assay is to detect structural chromosome aberrations, it is important to report polyploidy and/or endoreduplication when this is seen.

Reference mutagens are tested concurrently with the test item in order to demonstrate the sensitivity of the test system.

The assay is considered as acceptable, when all three experimental conditions are conducted: short term treatment **without** and **with** metabolic activation and long term treatment **without** metabolic activation. There is no requirement for verification of a clearly negative or positive result. In case the response is neither clearly negative nor clearly positive or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and / or further investigations. Scoring additional cells or performing a repeat experiment could be useful.

9.2. Justification for the Selection of the Test System

The OECD Guideline for Testing of Chemicals Section 4, No 473 – *"In Vitro* Mammalian Chromosome Aberration Test" [4] adopted 26 September, 2014 – recommends using a variety of cell lines or primary cell cultures (e.g. Chinese hamster fibroblasts, human or other mammalian peripheral blood lymphocytes).

9.3. Justification for the Selection of the Test Method

Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473 "*In vitro* Mammalian Chromosome Aberration Test" [4], adopted 26 September, 2014 – recommends the treatment of proliferating cells in the presence and absence of a metabolic activation system.

10. Materials and Methods

10.1. Characterisation of the Test Item

The identity of the test item was inspected upon delivery at the test facility (e.g. test item name, batch no. and additional data were compared with the label) based on the following specifications provided by the sponsor.

Name:	Soy Leghemoglobin Preparation
Composition	6.74% Soy Leghemoglobin (active ingredient)[1]
	8.11% other (inactive ingredient)
Batch No.:	16-015-101
Molecular Weight:	no data
Physical State:	liquid
Colour:	clear reddish brown
pH Value:	6.5 – 8.5
Active Component:	6.74% Soy Leghemoglobin
Purity:	no data
Expiry Date:	not applicable
Storage Conditions:	-20 to -80 °C
Safety Precautions:	The routine hygienic procedures will be sufficient to assure personnel health and safety.

10.2. Preparation of the Test Item

A solubility test was performed with different solvents and vehicles up to 5 mg/mL. According to the results of the solubility test the test item was dissolved in cell culture medium. To correct for 100% active ingredient (Soy Leghemoglobin), a factor of 14.837 was applied, based on the original value of 6.74% Soy Leghemoglobin provided by Impossible Foods[1], to reach the highest tested concentration of 5 mg/mL Soy Leghemoglobin. From this maximum concentration, separate dosing solutions were prepared prior to treatment by serial dilution and added to the cells. Therefore, all the dose concentrations refer to the concentration of the active ingredient. The treatment medium was compatible with the survival of the cells and the S9 activity.

10.3. Controls

Negative as well as positive controls were included in each experiment.

Negative Controls

Negative controls (treatment medium) were treated the same way as all dose groups.

[1] A GLP Certificate of Analysis was prepared by PSL (from PSL study numbers 43970 for the active ingredient) and gave a Percent Soy Leghemoglobin of 6.68%. This is within an acceptable margin of error of the analytical measurement. The original value provided by Impossible Foods was used to calculate study doses.

Positive Controls

Without metabolic activation

Name	EMS; ethylmethanesulfonate
CAS No	62-50-0
Supplier	Sigma
Catalogue No.	M 0880
Lot No.	BCBQ0451V
Dissolved in	nutrient medium
Final concentrations	400 and 900 µg/mL

The stability of the positive control substance in solution is proven by the mutagenic response in the expected range. The solution was prepared on the day of experiment.

Given that a high amount of historical control data was established at Eurofins Munich with EMS this substance was used instead of MMS (OECD Guideline for Testing of Chemicals No. 473 [4]) as positive control.

With metabolic activation

Name	CPA; cyclophosphamide
CAS No	50-18-0
Supplier	Sigma
Catalogue No.	C0768
Lot No.	SLBG4216
Dissolved in	nutrient medium
Final concentration	7.5 µg/mL

CPA displays a good stability at room temperature. At 25 °C only 3.5% of its potency is lost after 24 h [10]. The solution was aliquoted and stored at ≤ -15 °C. However the stability of CPA in solution was proven by the clastogenic response in the expected range.

10.4. Test System

10.4.1. Blood Collection

Human peripheral blood lymphocytes from healthy and non-smoking donors with no known recent exposure to genotoxic chemicals and radiation were used to examine the ability of chemicals to induce cytogenetic damage and thus to identify potential carcinogens or mutagens *in vitro*. For this study (in each experiment) blood was collected only from a single donor to reduce inter-individual variability.

Blood samples were drawn by venous puncture and collected in heparinized tubes. Before use the blood was stored under sterile conditions at 4 °C for a maximum of 4 h. Whole blood samples treated with an anti-coagulant (e. g. heparin) were pre-cultured in the presence of mitogen (phyto-haematogglutinin, PHA).

10.4.2. Culture Medium

Complete Culture Medium

RPMI 1640 medium supplemented with:

15	%	fetal bovine serum (FBS)
100 U/100	µg/mL	penicillin/streptomycin solution
0.24	g/mL	PHA-L

Also used for the long-term treatment and the post incubation.

Treatment Medium (short-term exposure)

Complete culture medium without FBS.

All incubations were done at 37 °C in humidified atmosphere with 5% CO_2.

10.4.3. Mammalian Microsomal Fraction S9 Homogenate

An advantage of using *in vitro* cell cultures is the accurate control of the concentration and exposure time of cells to the test item under study. However, due to the limited capacity of cells growing *in vitro* for metabolic activation of potential mutagens an exogenous metabolic activation system is necessary. Many substances only develop mutagenic potential when they are metabolized by the mammalian organism. Metabolic activation of substances can be achieved by supplementing the cell cultures with liver microsome preparations (S9 mix).

The S9 liver microsomal fraction was obtained from Trinova Biochem GmbH, Giessen, Germany. Male Sprague Dawley rats were induced with phenobarbital / β-naphthoflavone.

The following quality control determinations were performed by Trinova Biochem GmbH:

a) Alkoxyresorufin-0-dealkylase activities
b) Test for the presence of adventitious agents
c) Promutagen activation (including biological activity in the *Salmonella typhimurium* assay using 2-aminoanthracene and benzo[*a*]pyrene)

The following additional quality control determinations were performed by Eurofins Munich:

Biological activity in:

- the mouse lymphoma assay using benzo[*a*]pyrene
- the HPRT assay using 7,12-dimethylbenz[*a*]anthracene
- the chromosome aberration assay using cyclophosphamide

A stock of the supernatant containing the microsomes was frozen in aliquots of 5 mL and stored at ≤ -75 °C.

The protein concentration in the S9 preparation (Lot: 3513) was 42 mg/mL.

10.4.4. S9 Mix

An appropriate quantity of the S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. The final percentage of S9 mix in cell culture medium is 5% v/v.

Cofactors were added to the S9 mix to reach the concentrations below:

8	mM	$MgCl_2$
33	mM	KCl
5	mM	Glucose-6-phosphate
5	mM	NADP

in 100 mM sodium-phosphate-buffer pH 7.4. During the experiment the S9 mix was stored on ice.

10.5. Experimental Design

10.5.1. Culture Initiation

In the culture vessels 500 µL heparinized whole blood were added to 4.5 mL completed culture medium

10.5.2. Pre-Experiment for Toxicity

According to the relevant guidelines the highest recommended dose is 5000 µg/mL. The highest dose group evaluated in the pre-experiment was 5000 µg/mL Soy Leghemoglobin (active ingredient).

The following concentrations of Soy Leghemoglobin (active ingredient) were tested **without** and **with** S9 mix:

10, 50, 100, 250, 500, 1000, 2000, 3000, 4000 and 5000 µg/mL

10.5.3. Exposure Concentrations

On the basis of the data and the observations from the pre-experiment and taking into account the recommendations of the guidelines, the following concentrations were selected for the main experiments I and II

The dose group selection for microscopic analyses of chromosomal aberrations was based in accordance with the recommendations of the guidelines.

Table 3: Exposure concentrations

S9 Mix	Exp. interval	Prep. interval	Concentrations in µg/mL							
Experiment I										
-	4 h	24 h	200	**500**	**1000**	**2500**	**5000**			
+	4 h	24 h	200	500	**1000**	**2500**	**5000**			
Experiment II										
-	24 h	24 h	**100**	**200**	**500 (P)**	**1000 (P)**	2000 (P)	3000 (P)	4000 (P)	5000 (P)

Evaluated experimental points are shown in bold letters
P Precipitation was observed during preparation of the cells

10.6. Experimental Performance

10.6.1. Treatment

Experiment I

Short-term exposure 4 h (without and with S9 mix)

After 48 h the culture medium was replaced with serum-free medium containing the test item (**without** metabolic activation) and serum-free medium containing the test item with 50 µL/mL S9 mix (**with** metabolic activation). After 4 h the cells were spun down by gentle centrifugation for 10 min. The supernatant with the dissolved test item was discarded and the cells were resuspended in PBS. The washing procedure was repeated once as described. After washing, the cells were resuspended in complete cell culture medium (10.4.2 Culture Medium). The cells were prepared 24 h after the beginning of the treatment.

Experiment II

Long-term exposure 24 h (without S9 mix):

After 48 h the culture medium was replaced with complete medium (with 15% FBS) containing the test item without S9 mix. The treated cells were prepared at the end of the treatment.

10.6.2. Preparation of the Cultures

At least 2 h before harvesting, colcemid was added to the cultures (final concentration 0.2 µg/mL). The cultures were harvested by centrifugation 24 h after beginning of treatment. The supernatant was discarded and the cells were resuspended in approximately 5 mL hypotonic solution (0.4% KCl). The cell suspension was incubated at room temperature for 20 min. After removal of the hypotonic solution by centrifugation the cells were fixed with 3+1 methanol + glacial acetic acid. The fixation procedure was repeated twice. Slides were prepared by dropping the cell suspension onto a clean microscopic slide. The cells were stained with giemsa and according to the Fluorescent plus Giemsa technique, respectively. The slides were coverslipped using 2-3 drops of Eukitt(R). Afterwards they were air dried.

10.6.3. Proliferation Index

The negative control and the highest dose groups evaluated were treated in the presence of BrdU, parallel to the treatment groups, to reassure the proliferation index and/or replication time of the cultured lymphocytes. The proliferation index was determined by scoring the number of first, second and third metaphases in 100 cells per culture. The proliferation index (PI) was calculated at time point of preparation as:

$$PI = \frac{1\ (\%\ \text{cells in M1}) + 2\ (\%\ \text{cells in M2}) + 3\ (\%\ \text{cells in M3})}{100}$$

with
M1: first mitosis,
M2: second mitosis,
M3: third mitosis
initiating at the start of exposure.

10.6.4. Analysis of Metaphase Cells

All slides, including those of positive and negative controls were independently coded before microscopic analysis. Evaluation of the cultures was performed (according to standard protocol of the "Arbeitsgruppe der Industrie, Cytogenetik" [11]) using microscopes with 100x oil immersion objectives. As structural chromosomal aberrations breaks, fragments, deletions, exchanges and chromosomal disintegrations were recorded. Gaps were recorded as well but not included in the calculation of the aberration rates. The definition of a gap was as follows: an achromatic region (occurring in one or both chromatids) independent of its width. The remaining visible chromosome regions should not be dislocated neither longitudinally nor laterally. At least, if available, 300 well spread metaphases per concentration and validity controls were scored for cytogenetic damage. Metaphases with 46±2 centromeres regions were included in the analysis.

To describe a cytotoxic effect the mitotic index (% cells in mitosis; by counting the number of mitotic cells in 1000 cells) was determined (Table 5, Table 7). Additionally the number of polyploid cells was scored. Polyploid means a near tetraploid karyotype in the case of this aneuploid cell line.

10.7. Data Recording

The data generated were recorded in the raw data file. The results are presented in tables, including experimental groups with the test item, negative and positive controls. The experimental unit was the cell and therefore, the percentage of cells with structural aberration was evaluated. Different types of chromosome aberrations are listed with their numbers of frequencies for experimental and control groups. Gaps were recorded separately and reported but generally not included in the aberration frequency. Concurrent measurements of cytotoxicity were also recorded.

10.8. Acceptability of the Assay

The chromosomal aberration assay is considered acceptable if it meets the following criteria:

- the number of aberration found in the negative and/or solvent controls falls within the range of historical laboratory control data / is considered acceptable for addition to the laboratory historical negative control database.
- concurrent positive controls should induce responses that are compatible with those generated in the historical positive control data base and produce a statistically significant increase compared with the concurrent negative control
- the proliferation criteria in the solvent control should be similar to the corresponding negative control (where applicable)
- All three experimental conditions were tested unless one resulted in positive results
- Adequate number of cells and concentrations are analyzable

The criteria for the selection of top concentration are consistent with those described earlier (10.5.3)

10.9. Evaluation of Results

Providing that all acceptability criteria are fulfilled, a test chemical is considered to be clearly positive if, in any of the experimental conditions examined:

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) the increase is dose-related when evaluated with an appropriate trend test,

c) any of the results are outside the distribution of the historical negative control data

When all of these criteria are met, the test chemical is then considered able to induce chromosomal aberrations in cultured mammalian cells in this test system.

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test,

c) all results are inside the distribution of the historical negative control data.

The test chemical is then considered unable to induce chromosomal aberrations in cultured human peripheral blood lymphocyte cells in this test system.

11. Deviations from the Study Plan

There were the following deviations from the study plan:

- **Concerning:**

1. Project Staff Signatures (study plan, p. 2) and 4.3 Project Staff (study plan, p. 6)

Study Plan:

Study Director Dr. Hana Hofman-Hüther

Report:

Study Director Christine Tiessen

Reason:

Project handover

- **Concerning:**

Test item name (study plan, p. 1)

Study Plan:

Leghemoglobin

Report:

Soy Leghemoglobin Preparation

Reason:

Sponsor's request

- **Concerning:**

7.1 Characterisation of the Test Item (study plan, p.11)

Study Plan:

Active Component: 6.74% Leghemoglobin

Report:

Active Component: 6.74% Soy Leghemoglobin

Reason:

Sponsor's request

These deviations did not influence the quality or integrity of the present study.

12. Results and Discussion

12.1. Results

12.1.1. Pre-Experiment for Toxicity

According to the guidelines the highest recommended concentration was 5000 µg/mL Soy Leghemoglobin (active ingredient). The test item was dissolved in cell culture medium. No precipitation of the test item was noted. However, the colouring of the test item itself was dark-red. The highest concentration evaluated in the pre-experiment was 5000 µg/mL. The relative mitotic index was used as the parameter for evaluating toxicity. The concentrations evaluated in the main experiment are based on the results obtained in the pre-experiment (Table 4).

Table 4: Test for Cytotoxicity

Dose Group	Concentration [µg/mL]	Mitotic Index	relative [%]
without metabolic activation			
C	0	28.5	100
1	10	37.5	132
2	50	25	88
3	100	29	102
4	250	27	95
5	500	30.5	107
6	1000	29.5	104
7	2000	25	88
8	3000	20	70
9	4000	25	88
10	5000	25.5	89
with metabolic activation			
C	0	52.5	100
1	10	45.5	87
2	50	54.5	104
3	100	44.5	85
4	250	51	97
5	500	37	70
6	1000	48	91
7	2000	26	50
8	3000	41	78
9	4000	35.5	68
10	5000	36	69

The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the negativ control.

C: Control

12.1.2. Summary of Experiment I and Experiment II

Table 5: Experiment I - Summary of Cytotoxicity Data

Dose Group	Concentration [µg/ml]	Polyploid Cells			Mitotic Index Culture			relative
		1	2	Mean	1	2	Mean	[%]
without metabolic activation								
C	0	0	0	0	38	42	40	100
2	500	0	0	0	38	31	35	86
3	1000	0	0	0	29	26	28	69
4	2500	0	0	0	25	20	23	56
5	5000	0	0	0	28	15	22	54
EMS	900	0	0	0	37	13	25	63
with metabolic activation								
C	0	0	0	0	39	37	38	100
3	1000	0	0	0	42	42	42	111
4	2500	0	0	0	35	28	32	83
5	5000	0	0	0	41	39	40	105
CPA	7.5	0	0	0	38	29	34	88

The number of polyploid cells was determined in 150 cells per culture of each test group.
The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the negative controls.

C: Negative Control (Culture Medium)
EMS: Ethylmethanesulfonate
CPA: Cyclophosphamide

Table 6: Experiment I – Summary of Aberration Rates

Dose Group	Concen-tration [µg/ml]	Treatment Time	Fixation Interval	mean % aberrant cells	
				incl. Gaps	excl. Gaps
without metabolic activation					
C	0	4 h	24 h	**5.7**	**3.3**
2	500	4 h	24 h	**6.0**	**4.0**
3	1000	4 h	24 h	**7.3**	**4.3**
4	2500	4 h	24 h	**6.0**	**4.0**
5	5000	4 h	24 h	**6.7**	**1.7**
EMS	900	4 h	24 h	**18.5**	**16.0**
with metabolic activation					
C	0	4 h	24 h	**7.7**	**3.7**
3	1000	4 h	24 h	**7.3**	**4.3**
4	2500	4 h	24 h	**5.0**	**2.0**
5	5000	4 h	24 h	**5.0**	**2.7**
CPA	7.5	4 h	24 h	**18.5**	**15.5**

300 cells evaluated for each concentration, except for the positive controls EMS (200 cells) and CPA (200 cells)

C: Negative Control (Culture Medium)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
CPA: Positive Control (with metabolic activation: Cyclophosphamide)

Table 7: Experiment II - Summary of Cytotoxicity Data

Dose Group	Concentration [µg/ml]	Polyploid Cells			Mitotic Index Culture			relative
		1	2	Mean	1	2	Mean	[%]
without metabolic activation								
C	0	0	0	0	59	61	60	100
1	100	1	0	0.5	53	51	52	87
2	200	0	0	0	54	60	57	95
3 (P)	500	0	0	0	40	43	42	69
4 (P)	1000	0	0	0	22	41	32	53
5 (P)	2000	n.d	n.d	n.d	15	16	16	26
6 (P)	3000	n.d	n.d	n.d	8	8	8	13
7 (P)	4000	n.d	n.d	n.d	27	19	23	38
8 (P)	5000	n.d	n.d	n.d	18	32	25	42
EMS	400	0	0	0	38	19	29	48

The number of polyploid cells was determined in 150 cells per culture of each test group.
The mitotic index was determined in 1000 cells per culture of each test group.
The relative values of the mitotic index are related to the negative controls.

C: Negative Control (Culture Medium)
EMS: Ethylmethanesulfonate
n.d: not determined
P: Precipitation

Table 8: Experiment II - Summary of Aberration Rates

Dose Group	Concentration [µg/ml]	Treatment Time	Fixation Interval	mean % aberrant cells	
				incl. Gaps	excl. Gaps
without metabolic activation					
C	0	24 h	24 h	**4.3**	**2.3**
1	100	24 h	24 h	**6.0**	**2.7**
2	200	24 h	24 h	**6.7**	**3.3**
3 (P)	500	24 h	24 h	**3.3**	**2.0**
4 (P)	1000	24 h	24 h	**3.3**	**2.0**
EMS	400	24 h	24 h	**57.3**	**54.7**

300 cells evaluated for each concentration, except for the positive control EMS (75 cells)

C: Negative Control (Culture Medium)
EMS: Positive Control (without metabolic activation: Ethylmethanesulfonate)
P: Precipitation

12.1.3. Proliferation Index

Table 9: Experiment I - Proliferation Index determined by BrdU-Labeling

Dose Group	Concentration [µg/mL]	Treatment Time	Proliferation Index	1. Mitosis	2. Mitosis	3. Mitosis
without metabolic activation						
C	0	4 h	**1.16**	84	16	0
5	5000	4 h	**1.09**	91	9	0
with metabolic activation						
C	0	4 h	**1.12**	88	12	0
5	5000	4 h	**1.07**	93	7	0

C: Negative Control (Culture Medium)

Table 10: Experiment II - Proliferation Index determined by BrdU-Labeling

Dose Group	Concentration [µg/mL]	Treatment Time	Proliferation Index	1. Mitosis	2. Mitosis	3. Mitosis
without metabolic activation						
C	0	24 h	**1.56**	44	56	0
3	500	24 h	**1.23**	77	23	0
4	1000	24 h	**1.12**	88	12	0

C: Negative Control (Culture Medium)

12.2. Biometry

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the Fischer's exact test. The p value was used as a limit in judging for significance levels in comparison with the corresponding solvent control. Aberrant cells without gaps were only used for the calculation. Gaps are recorded separately and reported but generally not included in the total aberration frequency calculation according to the guideline.

Table 11: Biometry - Experiment I, without metabolic activation

Negative Control versus Test Group	Concentration [µg/mL]	Treatment Time [h]	Aberrant Cells (excl. gap)	Significance	p Value
C	0	4	10	-	1.0000
2	500	4	12	-	0.8286
3	1000	4	13	-	0.6716
4	2500	4	12	-	0.8286
5	5000	4	5	-	0.2956
EMS	900	4	32	+	<0.0001

+: significantly increased
-: not significant
EMS: Positive Control (Ethylmethanesulfonate)

Table 12: Biometry - Experiment I, with metabolic activation

Negative Control versus Test Group	Concentration [µg/mL]	Treatment Time [h]	Aberrant Cells (excl. gap)	Significance	p Value
C	0	4	11	-	1.0000
3	1000	4	13	-	0.8355
4	2500	4	6	-	0.3253
5	5000	4	8	-	0.6422
CPA	7.5	4	31	+	<0.0001

+: significantly increased
-: not significant
CPA: Positive Control (Cyclophosphamide)

Table 13: Biometry - Experiment II, without metabolic activation

Negative Control versus Test Group	Concentration [µg/mL]	Treatment Time [h]	Aberrant Cells (excl. gap)	Significance	p Value
C	0	24	7	-	1.0000
1	100	24	8	-	1.0000
2	200	24	10	-	0.6240
3	500	24	6	-	1.0000
4	1000	24	6	-	1.0000
EMS	400	24	41	+	<0.0001

+: significantly increased
-: not significant
EMS: Positive Control (Ethylmethanesulfonate)

Table 14: Biometry – Trend test

Statistical significance at the 5% level ($p < 0.05$) was evaluated by the χ^2 test for trend. The p value was used as a limit in judging for significance levels.

Experiment	Treatment Time [h]	Significance	P Value
Exp. I without metabolic activation	4	-	0.1222
Exp. I with metabolic activation	4	-	0.2315
Exp. II without metabolic activation	24	-	0.4083

+: significant
-: not significant
Statistical significance: statistical significant concentration-related increase in cells with chromosomal aberrations (χ^2 test for trend, $p < 0.05$).

12.3. Discussion

The test item Soy Leghemoglobin Preparation was investigated for a possible potential to induce structural chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 homogenate.

The selection of the concentrations used in experiment I and II was based on data from the solubility test and the pre-experiment which were performed according to the guidelines.

The chromosomes were prepared 24 h after start of treatment with the test item. The treatment interval in the pre- and main experiment (experiment I) was 4 h **without** and **with** metabolic activation. The treatment interval in experiment II was 24 h **without** metabolic activation. Duplicate cultures were set up per concentration in experiment I and II. Per culture 150 metaphases were scored for structural chromosomal aberrations (for exceptions see Tables).

Pre-Experiment (single culture)

Without and **with** metabolic activation, 4 h treatment, 24 h preparation interval:

10, 50, 100, 250, 500, 1000, 2000, 3000, 4000 and 5000 µg/mL

Experiment I (duplicate culture)

Without and **with** metabolic activation, 4 h treatment, 24 h preparation interval:

500, 1000, 2500 and 5000 µg/mL

Experiment II (duplicate culture)

Without metabolic activation, 24 h treatment, 24 h preparation interval:

100, 200, 500 and 1000 µg/mL

12.3.1. Precipitation

In the pre-experiment and experiment I precipitation was not observed after treatment of the lymphocytes with the different test item concentrations. However, the test item itself showed a dark-red colouring

In experiment II precipitation occurred in the concentrations 500 µg/mL and higher during the fixation of the cells. In contrast to experiment I, in the experiment with long-term treatment the test item is not removed by repeated washing steps, as the treatment period is stopped by the fixation step directly. When the cells were spread on the object slides the precipitation appeared as a greenish lacquer coat, visible by eye and with the aid of an inverted microscope. The evaluation of aberration rates was not affected.

12.3.2. Toxicity (Relative Mitotic Index)

In the pre-experiment without metabolic activation, cytotoxicity was not observed as the rel. mitotic index was not decreased below 70%. With metabolic activation, the concentrations 2000 µg/mL, 4000 µg/mL and 5000 µg/mL led to a reduction of the mitotic index to 50%, 68% and 69%, respectively. Given that the mitotic index of the concentration 3000 µg/mL was 78%, the decrease induced by the concentration 2000 µg/mL (50%) seemed to be an outlier. Because of this reason and due to the fact that the pre-experiment was not performed in duplicate cultures, the decrease to 50% was not regarded as biologically relevant. However, the highest concentrations 4000 µg/mL and 5000 µg/mL showed light cytotoxic effects.

In comparison to the pre-experiment, toxic effects (decrease below 70% rel. mitotic index) were seen at concentrations of 1000 µg/mL (69%), 2500 µg/mL (56%) and 5000 µg/mL (54%) in experiment I **without** metabolic activation (Table 5) and in experiment I **with** metabolic activation, no toxic effects (decrease below 70% rel. mitotic index) were observed. These divergent results are due to the use of different blood donors in experiment I and the pre-experiment. Overall, the results of the first

experiment are more precise than in the preliminary experiment of the dose range finding as two cultures were evaluated. Moreover, the added metabolic fraction in experiment I **with** metabolic activation, could led to a detoxification process. Therefore toxicity might be lower than in experiment I **without** metabolic activation.

In experiment II **without** metabolic activation toxic effects judged by the mitotic index were noted in the concentrations 500 µg/mL (69%), 1000 µg/mL (53%), 2000 µg/mL (26%), 3000 µg/mL (13%), 4000 µg/mL (38%) and 5000 µg/mL (42%) (Table 7). The stronger cytotoxic effects compared to experiment I can be attributed to the longer incubation period of 24 h. Although the mitotic index seemed to increase in the higher concentrations 4000 µg/mL and 5000 µg/mL, only concentrations up to 1000 µg/ml were evaluated for chromosomal aberrations as precipitation was noted from concentrations of 500 µg/mL and higher.

12.3.3. Toxicity (Proliferation Index)

The BrdU-technique was used for determining the proliferation index to detect a possible effect on the proliferation rate after treatment with the test item and thus indicating cell cycle delay. In the experiment I, the values of the proliferation index of the negative controls were 1.16 (**without** metabolic activation) and 1.12 (**with** metabolic activation) (Table 9). The proliferation index of the highest dose groups evaluated were 1.09 (5000 µg/mL) (**without** metabolic activation) and 1.07 (5000 µg/mL) (**with** metabolic activation). No biologically relevant decrease of the proliferation index was indicated.

In the experiment II, the values of the proliferation index of the negative controls were 1.56 (**without** metabolic activation) (Table 10). The proliferation index of the highest dose groups evaluated **without** metabolic activation (500 and 1500 µg/mL) were 1.23 and 1.12. A biologically relevant decrease to 79% at 500 µg/mL and 72% at 1000 µg/mL of the proliferation index was indicated. Therefore during the long-term experiment a mitotic delay was observed. A cell cycle delay might be the consequence of chromosomal aberrations or related to cytotoxicity. As only cytotoxicity was determined at these concentrations, the cell cycle delay seemed to be cytotoxicity related and concentration-dependent.

12.3.4. Clastogenicity

There are several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosome aberrations for at least one of the dose groups, which is higher than the laboratory negative control range (0.0% - 3.0% aberrant cells **without** metabolic activation and 0.0% - 3.7% aberrant cells **with** metabolic activation).

In experiment I **without** metabolic activation, aberration rates of 500 µg/mL (4.0%), 1000 µg/mL (4.3%) and 2500 µg/mL (4.0%) evaluated are slightly increased above the historical control range (Table 15). However, as the negative control is with 3.3% slightly above the control data, which indicates a raise of the basic level of chromosome aberrations, these effects are regarded as not biologically relevant. Moreover, this statement is in line with the result of the highest tested concentration of 5000 µg/mL (1.7%), which is within the historical control data. Reduced clastogenic effects due to cytotoxicity in the highest test item concentration of 5000 µg/mL (rel. mitotic index 54%) can be excluded as for the lower concentration of 2500 µg/mL a similar mitotic index was determined (rel. mitotic index 56%). The variation observed in the two highest concentrations (3.7% aberrant cells at 2500 µg/mL and 1.7% aberrant cells at 5000 µg/mL) is most likely due to the variability observed regularly in a biological test system. Additionally, neither a concentration dependent increase towards higher aberration rates nor a statistically significance was observed. In conclusion, in experiment I **without** metabolic activation the test item Soy Leghemoglobin Preparation did not induce structural chromosomal aberrations in human lymphocytes.

In experiment I **with** metabolic activation the negative control is with 3.7% chromosomal aberrations within the range of the historical negative control data (0-3.7%) (Table 15). The lowest concentration evaluated (1000 µg/mL) is slightly above the control range with 4.3% of chromosomal aberrations (Table 6). However, as the negative control is at the upper limit of the historical control data and test item concentrations 2500 µg/mL (2.0%) and 5000 µg/mL (2.7%) are within the control data and no

dose dependent increase or a trend towards higher aberration rates is obvious, this effect is regarded as not biologically relevant.

In experiment II no biologically relevant increase of the aberration rates was noted after treatment with the test item **without** metabolic activation (Table 8). The aberration rates of all dose groups treated with the test item were within the historical control data of the negative control (Table 16).

In the experiments I and II **without** and **with** metabolic activation no biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item as compared to the controls.

EMS (400 and 900 µg/mL) and CPA (7.5 µg/mL) were used as positive controls and induced distinct and biologically relevant increases in cells with structural chromosomal aberrations, thus proving the ability of the test system to indicate potential clastogenic effects.

The Fisher's exact test was performed to verify the results in the experiment. No statistically significant increase ($p < 0.05$) of cells with chromosomal aberrations was noted in the dose groups of the test item evaluated in experiment I and II **without** and **with** metabolic activation.

The χ^2 Test for trend was performed to test whether there is a concentration-related increase in chromosomal aberrations. No statistically significant increase was observed in experiment I **without** and **with** metabolic activation and in experiment II **without** metabolic activation.

12.3.5. Polyploid Cells

Table 5 and Table 7 show the occurrence of polyploid metaphases. No biologically relevant increase in the frequencies of polyploid cells was found after treatment with the test item.

13. Conclusion

In conclusion, it can be stated that during the described *in vitro* chromosomal aberration test and under the experimental conditions reported, the test item Soy Leghemoglobin Preparation did not induce structural chromosomal aberrations in human lymphocyte cells.

Therefore, Soy Leghemoglobin Preparation is considered to be non-clastogenic in this chromosome aberration test.

14. Distribution of the Report

1 original (paper):	Sponsor
1 copy (paper):	Eurofins BioPharma Product Testing Munich GmbH
1 copy (electronic):	Sponsor

15. References

15.1. Guidelines

[1] Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on August 28, 2013 (BGBl. I S. 3498).

[2] Konsens-Dokument der Bund-Länder-Arbeitsgruppe Gute Laborpraxis ("Consensus Document of the National and Länder Working Party on Good Laboratory Practice") on the archiving and storage of records and materials, 5 May 1998

[3] OECD Principles of Good Laboratory Practice (as revised in 1997); OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Co-operation and Development, Paris 1998

[4] Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 473, "*In vitro* Mammalian Chromosome Aberration Test", adopted 26 September, 2014.

[5] Commission Regulation (EC) No. 440/2008 B 10: "Mutagenicity – *In vitro* Mammalian Chromosome Aberration Test", dated May 30, 2008.

15.2. Literature

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15.3. Internal Eurofins Munich SOPs

Standard Operating Procedures (SOPs), No. 15-1-2, No. 15-2-7, No. 15-2-8

16. Appendix

16.1. Appendix 1: Historical Laboratory Control Data

Table 15: Historical Laboratory Control Data of the negative control (short term treatment) (2010 - 2015)

	NC Number of aberrant cells metabolic activation -		+	
	+ Gaps	- Gaps	+ Gaps	- Gaps
mean [%]	3.4	1.5	3.1	1.3
SD [%]	1.57	0.84	1.63	0.82
RSD [%]	46.4	55.6	52.3	62.0
min [%]	0.5	0.0	0.0	0.0
max [%]	6.5	3.0	9.7	3.7
n	35	35	62	62

NC: Negative Control
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.: minimum number of aberrant cells
max.: maximum number of aberrant cells
n: Number of assays
The historical data **without** metabolic activation comprise the 4 h and the 24 h treatment interval

Table 16: Historical Laboratory Control Data of the negative control (long term treatment) (2010 - 2015)

	NC Number of aberrant cells metabolic activation -	
	+ Gaps	- Gaps
mean [%]	3.0	1.1
SD [%]	1.54	0.90
RSD [%]	51.4	79.1
min [%]	0.5	0.0
max [%]	6.9	4.2
n	32	32

NC: Negative Control
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.: minimum number of aberrant cells
max.: maximum number of aberrant cells
n: Number of assays

Table 17: Historical Laboratory Control Data of the positive control (2010 - 2015)

	PC Number of aberrant cells metabolic activation			
	-		+	
	+ Gaps	- Gaps	+ Gaps	- Gaps
mean [%]	17.0	14.4	17.2	14.6
SD [%]	6.74	6.47	4.58	4.15
RSD [%]	39.7	45.0	26.6	28.4
min [%]	9.5	7.0	9.6	8.0
max [%]	41.0	38.1	31.0	26.7
n	68	68	62	62

PC: Positive Control (EMS **without** metabolic activation, CPA **with** metabolic activation)
mean: mean number of aberrant cells
SD: Standard Deviation
RSD: relative Standard Deviation
min.: minimum number of aberrant cells
max.: maximum number of aberrant cells
n: Number of assays

The historical data **without** metabolic activation comprise the 4 h and the 24 h treatment interval

16.2. Appendix 2: Raw Data

16.2.1. Main Experiment I

Table 18: Experiment I - Structural Chromosomal Aberrations, *without* metabolic activation: 4 h treatment, 24 h fixation period.

Dose Group	Concentration [µg/ml]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells incl. Gaps	Aberrant Cells excl. Gaps	Gaps		Types of Aberrations Found									
									Chromatid types				Chromosome types				Other	
							g	ig	b	f	d	ex	ib	if	id	cx	ma	cd
C	0	1	150	0	10	4	6	0	4	0	0	0	0	0	0	0	0	0
		2	150	0	7	6	2	0	3	0	0	0	0	3	0	0	0	0
		total	300	0	17	10	8	0	7	0	0	0	0	3	0	0	0	0
2	500	1	150	0	9	7	2	0	5	0	0	0	0	2	0	0	0	0
		2	150	0	9	5	3	2	1	0	0	0	0	4	0	0	0	0
		total	300	0	18	12	5	2	6	0	0	0	0	6	0	0	0	0
3	1000	1	150	0	11	6	5	1	5	0	1	0	0	0	0	0	0	0
		2	150	0	11	7	4	0	4	0	0	0	0	3	0	0	0	0
		total	300	0	22	13	9	1	9	0	1	0	0	3	0	0	0	0
4	2500	1	150	0	15	10	7	1	8	0	2	0	0	1	0	0	0	0
		2	150	0	3	2	1	0	2	0	0	0	0	0	0	0	0	0
		total	300	0	18	12	8	1	10	0	2	0	0	1	0	0	0	0
5	5000	1	150	0	13	3	11	0	2	0	1	0	0	0	0	0	0	0
		2	150	0	7	2	5	0	2	0	0	0	0	0	0	0	0	0
		total	300	0	20	5	16	0	4	0	1	0	0	0	0	0	0	0
EMS	900	1	150	0	19	14	5	0	4	0	0	2	0	8	0	0	1	0
		2	50	0	18	18	2	0	13	0	2	11	0	3	0	0	0	0
		total	200	0	37	32	7	0	17	0	2	13	0	11	0	0	1	0

C: Negative Control (Culture Medium)
EMS: Ethylmethanesulfonate

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

Table 19: Experiment I - Structural Chromosomal Aberrations, *with* metabolic activation: 4 h treatment, 24 h fixation period.

Dose Group	Concentration [µg/ml]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells		Gaps		Types of Aberrations Found									
					incl. Gaps	excl. Gaps			Chromatid types				Chromosome types				Other	
							g	ig	b	f	d	ex	ib	if	id	cx	ma	cd
C	0	1	150	0	10	5	5	0	3	0	1	0	0	1	0	0	0	0
		2	150	0	13	6	7	1	4	1	0	1	0	0	0	0	0	0
		total	300	0	23	11	12	1	7	1	1	1	0	1	0	0	0	0
3	1000	1	150	0	7	4	4	0	4	0	0	0	0	0	0	0	0	0
		2	150	0	15	9	7	0	7	0	1	0	0	1	0	0	0	0
		total	300	0	22	13	11	0	11	0	1	0	0	1	0	0	0	0
4	2500	1	150	0	9	2	7	1	0	0	0	0	0	1	0	1	0	0
		2	150	0	6	4	1	1	3	0	1	0	0	0	0	0	0	0
		total	300	0	15	6	8	2	3	0	1	0	0	1	0	1	0	0
5	5000	1	150	0	4	2	1	1	2	0	0	0	0	0	0	0	0	0
		2	150	0	11	6	5	1	5	0	0	1	0	1	0	0	0	0
		total	300	0	15	8	6	2	7	0	0	1	0	1	0	0	0	0
CPA	7.5	1	150	0	18	17	3	0	7	1	1	3	2	5	0	0	0	0
		2	50	0	19	14	6	1	17	0	0	1	0	5	0	0	0	0
		total	200	0	37	31	9	1	24	1	1	4	2	10	0	0	0	0

C: Negative Control (Culture Medium)
CPA: Cyclophosphamide

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

16.2.2. Main Experiment II

Table 20: Experiment II - Structural Chromosomal Aberrations, *without* metabolic activation: 24 h treatment, 24 h fixation period.

Dose Group	Concentration [µg/ml]	Culture	Scored Cells	Polyploid Cells	Aberrant Cells		Gaps		Types of Aberrations Found									
					incl. Gaps	excl. Gaps			Chromatid types				Chromosome types				Other	
							g	ig	b	f	d	ex	ib	if	id	cx	ma	cd
C	0	1	150	0	10	6	4	2	4	0	0	1	0	1	0	0	0	0
		2	150	0	3	1	1	1	1	0	0	0	0	0	0	0	0	0
		total	300	0	13	7	5	3	5	0	0	1	0	1	0	0	0	0
1	100	1	150	1	12	3	7	2	3	0	0	0	0	1	0	0	0	0
		2	150	0	6	5	1	0	4	0	0	0	0	1	0	0	0	0
		total	300	1	18	8	8	2	7	0	0	0	0	2	0	0	0	0
2	200	1	150	0	16	8	11	0	6	0	0	0	0	1	0	1	0	0
		2	150	0	4	2	1	1	1	0	0	0	0	1	0	0	0	0
		total	300	0	20	10	12	1	7	0	0	0	0	2	0	1	0	0
3	500	1	150	0	8	4	4	0	4	0	0	0	0	0	0	0	0	0
		2	150	0	2	2	1	0	2	0	0	0	0	0	0	0	0	0
		total	300	0	10	6	5	0	6	0	0	0	0	0	0	0	0	0
4	1000	1	150	0	7	3	4	0	3	0	0	0	0	0	0	0	0	0
		2	150	0	3	3	0	0	3	0	0	0	0	0	0	0	0	0
		total	300	0	10	6	4	0	6	0	0	0	0	0	0	0	0	0
EMS	400	1	50	0	23	21	14	3	27	0	0	4	0	5	0	0	2	0
		2	25	0	20	20	7	2	35	0	0	5	1	0	0	0	1	0
		total	75	0	43	41	21	5	62	0	0	9	1	5	0	0	3	0

C: Negative Control (Culture Medium)
EMS: Ethylmethanesulfonate

(abbreviations: g = gap; ig = iso-gap; b = break; ib = iso-break; f = fragment; if = iso-fragment; d = deletion; id = iso-deletion; ma = multiple aberration; ex = chromatid type exchange; cx = chromosome type exchange; cd = chromosomal disintegration)

16.3. Appendix 3: Certificate of Analysis

Product Safety Labs

CERTIFICATE OF ANALYSIS

Product: Soy Leghemoglobin Preparation

Lot #: PP-PGM2-16-015-101

PSL Reference No.: 160809-3D

Date of Analysis: September 1, 2016

Result:

Soy Leghemoglobin – 6.68%

Approval: (b) (6) 9/14/16

David Sinning
Analytical Services
Product Safety Labs

Date

QA Release: (b) (6) Sep 14, 2016

Rhonda Krick, B.S.
Quality Assurance
Product Safety Labs

Date

This material was analyzed in compliance with Good Laboratory Practice (40 CFR 160) standards. Data are reported in PSL GLP Study No. 43970

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Annex 6

UPDATED EXPERT COMMENTS ON POTENTIAL ALLERGENICITY OF SOYBEAN LEGHEMOGLOBIN

Steve L. Taylor, Ph.D.
Taylor Consulting LLC
Lincoln, NE

December 19, 2016

Impossible Foods has met with representatives from the Food & Drug Administration regarding its GRAS Notification (GRN540) for soy leghemoglobin. FDA representatives have shared several critical comments with Impossible Foods with respect to GRN540. Previously, I had submitted my expert opinion on the potential allergenicity of soy leghemoglobin (specifically, soy leghemoglobin preparation (LegH Prep), with soy leghemoglobin as its principal ingredient). Now, I wish to expand upon that previous opinion to address certain key concerns raised by FDA representatives. The concerns raised at various times by FDA regarding GRN540 and the potential allergenicity of soy leghemoglobin are listed below together with my responses based upon my scientific knowledge and expertise.

- FDA concern that Impossible Foods should perform a full allergenicity evaluation on soy leghemoglobin and develop a GRAS dossier patterned after GRN117

In one meeting between FDA and Impossible Foods, FDA compared GRN540 to GRN117, a notice on ice-structuring protein (ISP) that was advanced several years ago by Unilever. I also served as a consultant to Unilever and a member of the GRAS Panel for ISP. In my view, a major distinction exists between GRN540 and GRN117 that invalidates GRN117 as a model for the type of data that should be submitted by Impossible Foods on soy leghemoglobin. A key feature of GRN117 was that Unilever did not wish to label ISP as a fish protein. Accordingly, Unilever was obliged to conduct extensive studies to document that ISP was not an allergenic fish protein, and that its ingestion would be safe for fish-allergic consumers. The situation with soy leghemoglobin is the exact opposite. Impossible Foods fully intends to label soy leghemoglobin as a soy protein. Products with soy leghemoglobin also will be labeled as "Contains Soy" in accordance with FALCPA requirements. Thus, soy-allergic consumers will be advised by these label statements to avoid products containing soy leghemoglobin. In essence, Impossible Foods is conceding that soy leghemoglobin is a possible allergen from soy, even though there is no scientific evidence to suggest that this is the case.

- FDA concern that Impossible Foods should conduct clinical studies on soy-allergic individuals to determine if soy leghemoglobin is a soy allergen

Soy leghemoglobin is very unlikely to pose any risk to soy-allergic consumers. First, soy leghemoglobin is derived from the roots of the soybean plant and not the edible seeds. The known soy allergens are found in soybean seeds. Soy leghemoglobin bears no structural similarity to any of the known soy allergens. But beyond that, Impossible Foods is planning to identify soy leghemoglobin in its ingredient label as "leghemoglobin (soy)" and advise that products containing soy leghemoglobin should be labeled as "Contains Soy". Thus soy-allergic consumers will be alerted that they should avoid consumption of products containing soy leghemoglobin.

In my expert opinion, the state of the science on soybean allergens can be summarized in one word – confusing. Many soy proteins have been identified as potential allergens. Expert scientific consensus does not exist with respect to a list of all soy proteins that might be potential soy allergens. Consensus is emerging that Gly m 5 and Gly m 6 are the major soy allergens and these proteins are also the major seed storage proteins of soybean. Because of the confusing nature of the scientific evidence, the possible existence of other soy proteins as minor allergens cannot be excluded. Thus, in my expert opinion, it is the wisest course for Impossible Foods to reveal that the soy leghemoglobin ingredient is derived from soy. And in fact, Impossible Foods is recommending that the common or usual name for this ingredient should be "leghemoglobin (soy)".

Any FDA request that Impossible Foods should conduct clinical studies on the potential allergenicity of soy leghemoglobin is unreasonable in my opinion. While soybeans are widely considered as a commonly allergenic food, soy allergy appears to occur almost exclusively in young infants and is a transitory condition. The vast majority of soy-allergic infants outgrow their soy allergy by the age of 10 years (*Savage et al., 2010*). Finding suitable numbers of soy-allergic adults for an oral challenge study would be virtually impossible. My research group (Food Allergy Research & Resource Program) has been attempting to conduct a soy flour threshold study among adults (the IRB limited us to challenges of individuals age 16 or higher). This study has been ongoing for 11 years and we only have managed to locate 18 subjects on a worldwide basis. In my opinion, it would even be difficult to find a sufficient number of well-characterized soy-allergic subjects to be sources of blood serum to serum IgE-binding studies. Since Impossible Foods is advocating that this ingredient be clearly labeled as derived from soy, the necessity of providing clinical evidence of its potential allergenicity is very questionable in my opinion.

- FDA concern that Impossible Foods should evaluate the sensitizing potential of soy leghemoglobin as a novel protein

Impossible Foods has provided evidence of the potential sensitizing capacity of soy leghemoglobin within GRN540. Specifically, they provided evidence of the susceptibility of soy leghemoglobin to pepsin digestion. Soy leghemoglobin was rapidly hydrolyzed by pepsin, a characteristic that makes it less likely to retain any sensitizing capacity as the digested remnants enter the small intestine. While I would join other scientific experts in wishing that science could provide additional definitive and discriminatory tests to evaluate the potential allergenicity of novel proteins in the diet, this approach remains the only well-accepted procedure.

- FDA concern that Impossible Foods should evaluate the capacity of soy leghemoglobin to cross-react with other known allergens especially legume allergens

Impossible Foods has provided evidence of the potential allergenicity of soy leghemoglobin within GRN540. They provided evidence of sequence homology comparisons to a database of known allergen sequences (allergens from all sources, not just food). This approach is known to provide evidence of cross-reactive potential with known allergens from all sources especially when conservative bioinformatics criteria are used in the assessment as was done in this particular example. Specifically, this assessment did not reveal any sequence homologies between soy leghemoglobin and any known allergens from legume sources.

Cross-reactions within the legume botanical family are not especially common in the U.S. This fact is fortunate because more than 300 edible legume species exist in the human diet. Peanuts

are, by far, the most potent and prevalent cause of allergies within the legume family. Soybeans are also considered as commonly allergenic but soybean allergy is considerably less prevalent and typically less severe. Clinical cross-reactivity among various foods from the legume family is rare (*Bernhisel-Broadbent and Sampson, 1989*). However individuals allergic to a single legume often display positive skin prick tests to other legumes that they can safely ingest (*Bernhisel-Broadbent et al., 1989*). Over the years, many clinical investigators have errantly evaluated potential cross-reactivity among legumes only via the presence of cross-reactive IgE in patient sera or skin test cross-reactive to legume extracts (*Beslar, 2000*). As shown very conclusively (*Bernhisel-Broadbent and Sampson, 1989*), oral challenges are necessary to truly document cross-reactivity among legumes. In that pioneering study, only two of 69 patients (3%) sensitized to legumes (peanut, soybean, pea, green bean, lima bean) were reactive on oral challenge to two legumes (*Bernhisel-Broadbent and Sampson, 1989*). In both cases, these patients were primarily allergic to peanuts with histories of severe reactions and had mild reactions to soybeans. In contrast, 49 of the 69 subjects had positive skin tests or serum IgE tests to two or more legumes.

Similarly, among peanut-allergic individuals, oral challenges revealed the peanut allergy was the sole legume allergy in 94% of 142 subjects while only 8 of the 142 (5.6%) subjects reacted to other legumes on challenge: 4 to pea, 2 to soybean, and 2 to lentil (*Moneret-Vautrin et al., 1998*). Among 187 food-allergic children diagnosed by oral challenge, only 2 children (1.1%) were allergic to more than one legume (peanut-soy in one case; peanut-pea in the other) (*Bock and Atkins, 1990*). In the largest study reported to date in 793 persistent peanut-allergic subjects, 9.5% were considered allergic to other legumes by oral challenge including 48 to soy, 19 to pea, 7 to lentil, 4 to chickpea and 3 to green bean (*Neuman-Sunshine et al., 2012*)

Differing results were obtained in several other clinical studies. Peeters et al. (2009) evaluated 39 peanut-sensitized patients and found that 30/39 individuals were reactive on challenge to peanut while 12/30 subjects (40%) were also allergic to soybean, 6/30 subjects (20%) were also allergic to pea, and 8/30 subjects (26.7%) were also allergic to lupine. Similar results were found among soybean-allergic subjects where 21 of 35 individuals (60%) were also allergic to peanut (*Klemans et al., 2013*). These results might be ascribed to the selection of patients who were cross-reactive because especially in the study of Peeters et al. (2009), the focus of the study was lupine cross-reactivity.

Ibanez et al. (2000) studied a total of 66 legume-allergic subjects but did challenges to more than one legume on only 39 of these subjects. Of those 39 subjects, 21 (54%) reacted to two or more legumes. Of 15 patients challenged with lentil and pea, 11 (73%) reacted to both, 15 of 27 (56%) to lentil and chickpea, 9 of 16 (56%) to chickpea and pea, 8 of 15 (53%) to lentil, chickpea and pea, 3 of 5 (60%) to lentil and peanut and 2 of 5 (40%) to peanut and pea and 0 of 7 to peanut and chickpea.

These studies are the key references to legume cross-reactions that involve oral challenges to confirm that clinically significant cross-reactivity is actually occurring. Several of the studies suggest that cross-reactivity among various species of legumes is rather infrequent, while other studies suggest that certain cross-reactions among legumes are more common. In particular, cross-reactions among lentil, chickpea, and pea seem more common than cross-reactions with peanuts or soybeans.

In my opinion, based upon the prevalence and severity of peanut allergy, potential cross-reactions between soy leghemoglobin and peanut allergens should be the key area of potential concern. However, in that regard, the various peanut allergens are very well identified and characterized. No significant sequence homology exists between soy leghemoglobin and any of these peanut

allergens. Clinically significant cross-reactions between peanuts and soybeans occur infrequently even though some homology does exist between the vicilin and legumin allergens in peanuts and soybeans. The vicilins and legumins are seed storage proteins so some sequence homology might be expected. But, the similarities do not appear to lead to allergenic cross-reactivity in most patients with allergy to either peanut or soybean. Leghemoglobin is found in the root of the soybean plant and bears no structural resemblance or sequence homology to these seed storage proteins.

In my opinion, conducting clinical studies to determine if soy leghemoglobin elicits allergic reactions in peanut-allergic individuals is unwarranted because the results are quite predictable based upon bioinformatics comparisons. And, conducting clinical studies with soy leghemoglobin in individuals with allergies to other legumes is also unnecessary given that the legume allergens are found in the seeds while leghemoglobin is localized in the roots and because the existing evidence suggests that allergic cross-reactivity among legumes is limited to a few species that are not prevalent allergenic foods in the first place.

Conclusion

In my opinion, Impossible Foods has addressed all of the potential allergenicity issues associated with soybean leghemoglobin in a thorough fashion. The labeling of soy leghemoglobin as "leghemoglobin (soy)" will alert soy-allergic consumers to avoid this product. GRN540 addresses all of the potential allergenicity concerns. The available data in GRN540 document that soy leghemoglobin is unlikely to become a novel allergen and demonstrate that soy leghemoglobin is unlikely to cross-react with known allergens from various sources including other foods and legumes. Thus, in my expert opinion, additional testing as proposed by FDA is unnecessary.

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(b) (6)

Annex 7

University of Nebraska Lincoln

FOOD ALLERGY RESEARCH AND RESOURCE PROGRAM
Department of Food Science & Technology

10 October, 2016

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Summary of the Allergenicity and Toxicity Assessment of Soy Leghemoglobin Preparation for Food Use

My laboratory performed a weight of evidence assessment of the potential allergenicity and toxicity of leghemoglobin from soybean (*Glycine max*), expressed in *Pichia pastoris*, for food safety. The evaluation followed the principles of the CODEX Alimentarius Guidelines for risk assessment of foods derived from modern biotechnology (CAC/GL 44-2003). The assessment focused on the soy leghemoglobin protein, with the full 145 amino acid (AA) sequence listed in the NCBI protein database as GI:126241 (Accession #P02236.2). Additional bioinformatics was performed with sequences of the proteins from *P. pastoris* that are present in Soy Leghemoglobin Preparation.

Bioinformatics (sequence comparisons) were made using the AA sequence of the query protein (leghemoglobin) on AllergenOnline.org, version 16, with evaluation of Full-length (looking for sequence matches >50%), Sliding 80-mer (matches with >35% identity over 80) and 8 AA identity comparisons. The highest scoring overall alignments were ~ <26% identity to hemoglobins from a fly larvae (*Chironomus thummi*), which suggests overall an evolutionary relationship. However, it is highly unlikely there is any possibility of allergic cross-reactivity. The 80-mer match and 8 AA identity matches were negative. The sequence was also tested against NCBI Protein using BLASTP with keyword limits (allergen, allergy, toxin and toxic) as well as without keyword limit. There were no "statistically significant" alignments using "allergen". BLASTP alignments with "allergy" were very small and not likely to be important (29% identity over 93 AA with an E score of 7e-5 to *Lephospira yanagawae* and 43% identity over 31 AA with an E score of 0.002 to *Burkholderia multivorans*). Those matches are unlikely to represent cross-reactive matches and do not require additional testing.

Bioinformatics searches for "toxin" and "toxic" were also negative. Using the keyword "toxin" the highest scoring matches were to *Bordetella bronchiseptica* nitric oxide dioxygenase and *Bordetella pertusuis* with 35% identity over 31% coverage. There were no specific matches for toxic. Thus there is no important match to a toxin and no indication for toxicity testing.

Public information from peer-reviewed literature in PubMed was evaluated for evidence of allergy and toxicity associated with soybean protein known as "leghemoglobin". No relevant publications were identified.

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The bioinformatics analysis described above was also performed on 17 proteins of the recombinant host organism, *Pichia sp.* (*Komagataella sp.*). These proteins were identified by Impossible Foods and the Genome Center at the University of California, Davis as residual proteins constituting at least 1% of the total protein fraction of Soy Leghemoglobin Preparation. The 17 proteins were identified by LC-MS/MS and matched to the following proteins:

- Alpha aminoadipate reductase (1400 AA) had no significant alignments to allergens or toxins.
- Cobalamin-independent methionine synthase (768 AA) aligned to a pollen allergen, Sal k 3 of Russian thistle with 49% identity, and 80mer alignment and an 8 AA identity match. Yet Sal k 3 aligns with proteins of many edible foods that do not have shared allergy. There were no clear matches to toxic proteins.
- Aconitase (780 AA) showed no significant identity matches to any allergens. There were a number of statistically significant alignments to proteins in NCBI, but not to toxic proteins, only to enzymes that produce toxic metabolites. There were higher scoring matches to proteins without a label of "toxin".
- Transketolase (679 AA) did not have a significant match to any allergens. There were high scoring matches to proteins using BLASTP with "toxin" or "toxic" as key words, yet the proteins were only from toxic bacteria (e.g. *Bacillus cereus*), but without direct evidence of protein toxicity. There were no direct links for toxicity.
- Glycerol kinase (621 AA) did not have any matches to allergenic proteins. There were low scoring alignments to proteins in NCBI with the keywords "toxic" or "toxin". The aligned sequences were from *Bacillus thuringiensis*, an organism known to be toxic to a number of insects, but there is no direct link to toxic proteins.
- Catalase A (510 AA) had one statistically significant match to an allergen (E score 2.6 e-58), but only 37% identity over 475 AA. This indicates the proteins are apparently evolutionarily related, but not likely to have cross-reactivity as no 80 AA segment was higher than 35% identity and no 8 AA matches were found. The common enzyme was identified as highly similar to proteins from a number of organisms with high identity to toxic protein sources (*Bacillus sp., enterococcus sp., Streptomyces sp., Clostridium sp.*), but there is no direct link to toxic proteins.
- Glucose 6 phosphate dehydrogenase (G6PD, 504 AA) had an alignment of >35% identity (37%) for an 80 AA match to the German cockroach Bla g 3 allergen, but there are no reports of allergic cross-reactivity between fungi and cockroach. The protein did align with a number of G6PD proteins from "toxic" or "toxin" sources, homologues from organisms known to cause toxicity in, but not from the G6PD proteins.
- Hypothetical protein PAS (525 AA) had alignments to three "allergenic" proteins from two molds (*Davidiella sp.*, and *Aspergillus sp.*), and a storage mite (*Lepidoglyphus destructor*) with >35% identity. The proteins are not likely allergens. There were significant alignments with proteins from "toxic" sources (*Bacillus sp.*), but there is no evidence of direct protein toxicity.

- Mitochondrial aldehyde dehydrogenase (501 AA) had two high scoring matches to homologous sequences of two fungi (*Davidiella sp.*, and *Alternaria sp.*), but without direct evidence that these are cross-reactive allergens. There were also high scoring matches to a few homologous proteins associated with "toxin" in NCBI, but they were due to the source organism, *Bacillus thuringiensis*, and not due to direct toxicity.
- Delta-aminolevulinate dehydratase (341 AA) had only low scoring matches to proteins in AllergenOnline. There were high scoring matches to two proteins in *Candida albicans* with keyword matches to "allergen", but those are without proof of allergy. There were modest scoring matches to "toxins" in NCBI, but the proteins are not clear toxins.
- Mitochondria alcohol dehydrogenase (350AA) had a high identity match (76%) with the homologous protein of *Candida albicans*, Can f 1.0101. Yet there is high identity for the Can f 1.0101 protein and homologous proteins from many sources and no evidence of cross-reactivity. The protein also showed modest (36-42%) identity to proteins from bacterial sources of the same type that were identified with "toxin" as keyword limits. The bacteria are toxins, but there is no direct evidence of toxicity to the proteins.
- Malate dehydrogenase (342 AA) had a similar alignment with 51% identity to the malate dehydrogenase protein of *Malassezia furfur* (Mal f 4.0101) as a contact allergen. There is no evidence of cross-reactivity to homologous proteins of other sources. A 36% identity alignment was found with a short 80 AA segment of convicilin of *Pisim sativum* (pea), but again with no evidence of cross-reactivity. High scoring (50%) identity matches were noted for proteins identified in NCBI with "toxins" as a key word term, to proteins of the rat (*Rattus norveicus*) and bacteria (*Vibrio cholera* and *Escherichia coli*), but without direct evidence of protein toxicity.
- Putative protein unknown function (328 AA) had no significant allergen matches. Modest scoring matches were identified in NCBI to proteins listed in various bacteria using the keyword "toxins", but with no direct evidence of toxicity to the protein.
- Triosephosphate isomerase (248 AA) showed high scoring alignments (up to 53% identity) to triosephosphate-isomerase proteins from wheat, house dust mite and shrimp. The same proteins showed alignments of up to 63% identity over 80 AA. These proteins are minor airway allergens and not expected to represent food allergens. The protein aligned with 40-50% identity to homologous proteins from bacterial species listed in NCBI under "toxins" as keywords. No direct evidence of toxicity was found.
- Hypothetical protein cyclophilin (161 AA) matched cyclophilin proteins of diverse fungi, house dust mites and plant sources that have been identified as minor airway allergens. The identities were also found in the 80 AA searches. It is unlikely that these would represent risks of food allergy as cyclophilins are highly conserved across very diverse species. Homologous proteins were identified in NCBI using "toxins" as a keyword, but not direct evidence of protein toxicity was found.
- Cytosolic superoxide dismutase (154 AA) had identities of 53-57% to superoxide dismutase proteins of olive pollen, but the relevance of allergy is weak. There

were also modest to high scoring identities (up to 70%) with similar proteins of various bacteria with identities to "toxins", but without direct evidence of toxicity to the proteins.

- Mitochondria ATPase inhibitor (84 AA) had no significant matches to allergens or toxins.

Literature searches for associations of allergy with *P. pastoris* or *Komagataella sp.* and allergy and toxicity were found, but there were no clear associations with the proteins identified as proteins of interest. Thus, it appears the risks of allergy and toxicity for soy leghemoglobin and for the proteins from *Pichia pastoris* within Soy Leghemoglobin Preparation are not significant.

Finally we tested the stability of the Soy Leghemoglobin Preparation in a model simulated gastric digestion study that includes fixed concentration of protein to pepsin (enzyme) activity and evaluation of digestion resistance at times up to one hour at pH 2.0 and 37 °C. The assay conditions that were used have been published (Ofori-Anti et al., 2008) and used to evaluate proteins in genetically modified crops and novel ingredients. There is a positive correlation between the stability of abundant dietary proteins in this assay and food allergy. In addition, proteins that are rapidly digested by pepsin are unlikely to act as toxins in the digestive tract. Soy Leghemoglobin Preparation was rapidly digested in pepsin at pH 2.0 at both ratio of 1 µg in 10 units (as per standard protocol) and 1 µg in 1 unit pepsin activity (as an experimental protocol). Soy Leghemoglobin Preparation was rapidly digested in this assay to less than 10% residual protein in less than two minutes. No stable fragments were detected either, indicating low potential risk of allergy or toxicity.

My conclusion from this "weight of evidence" approach to dietary protein safety is that the Soy Leghemoglobin Preparation is very unlikely to present a risk of dietary allergy or toxicity to consumers.

Regards,

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Annex 8

Study Title

Bioinformatics Analysis of the Potential Allergenicity and Toxicity of Leghemoglobin from *Glycine max* for Food Use

Authors

Richard E. Goodman
Kwame Andoh-Kumi
Xiaoyun He

Study Completed On

10 October, 2016

Performing Laboratory

Food Allergy Research and Resource Program
Food Science and Technology
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1901 North 21st Street
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Laboratory Project ID

Study Number: REG ImpFoods1

Statement of No Data Confidentiality

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA Section 10(d)(l)(A), (B), or (C) and which pertains to a registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA section 10(g).

Company

Company Agent:

(b) (6)

Rachel Fraser, Ph.D.
Impossible Foods, Inc.

10/13/16
Date

Good Laboratory Practice Compliance Statement

This study was not conducted and reported in compliance with the requirements of the Good Laboratory Practice Standards (40 CFR Part 160) of the Code of Federal Regulations of the United States of America. This is a characterization assessment of the similarity of the introduced proteins to known and putative allergens based on source of the genes and the sequences of the proteins. There is no test system. However, raw data including PubMed searches and bioinformatics comparisons were archived in PDF format in the Authors laboratory with a copy given to the sponsor.

Applicant/Sponsor

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Title: Research Professor, FARRP, University of Nebraska—Lincoln
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Date: 10/13/16

Study Number: REG – ImpFoods1

Title: Bioinformatics Analysis of the Potential Allergenicity and Toxicity of Leghemoglobin from *Glycine max* for Food Use

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Study Start Date: 15 January, 2016

Study Completion Date: 10 October, 2016

Records Retention: All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.

Signature of Final Report Approval:

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Principle Investigator: Richard E. Goodman

10 October 2016
Date

SUMMARY

The Leghemoglobin (LegHb) protein which is naturally produced in soybean (*Glycine max*) has been produced as a recombinant protein in *Pichia pastoris* and supplied by Impossible Foods, Inc. of Redwood City, CA. The gene encodes the 145 amino acid protein sequence listed as Accession number P02236 in the UniProt protein database. The host, *Pichia pastoris* is also known by the synonym *Komagataella pastoris.*

Evaluation of potential risks of food allergy and toxicity associated with this product included a literature search of studies that might indicate possible risks, as well as a search of the amino acid sequence against the curated AllergenOnline.org database version 16 (January 2016), which is maintained at the University of Nebraska as well as against the NCBI Protein database using BLASTP with keyword limits for allergy and toxicity.

None of the results from the bioinformatics searches of the soy leghemoglobin amino acid sequence, compared to known and putative allergens or toxins, identified any significant sequence identity match to a protein likely to cause an adverse effect in consumers. The search results from the AllergenOnline database show that the likelihood of cross-reactivity for the soy leghemoglobin with the allergens is very low. And the search results from PubMed and BLASTP on allergens and toxic proteins suggest that the soy leghemoglobin has a low risk of food allergenicity or toxicity.

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Abbreviations

aa	Amino acid
AOL v16	http://www.AllergenOnline.com/ database version 16
BLASTP	Algorithm used to find local high scoring alignments between a pair of protein sequences (using databases on Entrez)
CODEX	CODEX Alimentarius Commission Guideline for the safety assessment of food derived from biotechnology
Entrez NCBI	A public genetic database maintained by the National Center for Biotechnology Information (NCBI) at the National Institutes of Health, Bethesda, MD. Protein entries in the Entrez search and retrieval system are maintained by the NCBI of the National Institutes of Health (U.S.A.)
FAO	Food and Agricultural Organization
FARRP	Food Allergy Research and Resource Program, University of Nebraska
FASTA3	Algorithm used to find local high scoring alignments between a pair or protein sequences (using the AllergenOnline database)
GE	Genetic engineering
GI	A unique identification number assigned by NCBI to each sequence in the database
LegHb	Soy Leghemoglobin
PubMed	A public information database of scientific journal articles and abstracts maintained by the National Library of Medicine, National Institutes of Health (U.S.A.)
8mer	Exact word search for segments of eight amino acid matches between the query protein and proteins in AllergenOnlin.org

1.0 Introduction

The Soy Leghemoglobin (LegHb) protein evaluated in this study is encoded by a gene originally derived from *Glycine max* (soybean). The gene was synthesized and transferred to *Pichia pastoris* for production of food, and the sequence was verified by Impossible Foods as accurate as produced in the recombinant host. This bioinformatics study is intended to evaluate potential risks of food allergy and food toxicology based on literature searches and bioinformatics comparisons of the expressed protein relative to those of known allergens and toxins.

Hemoglobins are ubiquitous proteins in nature. Hemoglobins have been shown to be expressed in bacteria, fungi, higher plants and animals (Everse, 2004). The structure and general function of hemoglobins are highly conserved throughout nature. Animal hemoglobins and myoglobins have been widely consumed in the human diet throughout history through meat, poultry, and fish products. Plant hemoglobins are also consumed in the human diet. Sprouted barley, which is widely used in the beverage industry (malted barley) and in the baking industry (malted barley flour), has been shown to express hemoglobin 1 day after imbibition (Duff et al., 1998). Since sprouted barley is widely used in the beverage industry (malted barley) and in the baking industry (malted barley flour), dietary exposure is common. Thus, it is clear that various heme proteins of plant and animal origin are widely consumed in the human diet.

Legume nodules are unique symbiotic organs where atmospheric N_2 is reduced to ammonia and assimilated by the plant in exchange for photosynthetically produced sugars. Nodule function requires leghemoglobin (LegHb), hemeproteins that occur at concentrations of 1–5 mM in the cytosol of nodule host cells. These proteins transport and deliver O_2 to the symbiosomes at a low but steady concentration that allows efficient bacteroid respiration while preventing nitrogenase inactivation (Appleby and Bergersen, 1980). Generally, there are several LegHb isoproteins in the nodules. Soybean nodules contain four major LegHbs (Fuchsman and Appleby, 1979).

Impossible Foods, Inc. has proposed using purified soy leghemoglobin (>65% purity), produced as a recombinant protein in *Pichia pastoris* to enhance the flavor of meat analogue products. They are interested in evaluating the potential allergenicity and toxicity of the soy leghemoglobin to ensure product safety.

2.0 Purpose

The purpose of this study is to perform an evaluation or screening of the potential allergenicity and toxicity of the soy leghemoglobin based on published literature about the source of the gene and bioinformatics (sequence comparisons) of leghemoglobin with known allergens and toxins. The intent is to guide decisions regarding whether additional safety tests would be needed for the protein if used in food products.

3.0 Methods

3.1 Scientific literature search strategies. The PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) maintained by the U.S. National Library of Medicine was used as the primary data source for scientific literature on allergy and toxicity. The primary question is whether the source of the gene is a common cause of allergy or toxicity. The data (authors, publication, date and abstracts) from searches were saved to files for review. All publication abstracts were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further by reading the journal articles.

3.1.1 Search for allergenicity. Search terms "*Glycine max*" as well as "*Glycine max*" AND "allergen" were used on 07 March, 2016. Further searches with "*Glycine max*" AND "allergen" AND "leghemoglobin" as well as "*Glycine max*" AND "allergy" AND "heme" were conducted on 07 March, 2016.

3.1.2 Search for toxicity. Search terms "*Glycine max*" AND "toxin" as well as "*Glycine max*" AND "toxic" were used on 07 March, 2016. Second search "*Glycine max*" AND "toxi*" AND "leghemoglobin" as well as "*Glycine max*" AND "toxi*" AND "heme" were conducted on 07 March, 2016.

3.2 Sequence database search strategies.
The AllergenOnline version 16 (http://www.allergenonline.org/) and the NCBI Entrez Protein (http://www.ncbi.nlm.nih.gov/BLAST/) databases were used as the protein amino acid data sources for the sequence comparisons for allergens and toxins on Feb 27 to 29, 2016. The AllergenOnline database was updated in 27 January 2016 and is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. Protein entries in the Entrez search and retrieval system is compiled and maintained by the NCBI of the National Institutes of Health (U.S.A.). The database is potentially updated or modified daily, and therefore the date of sequence searches by

BLASTP is relevant to the dataset used in the BLASTP searches. BLASTP and FASTA3 are unique computer algorithms that provide similar local alignments and results if the appropriate scoring matrices and criteria are used.

3.2.1 FASTA3 overall search of AllergenOnline. The potential sequential and inferred structural similarities of the soy leghemoglobin were evaluated using version 16 of AllergenOnline.org with *E* scores of 10 and 1.0.

3.2.2 FASTA3 of AllergenOnline by 80 aa segments. This short (80-amino acid) segment search is based on the recommendation of Codex (2003). The rationale is that this might help in identifying structural motifs, much shorter than the intact protein, which might contain a conformational IgE binding epitope. It should also help to identify potentially cross-reactive proteins that are not true homologues of an allergen that have significant local identities that might provide an immunological target for IgE antibodies in those with allergies to the matched allergen. A match of >35% with a known allergen will suggest further testing for possible cross-reactivity although matches using the sliding 80 amino acid window search that are not also identified by overall FASTA search may represent an irrelevant alignment. Thus evaluation of *E* scores and relative comparison of matched sequences with the NCBI database is sometimes warranted.

3.2.3 Exact Word match of AllergenOnline by 8-contiguous aa.
A word/string search routine on AllergenOnline.org was used to identify any eight contiguous amino acid segment of soy leghemoglobin sequence that exactly matched any 8 amino acid segment of any of the allergen sequences in database. The rationale for identifying identity matches in very short sequences is an assumption that individual epitopes may be represented by peptide segments as short as eight amino acids (Metcalfe et al., 1996).

3.2.4 BLASTP of NCBI Entrez without keyword limit. The BLASTP is available on the NCBI Entrez website (http://www.ncbi.nlm.nih.gov/BLAST/). The current version is BLASTP 2.3.0 (22 Dec., 2015). The purpose of this BLASTP search is to compare the soy leghemoglobin to all known protein sequences to evaluate whether there are other similar proteins from other organisms that might provide information of safe exposure to homologues of this protein.

3.2.5 BLASTP of NCBI Entrez with "allergen" and "allergy" as keywords limit. BLASTP search was used comparing soy leghemoglobin sequence against the entire Entrez Protein database, with a limit option selected to query entries for "allergen" or "allergy", to align only with proteins identified as allergens or allergy. The purpose of this BLASTP search is to ensure that a significant match with a newly discovered allergenic sequence that has not yet been

entered into AllergenOnline is not overlooked. Evaluation of the *E* value, the length of the alignment and the percent identity of any identified match is necessary to judge the significance of any alignment using BLASTP.

3.2.6 BLASTP of NCBI Entrez with "toxin" and "toxic" as keywords limit. The purpose of this BLASTP search is to identify matches to known toxic proteins (toxins) and if alignments share significant identities, to determine potential risks that would require further testing.

4.0 Results and Discussion. The summary results for the PubMed search using search terms, and the amino acid sequence of soy leghemoglobin, are presented here.

4.1 PubMed Searches. The scientific literature database, PubMed, was searched for evidence that the leghemoglobin from *Glycine max* are likely source of allergy or toxicity. The search did not reveal evidence that the leghemoglobin from *Glycine max* represents food safety risk. Summary of all information is present in Table 1.

4.1.1 Allergenicity. A search of PubMed using only the organism name, "*Glycine max*", returned 21,936 articles. Restricting the search of PubMed by including both "*Glycine max*" AND "allergen" returned 381 references. A second search with terms "*Glycine max*" AND "allergen" AND "leghemoglobin" had no references. Further searches with "*Glycine max*" AND "allergy" AND "heme" yielded one reference on impairment of carotenoid and flavonoid biosynthesis due to mutation of Arabidopsis HY1 which is not relevant to the topic under review. It can be concluded from the literature search that the leghemoglobin from *Glycine max* does not raise a concern of possible allergy.

4.1.2 Toxicity. The search of PubMed using both "*Glycine max*" AND "toxin" returned 419 references, using both "*Glycine max*" AND "toxic" returned 342 references. A second search with terms "*Glycine max*" AND "toxi*" AND "leghemoglobin" yielded one reference on nitration of leghemoglobin in functional legume nodules in a tyrosine residue within the heme cavity by a nitrite/peroxide-dependent mechanism which is not relevant to the topic under review. Further searches with "*Glycine max*" AND "toxi*" AND "heme" yielded 8 references. None of the publications implicated any natural protein expressed by the organisms of interest as a toxin. The majority of the papers reported on the effect of heme-peroxidases or heme oxygenase system, disordering of these systems may cause adverse effect on plants or animals or human because of changing of hydrogen peroxide level. Thus, based on literature search there is no reason to suspect the leghemoglobin produced from *Glycine max* would elicit a toxic effect on consumers.

Table 1 Pubmed search results (Summary table)

Protein Source	Search results			
	Glycine max only	*Glycine max* AND Leghemoglobin	*Glycine max* AND allergen/allerg*	*Glycine max* AND toxi*
Glycine max (Soybean)	21936 articles were found.	201 articles found on leghemoglobin. When allergen was added to the search criteria, no reference came up.	381 articles found. Further searches with leghemoglobin added to the search criteria yielded no results. One article was sited when searched with Soy AND allergy AND heme but was not relevant to the topic.	419 articles found for toxin and 342 articles found for toxic. An additional criterion of "leghemoglobin" added to the search criteria yielded one result but was not relevant to the topic. An additional criterion of "heme" added to the search criteria yielded 8 results but were not relevant to the topic.

4.2 Sequence comparison of the LegHb to allergens and toxins. The amino acid sequence of soy leghemoglobin (Table 2) was compared to known allergens using both a full-length FASTA alignment search and a sliding window of 80 comparisons against AllergenOnline.org, version 16. Additionally, a BLASTP search was performed against the NCBI database using keyword search limits of "allergen" / "allergy" and "toxin"/ "toxic".

Table 2 Amino acid sequence of the soy leghemoglobin

Organism	Hemoglobin class	Native protein sequence
Glycine max	leghemoglobin (legHb) GI:126241	MGAFTEKQEALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKLT GHAEKLFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAVGD KWSDELSSAWEVAYDELAAAIKKAF

4.2.1 Full length FASTA3 vs. AllergenOnline with LegHb. Results of the full length FASTA3 searches of the soy leghemoglobin against AllergenOnline version 16 did not identify any significant alignment with an allergen. Scoring results for the soy leghemoglobin showing alignments with *E* scores less than 1.0 are shown in Table 3 and demonstrate no significant matches with any allergen. Their identities (%) are markedly below the level that is likely to indicate cross-reactivity (< 50% identity, Aalberse, 2000) and it is also below the 35% identity level suggested by Codex (2003) as a match

that may possibly be cross-reactive. Thus, there is only a small likelihood that the soy leghemoglobin is sufficiently similar to an allergen to suspect they might trigger allergic responses in allergic subjects due to cross-reactivity.

Table 3. Overall FASTA3 search of AllergenOnline with the soy leghemoglobin. Only the alignments with *E* scores less than 1.0 of known and putative allergens in AllergenOnline version 16, compared to the soy leghemoglobin, using FASTA3 are listed since none of the results were significant. The matched proteins are very distant homologues of hemoglobin, occupational allergens.

Query sequence	Sequence GI #	Organism	Description	Length aa	*E* score	% Identity	aa Alignment length
Soy Leghemoglobin (legHb) GI:126241 *Glycine max*	56405052	*Chironomus thummi thummi*	Globin (Insect haemoglobin)	161	0.00024	26.2	145
	121244*	*Chironomus thummi thummi*	Globin	161	0.00043	25.9	147
	56405054	*Chironomus thummi thummi*	Globin	161	0.0009	25.0	144
	121248	*Chironomus thummi thummi*	Globin	161	0.0012	25.0	144
	121249	*Chironomus thummi thummi*	Globin	162	0.0012	25.2	151
	2506460	*Chironomus thummi thummi*	Globin	158	0.0039	24.2	149
	1707908	*Chironomus thummi thummi*	Globin	160	0.0053	25.2	139
	121259	*Chironomus thummi thummi*	Globin	151	0.0068	26.9	119
	121237	*Chironomus thummi thummi*	Globin	151	0.019	26.3	137
	2506461	*Chironomus thummi thummi*	Globin	162	0.049	24.0	150
	1707911	*Chironomus thummi thummi*	Globin	161	0.057	22.9	140
	121256	*Chironomus thummi thummi*	Globin	151	0.32	22.1	149

4.2.2 Sliding 80-amino acid window FASTA3 vs. AllergenOnline with LegHb. Results of the comparisons of the soy leghemoglobin sequence against all of the sequences in AllergenOnline.org version 16 database have no results. Thus, the soy leghemoglobin showed no alignment over the Codex threshold. The risk of cross-reactions for allergic consumers is very low.

Table 4. Scanning 80-mer sliding window search results for soy leghemoglobin

80mer Sliding Window Search Results

Database	AllergenOnline Database v16 (January 27, 2016)
Input Query	>Leghem Impossible Foods 1 March 2016 MGAFTEKQEALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKLT GHAEKLFGLVRDSAGQLKANGTVVADAALGSIHAQKAITDPQFVVVKEALLKTIKEAVGD KWSDELSSAWEVAYDELAAAIKKAF
Length	143
Number of 80 mers	66
Number of Sequences with hits	0

No Matches of Greater than 35% Identity Found

AllergenOnline Database v16 (January 27, 2016)

4.2.3 Eight-contiguous amino acid exact word search with LegHb. There were no matches of eight amino acid segments between soy leghemoglobin and any allergen in the AllergenOnline.org version 16 database, further demonstrating a lack of risk of food allergy.

Table 5. Eight-contignous amino acid search results for soy leghemoglobin

Query sequence	MGAFTEKQEALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLSNGVDPSNPKLTGHAEKLFGLVRDSAGQLKANGTVVADAALGSIHA QKAITDPQFVVVKEALLKTIKEAVGDKWSDELSSAWEVAYDELAAAIKKAF
Number of 8mers	138
Results	**No sequences found with an exact 8mer match**

4.2.4 BLASTP of NCBI without keyword limit with LegHb. The full-length of the soy leghemoglobin was compared to all sequences in NCBI-Entrez database. The scoring alignments with *E* scores of the top 10 non-heme protein alignments identified by BLASTP were considered in some detail to determine if there is significant homology to proteins of sources with likely safe human exposure or unsafe (allergenic or toxic) exposure. The plant leghemoglobin was most closely related to other eukaryotic hemoglobins and were about 26% or more identical to some chordate leghemoglobins. These proteins are clearly evolutionarily related to oxygen carrying proteins from diverse sources, including organisms that humans are exposed to without harm and some organisms that they are harmed by. Thus, the results from BLASTP comparison to all proteins were neutral, but the ubiquitous nature of leghemoglobin without obvious indications of harm suggesting they are generally safe.

4.2.5 BLASTP of NCBI Entrez using "allergen" and "allergy". The full-length amino acid sequence of the soy leghemoglobin was compared to sequences in NCBI Entrez, which were designated as "allergen" or "allergy" in the NCBI database on 27 Feb, 2016. The alignment results with keyword "allergen" returned no results with an *E* score of less than 10. The aligned matches with keyword "allergy" (Tables 6) were not significant as judged by low identity matches (<50% identity) with partial protein alignments. The results showed that the soy leghemoglobin sequence had lower *E* scores with adenylate / guanylate cyclase catalytic domain protein from *Leptospira yanagawae serovar Saopaulo* (*E*=7e-05) and with nitric oxide dioxygenase from some strains of bacterial, such as ***Burkholderia multivorans, Bordetella pertussis, Achromobacter piechaudii, Bordetella bronchiseptica etc.*** (with E scores vary from 0.002 to 0.19). While none of them had more than 50% identity over one-half length of the query sequence. These proteins or enzymes were labeled by "allergy" because the sequences of them were obtained from a project funded in whole or in part with federal funds from the National Institute of Allergy and Infectious Diseases. No evidence showed that these proteins were allergens. For example, the function of adenylate / guanylate cyclase catalytic domain protein is 2Fe-2S iron-sulfur cluster binding domain. And the function of nitric oxide dioxygenase is bifunctional nitric oxide dioxygenase / dihydropteridine reductase. The aligned proteins would not be considered homologues of the soy leghemoglobin and the probability of cross-reactivity is extremely small based on observations of Aalberse (2000) and Goodman et al. (2008).

Table 6. BLASTP of NCBI Entrez with leghemoglobin using the keyword "allergy". The scoring alignments with the top 2 aligned proteins are shown for the soy leghemoglobin protein vs. proteins labeled with the keyword "allergy" in the NCBI Entrez database on 27th Feb, 2016, using BLASTP. The sequence identities are low and / or the length of alignments are very short, indicting unlikely homology and that the overall structure is unlikely to be similar. Thus even if the NCBI sequence is a proven allergen, there is very little likelihood of cross-reactivity by the heme protein.

Sequence GI#	Organism	Description	Length aa	E score	% identity	Query Cover%
EOQ89444.1	*Leptospira yanagawae serovar Saopaulo*	adenylate/guanylate cyclase catalytic domain protein	440	7e-05	29	93
EJO56091.1	*Burkholderia multivorans*	nitric oxide dioxygenase	403	0.002	43	31

4.2.6 BLASTP of NCBI Entrez with "toxin" and "toxic". The full-length sequence of the soy leghemoglobin was compared to sequences in NCBI-Entrez, which were designated as "toxin" or "toxic" in the NCBI database on 28 Feb, 2016. The alignment results with keyword "toxic" returned no results with an *E* score of less than 10. The aligned proteins with keyword "toxin" are shown in Table 7. Two identity matches were found, with homologues of nitric oxide dioxygenase from *Bordetella bronchiseptica* and dihydropteridine reductase from *Bordetella pertussis* (Tables 7). They are identified with the BLASTP using a toxin keyword as they are from "toxic" organisms. The sequence identities are low (35%) and the length of alignments are very short (31%), indicting unlikely homology and that the overall structure is unlikely to be similar. There does not appear to be a basis to suspect that the soy leghemoglobin are likely toxins.

Table 7. BLASTP of NCBI Entrez with leghemoglobin using the keyword "toxin". The best scoring alignments to putative toxins shown in the NCBI Entrez database on 28th Feb, 2016, were identified by BLASTP with the full-length sequence of the soy leghemoglobin.

Sequence GI#	Organism	Description	Length aa	E score	% identity	Query Cover%
BAO69648.1	*Bordetella bronchiseptica*	nitric oxide dioxygenase	402	0.027	35	31
ALX21654.1	*Bordetella pertussis*	dihydropteridine reductase	402	0.028	35	31

4.3 Bioinformatics summary for the leghemoglobin. None of the results from the bioinformatics searches of the soy leghemoglobin amino acid sequence, compared to known and putative allergens or toxins, suggested any clear and significant sequence similarity that suggests potential adverse effects for consumers. The search results from the AllergenOnline database show that the likelihood of cross-reactivity for the soy leghemoglobin with the allergens is very low. And the search results from PubMed and BLASTP on allergens and toxic proteins suggest that the soy leghemoglobin has a low risk of food allergenicity or toxicity.

5.0 Conclusions

Bioinformatics analyses were performed on leghemoglobin from soy (*Glycine max*) to evaluate whether there might be some safety concerns for foods produced with this protein included as ingredients.

Based on the evidence and my knowledge of cross-reactive IgE binding, there is not a scientifically justifiable reason to perform serum IgE binding studies with leghemoglobin of *Glycine max* as no significant sequence identity matches were found to any known allergens. Therefore no "at-risk" population of allergic subjects could be identified to evaluate potential cross-reactivity and risks of allergy or allergenic cross-reactivity are low (Goodman, 2008).

Sequence comparisons of the soy leghemoglobin to known toxins identified some statistically significant, but short and modest identity matches to two proteins from bacterial species that are pathogenic or toxic, yet the homologous proteins have not been identified as toxins. Thus, *in vitro* and *in vivo* toxicity testing should not be required to evaluate food safety in our opinion.

BLASTP alignments without keyword selection did identify a number of important high identity matches to other leghemoglobins (hemoglobins from legumes). These are homologues of proteins that carry oxygen necessary for microbes in root nodules to fix nitrogen. We could not find evidence that these proteins are toxic to mammals.

6.0 References

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APPENDIX 1. AllergenOnline.org Database version 16, January 2016 (105 pages)

Annex 9

Study Title

Bioinformatics Analysis of the Potential Allergenicity and Toxicity of *Pichia pastoris* Proteins for Food Use

Author

Richard E. Goodman

Study Completed On

10 October, 2016

Performing Laboratory

Food Allergy Research and Resource Program
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Laboratory Project ID

Study Number: REG ImpFoods2

Statement of No Data Confidentiality

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA Section 10(d)(l)(A), (B), or (C) and which pertains to a registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA section 10(g).

Company

Company Agent:

(b) (6)

Rachel Fraser, Ph.D.
Impossible Foods, Inc.

10/13/16
Date

Good Laboratory Practice Compliance Statement

This study was not conducted and reported in compliance with the requirements of the Good Laboratory Practice Standards (40 CFR Part 160) of the Code of Federal Regulations of the United States of America. This is a characterization assessment of the similarity of the introduced proteins to known and putative allergens based on source of the genes and the sequences of the proteins. There is no test system. However, raw data including PubMed searches and bioinformatics comparisons were archived in PDF format in the Authors laboratory with a copy given to the sponsor.

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Study Number: REG ImpFoods2

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Study Start Date: 15 January, 2016

Study Completion Date: 10 October, 2016

Records Retention: All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.

Signature of Final Report Approval:

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10 October, 2016

Principle Investigator: Richard E. Goodman Date

SUMMARY

Pichia pastoris is being used as a recombinant host for efficient expression of a recombinant food protein using a leghemoglobin gene derived from soybean. The product, referred to as Soy Leghemoglobin Preparation, is intended for food use to enhance the flavor profile of meat analogues. Soy Leghemoglobin Preparation is being developed by Impossible Foods and 17 *P. pastoris* proteins were identified by Impossible Foods, Inc. of Redwood City, CA as being present in the final product. The 17 proteins were identified using proteomic analysis from 10 stainable protein bands in a one-dimensional SDS-PAGE as the most abundant residual proteins from the yeast in the Soy Leghemoglobin Preparation. Each of the 17 *Pichia* proteins represented 1% or more of the total protein content of Soy Leghemoglobin Preparation. The *P. pastoris* proteins were identified by LC-MS/MS and the highest matching peptide fragments identified proteins by the highest BLASTP alignment to proteins of *P. pastoris*.

Evaluation of potential risks of food allergy and toxicity associated with the proteins from *Pichia pastoris* within Soy Leghemoglobin Preparation included a literature search for studies that might indicate possible risks of allergenicity or toxicity, as well as a search of the amino acid sequences against the curated AllergenOnline.org database version 16 (January 2016). AllergenOnline is maintained at the University of Nebraska to evaluate potential risks of allergic cross-reactivity. Additional sequence comparisons were performed using the NCBI Protein database using BLASTP with keyword limits for allergy and toxicity.

The results of these evaluations indicate that the proteins from *Pichia pastoris* are unlikely to present any unique and unacceptable risks of allergy or allergic cross-reactivity, compared to risks presented by foods containing ingredients from other yeasts and molds approved for food use. In addition, no information was identified that would suggest these proteins from *P. pastoris* are potential toxins or would result in the production of toxic metabolites. My conclusion is that no further tests should be required to demonstrate the safety of foods produced using the Soy Leghemoglobin Preparation, which includes modest concentrations of the identified *P. pastoris* proteins.

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GI	A unique identification number assigned by NCBI to each sequence in the database
LegHb	Soy Leghemoglobin
PubMed	A public information database of scientific journal articles and abstracts maintained by the National Library of Medicine, National Institutes of Health (U.S.A.)
8mer	Exact word search for segments of eight amino acid matches between the query protein and proteins in AllergenOnlin.org

1.0 Introduction

The soy Leghemoglobin (LegHb) protein evaluated in study REG ImpFoods1 was expressed in *Pichia pastoris* for production of food. As evaluated, LegHb is not expected to present any specific risks of allergy to toxicity for those consuming the food product. In this study Impossible Foods, Inc. provided the primary sequences of proteins from *P. pastoris* that are present as minor proteins in the Soy Leghemoglobin Preparation due to normal expression by the recombinant yeast host. This bioinformatics study is intended to evaluate potential risks of food allergy and food toxicology of the 17 most abundant endogenous *Pichia* proteins in the Soy Leghemoglobin Preparation food product.

The same bioinformatics methods used to evaluate the potential risks of food allergy and toxicity for LegHb were used in this study to evaluate each of the 17 proteins from *P. pastoris*. The raw data has been archived at the University of Nebraska. This report provides a summary of the findings for each of the seventeen proteins.

2.0 Purpose

The purpose of this study is to perform an evaluation or screening of the potential allergenicity and toxicity of the *Pichia* proteins present in the Soy Leghemoglobin Preparation based on published literature about the source of the gene and bioinformatics (sequence comparisons) with known allergens and toxins. The intent is to guide decisions regarding whether additional safety tests would be needed for the proteins if used in food products.

3.0 Methods

3.1 Scientific literature search strategies. The PubMed database (http://www.ncbi.nlm.nih.gov/pubmed) maintained by the U.S. National Library of Medicine was used as the primary data source for scientific literature on allergy and toxicity. The primary question is whether the source of the gene is a common cause of allergy or toxicity. The data (authors, publication, date and abstracts) from searches were saved to files for review. Publication abstracts of publications identified in the searches were manually reviewed and any likely relevant publications suggesting adverse health risks were investigated further by reading the journal articles. Simple searches for the organism name *Pichia pastoris* retrieved 5244 references. A search for *Komagataella phaffii* only retrieved 6 entries. None of the publications on *K. phaffii* were related to allergens or toxins of the organism. Many of the *P. pastoris* references were about cloning genes from various sources into the yeast for eukaryotic expression. The search had to be more specific as described below.

3.1.1 Search for allergenicity. Search terms "*Pichia pastoris*" AND "allergen" NOT "recombinant" were used on 1 July, 2016, reducing the articles identified to 4 but those were about recombinant proteins expressed in the yeast. Therefore new searches used simply the organism (Pichia pastoris) AND the individual protein names (alpha aminoadipate reductase, cobalamin-independent methionine synthase, aconitase, transketolase, glycerol kinase, catalase A, glucose 6 phosphate dehydrogenase (G6PD), PAS, mitochondrial aldehyde dehydrogenase, delta-aminolevulinate dehydratase, mitochondrial alcohol dehydrogenase, malate dehydrogenase, putative protein, triosephoasphate isomerase, cyclophilin, cytosolic superoxide dismutase and mitochondrial ATPase inhibitor. In some cases where too many publications were identified, additional keywords of allergen or allergy were added.

3.1.2 Search for toxicity. Search terms "*Pichia pastoris*" AND "toxin" were used on 1 July, 2016, returning 185 publications. Alternatively toxic was used in place of toxin, still returning 72 publications. Therefore the alternative of using Pichia pastoris AND the name of each protein was used: alpha aminoadipate reductase, cobalamin-independent methionine synthase, aconitase, transketolase, glycerol kinase, catalase A, glucose 6 phosphate dehydrogenase (G6PD), PAS, mitochondrial aldehyde dehydrogenase, delta-aminolevulinate dehydratase, mitochondrial alcohol dehydrogenase, malate dehydrogenase, putative protein, triosephoasphate isomerase, cyclophilin, cytosolic superoxide dismutase and mitochondril ATPase inhibitor and in some cases "AND toxin" or "AND toxic" were added to focus the search. Abstracts and when needed, full-publications were reviewed.

3.2 Sequence database search strategies.

The AllergenOnline version 16 (http://www.allergenonline.org/) and the NCBI Entrez Protein (http://www.ncbi.nlm.nih.gov/BLAST/) databases were used as the protein amino acid data sources for the sequence comparisons for allergens and toxins on 27 May, 2016. The AllergenOnline database was updated in 27 January 2016 and is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. Protein entries in the Entrez search and retrieval system is compiled and maintained by the NCBI of the National Institutes of Health (U.S.A.). The database is potentially updated or modified daily, and therefore the date of sequence searches by BLASTP is relevant to the dataset used in the BLASTP searches. BLASTP and FASTA3 are unique computer algorithms that provide similar local alignments and results if the appropriate scoring matrices and criteria are used.

3.2.1 FASTA3 overall search of AllergenOnline. The potential sequential and inferred structural similarities of the 17 proteins from *Pichia pastoris* were evaluated using version 16 of AllergenOnline.org with *E* scores of 10 and 1.0.

3.2.2 FASTA3 of AllergenOnline by 80 aa segments. This short (80-amino acid) segment search is based on the recommendation of Codex (2003). The rationale is that this might help in identifying structural motifs, much shorter than the intact protein, which might contain a conformational IgE binding epitope. It should also help to identify potentially cross-reactive proteins that are not true homologues of an allergen that have significant local identities that might provide an immunological target for IgE antibodies in those with allergies to the matched allergen. A match of >35% with a known allergen will suggest further testing for possible cross-reactivity although matches using the sliding 80 amino acid window search that are not also identified by overall FASTA search may represent an irrelevant alignment. Thus evaluation of *E* scores and relative comparison of matched sequences with the NCBI database is sometimes warranted.

3.2.3 Exact Word match of AllergenOnline by 8-contiguous AA.
A word/string search routine on AllergenOnline.org was used to identify any eight contiguous amino acid segment of the query protein sequence that exactly matched any 8 amino acid segment of any of the allergen sequences in database. The rationale for identifying identity matches in very short sequences is an assumption that individual epitopes may be represented by peptide segments as short as eight amino acids (Metcalfe et al., 1996).

3.2.4 BLASTP of NCBI Entrez without keyword limit. The BLASTP is available on the NCBI Entrez website (http://www.ncbi.nlm.nih.gov/BLAST/). The current version is BLASTP 2.3.0 (22 Dec., 2015). The purpose of this BLASTP search is to compare the leghemoglobin to all known protein sequences to evaluate whether there are other similar proteins from other organisms that might provide information of safe exposure to homologues of this protein.

3.2.5 BLASTP of NCBI Entrez with "allergen" and "allergy" as keyword limits. BLASTP search was used comparing leghemoglobin sequence against the entire Entrez Protein database, with a limit option selected to query entries for "allergen" or "allergy", to align only with proteins identified as allergens or allergy. The purpose of this BLASTP search is to ensure that a significant match with a newly discovered allergenic sequence that has not yet been entered into AllergenOnline is not overlooked. Evaluation of the *E* value, the length of the alignment and the percent identity of any identified match is necessary to judge the significance of any alignment using BLASTP.

3.2.6 BLASTP of NCBI Entrez with "toxin" and "toxic" as keyword limits. The purpose of this BLASTP search is to identify matches to known toxic proteins (toxins) and if alignments share significant identities, to determine potential risks that would require further testing.

4.0 Results and Discussion. The summary results for the PubMed search using search terms, and the amino acid sequence of 17 proteins from *Pichia pastoris*, are presented here. Impossible Foods, Inc. contracted the UC Davis Genome Center to identify 17 proteins from 10 Coomassie stained bands from the SDS-PAGE gel shown in Figure 1. The UC Davis Genome Center used LC-MS/MS to identify the proteins in bands that represent ~ 1% of the total protein abundance. The protein identities and sequences are listed in Table 1.

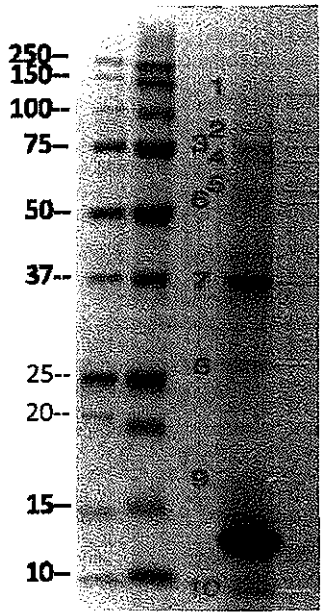

Figure 1. Coomassie blue stained gel of a Soy Leghemoglobin Preparation final product with residual *Pichia pastoris* proteins. The Coomassie stained SDS-PAGE gel image is from Impossible Foods, Inc. from PP-PGM2-15-320-101 production batch of Soy Leghemoglobin Preparation. Soy leghemoglobin is the broad stained band at 12 to 13 kDa. The stained proteins marked with orange bands and numbered from 1 (~ 140 kDa) to 10 (~ 8 kDa) are proteins from *P. pastoris* that represent ≥1% of the total protein fraction of Soy Leghemoglobin Preparation. Their identities were determined by LC-MS/MS with matches to full-matched sequences from NCBI listed in Table 1.

Table 1 Amino acid sequences of 17 proteins from *Pichia pastoris* (synon. *Komagataella phaffi*) from Soy Leghemoglobin Preparation. The protein sequences shown here were identified by LC-MS/MS as sequences in NCBI Protein database matching peptide segments from the 10 SDS-PAGE gel bands from *Pichia pastoris* proteins shown in Figure 1. Multiple protein matches were identified for bands 2, 5, 7, and 9. All protein hits were analyzed by literature search and sequence homology. The proteomics work was performed by Impossible Foods, Inc.

Organism	Protein, GI and band#	Native protein sequence
Komagataella phaffii *(Pichia pastoris)*	Alpha aminoadipate reductase GI:238030060 Protein band #1 – 1400 AA	MSEENLNYWANILDGPTLSVLPRDYNRPVAGKVIEANKTFDISDILPFLNKANEPSVTQFTAPLAVFAVL VYRLTGDDDIVILTDSPKKQNLPFVVRLQVDPSKSFVDVSKQVGEQYLESLERATPLKDIVTHLKESKQL PNYPPIFRLSFQTAKKVQQLSTLVEGSTRDLAIPLENNTSINIYYNSLLYTHNRIAYFSQQFSSFIDEVN KAPETPIGKISLLTEQQSKLLPDPTANLDWSGYRGAIQDIFSDNAEKFPDRTCVVETKSFLNPNSQTRTF TYKQIDQASNIVGNYLVHTGIKRGDVVMIYAYRGVDLMVAVMGVLKAGATFSVIDPAYPPARQNVYLQVA KPAGLIVLEKAGVLDQLVEDYIKNELSLVSRISNLKIEADGNVLGGDVDGKDALYDYQQFKTRRTGVLVG PDSNPTLSFTSGSEGIPKGVLGRHFSLAYYFPWMSKTFNLSENDKFTMLSGIAHDPIQRDMFTPLFLGAQ LLIPTSDDIGTPGKLAEWMQTYGATVTHLTPAMGQLLSAQATKEIPSLHHAFFVGDILTKRDCLRLQTIA QNVNIINMYGTTETQRAVSYFEIPSRAQDSTFLEVQKDIMPAGKGMHNVQLLVVNRHDRSRTCAIGEVGE IYVRAAGLAEQYRGQPDLNKEKFVPNWFVSPSKWVEEDKKISKDEPWREFYLGPRDRLYRTGDLGRYLPT GDCEVSGRADDQVKIRGFRIELGEIDTHISRHPLIRQNVTLVRRDKDEEPILISYVVPKETPELENFKSS SDDLDDLNDPIVKSLLLYRELIKDLKAHLKKTLASYAIPTIIVPMAKLPLNPNGKVDKPKLPFPDTVQLA AVAQKSSAEVDDSEFTTTELQIKDLWLQVLPNPPASISLEDSFFDLGGHSILATRMIFELRRKLAVDLPL GTIFKHPTVKLFAAEVDRVKNGDEVQFADNKQESTSAGSDEQVVDYFQDAKDLVSSQLLDSYKSRLALSN AELINIFLTGATGFLGSYILKDLLERDLDVQVYAHVRAKDEESGLERLRNTGKVYGIWNEEWTSRIKVVI ADLSKDKLGLSGEKYAELANTIDLIIHNGALVHWVYPYSKLRDANVISTINVLNLAASGKPKQFGFVSST STLDTEHYITLSDTLTEQGEDGIPESDDLLGSSKGLGTGYGQSKWAAEYIIRRAFERGLRGAIIRPGYVT GHSRTGACNTDDFLLRMLKGCAELGKLPNISNTVNMVPVDEVALVVTASSLEPTAEEGHCVVQVTGHPRI RFNEFLNALNDYGYEVKLTDYVEWKRDLERFVVDQSKDSALYPLLHFVLDNLPQDTKAPELDDKNAKDIL SGDTRWTGYDGSKGRGVDSAQTGIYIAYLIKTGFLPPPSKEGKKPLPEIEISEESLKLIKEGAGARTSAA
Komagataella phaffii *(Pichia pastoris)*	Cobalamin-independent methionine synthase GI:238030843 Protein band #2a – 768 AA	MVQSSVLGFPRIGAERELKKTTEAYWSGKVGKDELFKVGKEIRENNWKLQKAAGVDVIASNDFSYYDQVL DLSLLFNAIPERYTKYELDPIDTLFAMGRGLQRKATDSEKAVDVTALEMVKWFDSNYHYVRPTFSHSTEF KLNGQKPVDEYLEAKKLGIETRPVVVGPVSYLFLGKADKDSLDLEPISLLEKILPVYAELLAKLSAAGAT SVQIDEPILVLDLPEKVQAAFKTAYEYLANAKNIPKLVVASYFGDVRPNLASIKGLPVHGFHFDFVRAPE QFDEVVAALTAEQVLSVGIIDGRNIWKADFSEAVAFVEKAIAALGKDRVIVATSSSLLHTPVDLTNEKKL DSEIKNWFSFATQKLDEVVVVAKAVSGEDVKEALSVNAAAIKSRKDSAITNDADVQKKVDSINEKLSSRA AAFPERLAAQKGKFNLPLFPTTTIGSFPQTKDIRINRNKFTKGEITAEQYDTFIKSEIEKVVRFQEEIGL DVLVHGEPERNDMVQYFGEQLKGFAFTTNGWVQSYGSRYVRPPVVVGDVSRPHAMSVKESVYAQSITKKP MKGMLTGPITVLRWSFPRNDVSQKVQALQLGLALRDEVNDLEAASVEVIQVDEPAIREGLPLRSGQERSD YLKYAAESFRIATSGVKNTTQIHSHFCYSDLDPNHIKALDADVVSIEFSKKDDPNYIQEFSNYPNHIGLG LFDIBSPRIPSKEEFIARIGEILKVYPADKFWVNPDCGLKTRGWEEVRASLTNMVEAAKTYREKYAQN
Komagataella phaffii *(Pichia pastoris)*	Aconitase GI:254564667 Protein band #2b – 780 AA	MLSARRVLAKINSRGLATVSGLTKDSLVEMNLLEKGNYINYKQQLDNVNIVKERLGRPLTYAEKLLYGHL DKPHEQDIERGVSYLKLRPDRIACQDATAQMAILQFMSAGMPSVATPTTVHCDHLIQAQKGGAADLERAI RLNKEVYDFLATACARYNIGFWKPGSGIIHQIVLENYAFPGELLIGTDSHTPNAGGLGQLAIGVGGADAV DVMAGLPWELKAPKIIGVKLTGRMNGWTSPKDIILKLAGITTVKGGTGAIVEYFGDGVDTFSCTGMATIC NMGAEIGATTSVFPFNNSMVDFLDATGRSEIGEFAKVFQKEYLSADPGCEYDQVIEIDLNTLEPHINGPF TPDLATPVSKMKEVAVANDWPLEVKVGLIGSCTNSSYEDMTRAASIIEDAASHGVKARSLYTVTPGSEQI RATIARDGQLKTFTDFGGSVLANACGPCIGQWDRQDIKKGDKNTIVSSFNRNFTSRNDGNPATHAFVASP EMVTAYAIAGDLRFNPLTDKLKDKDGNEFLLKDPVGVGLPVRGYDPGENTYQAPPEDRASVEVVISPSSD

		RLQRLTPFQPWDGKDAERLPILIKSVGKTTTDHISMAGPWLKYRGHLQNISNNYMIGAINAENGEANNVX NHYTGVYSGVPDTAAAYRDNGVKWVVIGGENFGEGSSREHAALEPRYLGGFAIITKSFARIHETNLKKQG LLPLNFTDPAAYDRIQPDDEVDILGLTELAPGKNVTLRVHPADGSPTWETPLSHTYNAEQIEWFKYGSAL NNMAAVKASK
Komagataella phaffii *(Pichia pastoris)*	Transketolase GI:238030057 Protein band #3 – 679 AA	MSDLLAINTIRLLAVDTVAKANSGHPGAPMGLAPAAHTLFKQMRFNPRNPAWINRDRFVLSNGHACALLY TMLFLYGYDYTIDDLKSFRQLNSKTPGHPEAELPGVEVTTGPLGQGIANAVGLAIAQAQLAATYNKPNYE LFSNYTYAFLGDGCLQEGVAQEAISLAGHLGDGLKLIAFWDDNQISIDGDTNVSFTEDVPAKFRAQGWEV LSVKDGNDDLEGIAAALAKAKTTNKPTLIRLPTIIGYGSLQQGTHGVHGSPLKPDDIKQLKKKFGFDPEQ NFVVPREVTESYAKHVADNQQVEVEWNKLLTAYTKEYPELGQELHRRLDGKLPENWQKALPTYTVDDKPV ASRKLSEIVLTSIEKELPELVGGSADLTGSNLTRWPDAVDFQPKSTGLGDFSGRYFRFGVREHGMGAIIN GISAYGANFKAYGGTFLNFVSYASGAVRLSALSGHPIIWVATHDSIGLGEDGPTHQPIETLAHLRALPNL MVWRPADGNETSAAYLRAIESKHTPSIIALTRQNLPQLEHSSIEKAAKGGYTVYPVENPDIILVASGSEV SIAIDGAKKLGTEGVKASVVSIPDFFTFDSQARSYQLSVLPDGVPIMSVEVMSTFGWSKYSHEQFGINRF GISGPGPEIYKFFEFTAEGVADRASKVVQFYKGKELLSPLNKAFESVHA
Komagataella phaffii *(Pichia pastoris)*	Glycerol kinase GI:238034027 Protein band #4 – 621 AA	MGKDYTPLVATIDIGTTSTRAILFDYHGQEVAKHQIEYSTSAQDDIKRKRSQIISSEGISLTVSDDLEVE SVDNKAGPTLQFPQPGWVECRPSHILANAVQCLAACLVTMENKNLDRDEKNKYKLISIGVANMRETTVVW SKKTGKPLYNGIVWNDTRNNDIVDEYTAKYSEKEREEMRTLCGCPISTYFSATKFRWLLKHVPEVKQAYD NADGDLMFGTIDSWLIYHLTNEKSHVTDVTNASRTNFMNIETNKYDDRLLKFWDVDTSKVILPEIRSSAE VYGHFKVPHLESIGYVESYLTDDALALLETIEGAPLAGCLGDQSASLVGQLAVRKGDAKCTYGTGAFLLY NTGDQTLISEHGALTTVGYWFPGLDESEDGKHSSKPQYALEGSIAVAGSVVQWLRDNLRLISKAQDVGPL ASQVDNSGGVVFVPAFSGLFAPYWDSNSRGTIFGLTQYTSASHIARAALEGVCFQTRAILKAMISDAGAS ADFLEESSKATGHNPLSVLAVDGGMSKSDEMMQIQADILGPCVTVRRSINPECTALGAAIAAGFGVFKED RIWGSLKECTEAILEGNKMYLAAGNTSLDFKATLSDEVRRKEWRLWENAIAKAKGWLKDTA
Komagataella phaffii *(Pichia pastoris)*	Catalase A GI:254569930 Protein band #5a – 510 AA	MSQPPKWTTSNGAPVSDVFATERATFDNANHANNAPKVGPLLLQDFQLIDSLAHFDRERIPERVVHAKGA GAFGEFEVTDDISDVCAAKFLDTIGKTRIFTRFSTVGGEKGSADSARDPRGFSTKFYTEEGNLDLVYNN TPIFFIRDPSKFPHFIHTQKRNPATNLKDANMFWDYLVNNQESIHQVMYLFSDRGTPASLRKMNGYSGHT YRWYNKKGEWVYVQVHFKSDLGVVNFNNEEAGKLAGEDPDYHTGDLFNAIERGEYPSWTCYIQTMTQEQA AKQPFSVFDLTKVWPHKDFPLRRFGKFTLNENPKNYFAEVEQAAFSPSHTIPSMQPSADPVLQSRLFSYP DTHRHRLGVNYQQIPVNCPVAPVFTPQMRDGSMTVNGNLGSTPNYRSSFCPFSTEAQIQTNSHTPEEVLA AHTEKFHWGGILDSKSYDFEQPRALWKVFGKTPGQQRNFCHNVAVHVAAANHEIQDRVFEYFSKVYPEIG DQIRKEVLQLSPRGDSAARL
Komagataella phaffii *(Pichia pastoris)*	Glucose 6 phosphate dehydrogenase GI:238031000 Protein band #5b – 504 AA	MTDTKAVSFVGHTAIVVFGASGDLAKKKTFPALFGLYREGYLSNKVKIIGYARSKLDDKEFKDRIVGYFK TKNKGDEDKVQEFLKLCSYISAPYDKPDGYEKLNETINEFEKENNVEQSNRLFYLALPPSVFIPVATEVK KYVHPGSKGIARIIVEKPFGHDLQSAEELLNALKPIWKEEELFRIDHYLGKEMVKNLLAFRFGNAFINAS WDNRHISCIQISFKEPFGTEGRGGYFDSIGIIRDVIQNHLLQVLTLLTMERPVSNDPEAVRDEKVRILKS ISELDLNDVLVGQYGKSEDGKKPAYVDDETVKPGSKCVTFAAIGLSINTERWEGVPIILRAGKALNEGKV EIRVQYKQSTGFLNDIQRNELVIRVQPNEAMYMKLNSKVPGVSQKTTVTELDLTYKDRYENFYIPEAYES LIRDAMKGDHSNFVRDDELIQSWKIFTPLLYHLEGPDAPAPEIYPYGSRGPASLTKFLQDHDYFFESRDN YQWPVTRPDVLHKM

Komagataella phaffii *(Pichia pastoris)*	Hypothetical protein PAS GI:238031215 Protein band #5c – 525 AA	MLRTSPATKKALKSQINAFNVAALRFYSSLPLQVPITLPNGRTYNQPTGLFINNEFVPSKQGKTFAVLNP STEEEITEVYESREDDVELAVAAAQKAFDSTWSTQDPAERGKVLNKLADLIEEHSETLAAIESLDNGKAI SSARGDVGLVVAYLKSCAGWADKVFGRVVETGSSHFNYVRREPLGVCGQIIPWNFPLLMWSWKVGPALAT GNTVVLKTAESTPLSALYVSQLVKEAGIPAGVHNIVSGFGKITGEAIATHPKIRKVAFTGSTATGRHIMK AAAESNLKKVTLELGGKSPNIVFNDANIKQAVANIILGIYYNSGEVCCAGSRVYVQSGIYDELLAEFKTA AENVKVGNPFDEDTFQGAQTSQQQLEKILGFVERGKEDGATLITGGGRLGDKGYFVQPTIFGDVTPEMEI VKEEIFGPVVTISKFDTIDEVVDLANDSQYGLAAGIHSDDINKVIDVAARIKSGTVWVNTYNDFHQMVPF GGFGQSGIGREMGVEALENYTQYKAIRVKINHKNE
Komagataella phaffii *(Pichia pastoris)*	Mitochondrial aldehyde dehydrogenase GI:238033249 Protein band #6 – 501 AA	MTFAPPLEFEIDLPNGLKYTQPLGLFINNEFVEGVEGKLLPVINPCDETKITQVWEASAADVDRAVDAAE DAFNNSVWATQDPLERGKLMNKLADLIDRDFNILAGIESIDNGKAYTSAQGDVTLAVNYIRSCAGWADKI LGNVVDSGNTBLNLVKREPLGVVGQIIPWNFPLLMLAWKLGPALATGNTVVLKTAESTPLSGLYVAKLIK EAGFPPGVVNILSGFGNPAGAAIAAHPRIKKIAFTGSTATGRKIMEAAAKSNLKKVTLELGGKSPNIVFE DADIQKTIHNIILGIFFNSGEVCCAGSRVYIQDTVYEEVLEAFKKETDNVKVGGPFEEGVFQGPQTSELQ LNRILSYIKHGKDEGARVITGGSRYRNRGYYIKPTIFADVTEDMKIVKEEIFGPVVTITKFSTVDEVVGY ANNTNYGLAAGIHTNNLNKAIDVASRIKAGVVWINTYNDFHHMVPFGGYGESGIGRELGAEALDNYTQAK AIRIAYTFEHK
Komagataella phaffii *(Pichia pastoris)*	Delta-aminolevulinate dehydratase GI:238033645 Protein band #7a – 341 AA	MVSKAEYLDDHPTQISSILSGGYNHPLLREWQSERQLNKNMFIFPLFVTDRPDEEELIPSLPNIKRFGVN KLIPYVGGLVSKGLRAVILFGVPLKPGVKDEEGTAADDPEGPVIQAIKHLRKNFPDLYIITDVCLCEYTS HGHCGILYEDGTINRELSVRRIAAVAVKYAQAGANSVAPSDMTDGRIRDIKEGLLSAGLAHKTFVMSYAA KFSGNLYGPFRDAAGSCPSQGDRKCYQLPSGGKGLAHRALIRDMNEGTDGIIVKPSTFYLDIVADAYQLC KDYPICCYQVSGEYAMLHAAAEKNIVDLKSIAFEAHQGFLRAGARLIISYFTPEFLEWLSE
Komagataella phaffii *(Pichia pastoris)*	Mitochondrial alcohol dehydrogenase isozyme III GI: 238031179 Protein band #7b-350 AA	MSPTIPTTQKAVIFETNGGPLEYKDIPVPKPKSNELLINVKYSGVCHTDLHAWKGDWPLDNKLPLVGGHE GAGVVVAYGENVTGWEIGDYAGIKWLNGSCLNCEYCIQGAESSCAKADLSGFTHDGSFQQYATADATQAA RIPKEADLAEVAPILCAGITVYKALKTADLRIGQWVAISGAGGGLGSLAVQYAKALGLRVLGIDGGADKG EFVKSLGAEVFVDFTKTKDVVAEVQKLTNGGPHGVINVSVSPHAINQSVQYVRTLGKVVLVGLPSGAVVN SDVFWHVLKSIEIKGSYVGNREDSAEAIDLFTRGLVKAPIKIIGLSELAKVYEQMEAGAIIGRYVVDTSK
Komagataella phaffii *(Pichia pastoris)*	Malate dehydrogenase GI: 238034064 Protein band #7c-342 AA	MVKVTVCGAAGGIGQPLSLMFKLNPYVTTLALYDVVNVPGVGKDLSHIDTDTKLESYLPENDGLEKALTG SDLVIIPAGVPRKPGMTRDDLFAINAGIIRDLANGIAQFAPSAFVLVISNPVNSTVPIVAEILKRNNVFN PQKLFGVTTLDCVRANTFVAELSKDKEASAFDTRVLGGHSGETIVPVFSQSAPEVYKELSDEQKAALVHR VQFGGDEVVKAKNGAGSATLSMAYAGYKLGHALLAAINDTPNIIESTFVYLKDSKIKGAAEAFKYINEKL KDSDSSDVDFFALPVVLSSNGIEEIKWDILEKVDAKETELLKIATGQLSKNIARGTAFIAGN

Komagataella phaffii *(Pichia pastoris)*	Putative protein, unknown function GI:238033788 Protein band #7d-328 AA	MVVAIEGGTGLGLMNLTWKPTPTPIDDAIETIRYAVEEAGVRYLNGGEFYNFPLDSNLNLQYIQEFAKRY PELYKKVSLSVKGAVSLVDVSPDSSPENLEKSISNITKHLPNNFLPIFEPARIDKRYSIEETIKNLSKFV EDGRIGGISLSEVGADTIRRAAKVAPIACVEVEFSLLTRDILHNGVLAACEDLNIPIIAYSPLGRGFLTG TINSKADIPEGDIRLSLERFNDDEVIEHNLKLVRGLKKIADKRGVTLAQLSLAWLRKFGDKHVKVLPIPS CSSPRRVAENTKEISLTDSEFQEITDFAESVPIKGGRYNKASEAVLNG
Komagataella phaffii *(Pichia pastoris)*	Triose phosphate isomerase GI:238032989 Protein band #8 – 248 AA	MARTFFVGGNFKMNGSKKSIHEIIERLNNTKLPENVEVVIAPPAPYLQQAVTENKQKTVYVSAQNSFDKA SGAYTGEVSVEALRDLGVPYVILGHSERRTINKEDDAFIASKTKFALDQGLKVILCIGETLEEKQANITL DVVKRQLQAVVDVVSDWTNIVVAYEPVWAIGTGLAATPSDAQDVHKQIRDFLATVIGKDQAEKVRILYGG SVNGKNAVEFRDKADVDGFLVGGASLKPEFVDIINSRN
Komagataella phaffii *(Pichia pastoris)*	Hypothetical protein (cyclophilin) PP7435 GI:328350030 Protein band #9a – 161 AA	MTKTFFDVSSNDQPLGRIVFELYDDVPKTIENFRALCTGEKGYGYKDSIFHRVIPQFMLQGGDFTKFNGT GGKSIYGEKFADENFIHKHTKPGLLSMANAGPNTNGSQFFITTVPCPWLDGKHVVFGEVVDGLDVVSKIE TLGSSSGATKTQLKITNSGEL
Komagataella phaffii *(Pichia pastoris)*	Cytosolic superoxide dismutase GI:238034030 Protein band #9b – 154 AA	MVKAVAVLRGDSTVGGTVVFEQSSESSPTTITYDIKGNSPNAERGFHIHQFGDNTNGCTSAGPHFNPFGK THGAPTDEARHVGDLGNVKTDAEGVAKGVITDNQVKLIGETSILGRTVVIHDGTDDLGKGGHADSLKTGN AGGRPACGVIGLAA
Komagataella phaffii *(Pichia pastoris)*	Mitochondria ATPase Inhibitor GI:238029769 Protein band #10 – 84 AA	MFQRTTATLVRQNKAIARFYSEGSTGAPRSDGSGDAFTKREKAQEDFYIKKHQAEQLAKLREQLKNQKEH LQNLEKEINNIKEK

4.1 PubMed Searches. The scientific literature database, PubMed, was searched for evidence that any of the proteins from *Pichia pastoris*, and specifically the 17 proteins identified by Impossible Foods, Inc. are likely source of allergy or toxicity. The search did not reveal evidence that the *Pichia pastoris* or the 17 proteins from the species represent food safety risks. Summary of all information is present in Table 2.

4.1.1 Allergenicity. A search of PubMed using only the organism name, "*Pichia pastoris*" AND "allergen" returned 128 articles. A quick review of the summary entries showed that they all appear to be related to the use of the yeast as a recombinant vector for expressing a wide variety of proteins from various eukaryotic sources. Adding a third term, NOT "recombinant" reduced the returns to five and a careful read of the results demonstrated no link to allergy related to endogenous proteins from *Pichia pastoris*. However, all five only appear to be related to recombinant proteins in the yeast, therefore the search exclusion was not appropriate. A search with the newer name of the species, *Komagataella phaffii* without any other search term returned only six publications, all related to the taxonomy or genomics cloning of the yeast species (synon. *Pichia pastoris*) or the use of this species as a recombinant vector. To more fully evaluate the potential risks of allergy, the names of each of the identified 17 *P. pastoris* proteins were searched in combination with *Pichia pastoris* using the "AND" function. The identified references and/or abstracts were saved in an archive. Review of the information failed to identify any study data to suggest possible allergenicity of the endogenous proteins identified by Impossible Foods in their proteomic analysis. Thus there is no evidence that endogenous proteins from *Pichia pastoris* are allergens.

4.1.2 Toxicity. The search of PubMed using both "*Pichia pastoris*" AND "toxin" returned 185 references, however, most appear to relate to expressing various eukaryotic genes to produce recombinant proteins. The list was reduced to 26 by excluding "recombinant". One of the publications specifically looked for protein toxin expression by *P. pastoris*, Banerjee and Verma (2000). They tested various strains of the yeast to produce protein toxins that would kill other yeasts. They failed to detect any evidence of toxin production. There were no other publications that seemed to relate to toxins except for heterologously expressed genes from other organisms. Thus, based on literature search there is no reason to suspect that *Pichia pastoris* produces toxic proteins.

Table 2 Pubmed search results (Summary table)

Protein Source	Search results			
	Pichia pastoris* or *Komagataella phaffii	***Pichia pastoris* AND allergen**	***Pichia pastoris* AND toxin**	**Individual endogenous (17) *Pichia* proteins**
Pichia pastoris *synon. Komagataella phaffii* (recombinant yeast host)	5243 articles were found.	128 articles, most relate to expression of heterologous genes and proteins. 5 articles when NOT recombinant was added. --- Six references were found for *Komagataella phaffii*, all related to heterologous expression or to taxonomic relationships with *P. pastoris* or other *Komagataella sp.*	185 articles were found. Adding NOT recombinant reduced the number to 26. Carefully reading the abstracts demonstrated that only one considered possible protein toxins expressed by the yeast, *Pichia pastoris*. The paper (Banerjee and Verma, 2000) demonstrated that no toxic proteins were identified in *P. pastoris*.	Searches with the following individual identified proteins did NOT identify any publications that described allergenicity or toxicity. Alpha-aminoadipate reductase: Zero articles Cobalamin-independent methionine synthase, NOT recombinant: Four articles Aconitase: Two articles Transketolase: Five articles Glycerol kinase: Five articles Catalase A: Twenty one articles G6PD: One article Hypothetical PAS: Twenty one articles Mitochondrial aldehyde dehydrogenase: One article Delta-aminolevulinate dehydrogenase: One article Mitochondrial alcohol dehydrogenase: Two articles Malate dehydrogenase: One article Unknown function (possibly pyridoxal reductase: Zero Triosphosphate isomerase: Zero Cyclophilin: Zero Cytosolic superoxide dismutase: Three articles Mitochondria ATPase inhibitor: Zero

4.2 Sequence comparisons of the 17 *Pichia pastoris* proteins to allergens and toxins. The amino acid sequence of each of the 17 endogenous proteins of *Pichia pastoris* identified by Impossible Foods from the 10 highest intensity bands were compared to known allergens using both full-length FASTA3 alignment searches and a sliding window of 80 comparison against AllergenOnline.org, version 16 as well as an exact 8AA word search. Additionally, a BLASTP search was performed against the NCBI database using keyword search limits of "allergen" / "allergy" and "toxin"/ "toxic". Results are presented in summary form in Tables 3 to19 for proteins 1 through 17.

Table 3. Bioinformatics Summary Results of Protein Band #1. The 1400 AA protein, Alpha aminoadipate reductase was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	No match to any protein in AllergenOnline version 16 with an E score of < 1. Four low identity short segment matches with E scores between 1 and 10.
AllergenOnline 80mer FASTA3	No match to any protein in AllergenOnline version 16, with identity of >35% over any 80 AA segment.
AllergenOnline Exact 8 AA	No exact 8mer match with any protein in AllergenOnline version 16.
NCBI BLASTP Allergen	Two low scoring identity matches to non-homologous, hypothetical proteins with *E* scores >1 e-05, and alignments of < 25% of the length and < 30% identity.
NCBI BLASTP Toxin or Toxic	A number of low scoring identity matches to homologous proteins (small E scores) that are proteins from organisms that are classified as toxic to insects (e.g. Bacillus thuringiensis), but no significant alignment to an obvious toxin or toxic proteins.
NCBI BLASTP No keyword	Many high scoring identity matches with protein homologous proteins from other yeasts and molds. The highest scoring match to a *Saccharomyces sp.* protein has an *E* score = 0.0, and 60% identity in a 1418 AA alignment.
Conclusion: No risk of Allergy or toxicity identified for protein #1	

Table 4. Bioinformatics Summary Results of Protein Band #2a. The 768 AA Cobalamin-independent methionine synthase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	One significant match to a protein in AllergenOnline version 16. The protein is Sal k 3 (Assarehzadegan et al., 2011), a pollen allergen from Russian thistle (*Salsola cali*), with an E score of 1e-158, 49% identity over the full length. The Sal k 3 is unique in identification of an allergen, yet a BLAST with Sal k 3 reveals highly homologous proteins in many dicotyledonous plants ranging from pigweed, to mustards, cotton, strawberries, soybeans and peaches with much higher identities than achieved by *Pichia pastoris* protein band #2. It is not clear how important Sal k 3 is as an allergen and the large protein is likely to be heat-denatured and digested rapidly by multiple proteases.
AllergenOnline 80mer FASTA3	The only protein in AllergenOnline version 16 is Sal k 3, with a best 80 AA alignment of 77.5%.
AllergenOnline Exact 8 AA	The only protein with an exact 8mer match is Sal k 3.
NCBI BLASTP Allergen	Two low scoring identity matches to non-homologous, hypothetical proteins with *E* scores >1 e-05, and alignments of < 25% of the length and < 30% identity.
NCBI BLASTP Toxin or Toxic	A number of low scoring identity matches to homologous proteins (small *E* scores) that are proteins from organisms that are classified as toxic to insects (e.g. *Bacillus thuringiensis*), but no significant alignment to an obvious toxin or toxic proteins.
NCBI BLASTP No keyword	Many high scoring identity matches with homologous proteins from other yeasts and molds. The highest scoring match to a *Saccharomyces sp.* protein has an *E* score = 0.0, and 77% identity in a 765 AA alignment.
Conclusion: There is a very minimal risk of allergy based on the modest alignment with Sal k 3. However, Sal k 3 has many higher scoring alignments with proteins in commonly consumed plant foods, and no publication of allergy except airway allergy and skin test reactivity to Sal k 3. Thus the risk of allergy is likely to be very low. There was no significant match to a toxin, so the likely risk of toxicity is extremely low.	

Table 5. Bioinformatics Summary Results of Protein Band #2b. The 780 AA Aconitase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Two alignments with *E* scores between 0.1 and 1 (*Aspergillus sp.* endo-chitosane 238 AA, 31% identity over 80 AA; *Sarcoptes scabiei* inactive cysteine protease, 340 AA, 25% identity over 139 AA), and 10 alignments with *E* scores between 4 and 10, various proteases with low identity scores (24% to 37% identity) with proteins in AllergenOnline.org version 16.
AllergenOnline 80mer FASTA3	No matches of >35% identity over 80 AA with any protein in AllergenOnline.org version 16.
AllergenOnline Exact 8 AA	No exact 8 AA match with any protein in AllergenOnline.org version 16.
NCBI BLASTP Allergen	One very low scoring alignment with an alkyl hydroperoxide annotated as a "Mal allergen" (no reference) from an Antarctic coastal bacteria, genomic sequence. The E score was 0.86, 35% identity over 72 AA and three other irrelevant lower identity alignments.
NCBI BLASTP Toxin or Toxic	A number of statistically significant alignments with *E* scores smaller than 1e-50, one with 0.0. However most of the alignments are modest identities of 50% or less, indicating homology. Most of the source organisms are toxic, but no obvious reference to toxicity of the proteins. There was one higher scoring alignment of 66% with E score 0.0, to a bovine homologue of the aconitase enzyme (GI:157831069, bovine 4-hydroxy-trans-aconitate) in crystal structure with a "toxic" substrate, fluorocitrate, that disrupts the tricarboxylic acid cycle (Lauble et al., 1996), but the enzyme is not toxic, the chemical is toxic to the enzyme and thereby impacts the consumer of the compound.
NCBI BLASTP No keyword	Many high scoring identity matches with protein homologous proteins from other yeasts and molds. The highest scoring match to a *Saccharomyces sp.* protein has an *E* score = 0.0, and 81% identity in a 775 AA alignment.
Conclusion: There is a very minimal risk of allergy based on the modest alignment with endo-chitosane and inactive protease found in full-length alignment. Thus the risk of allergy is likely to be very low. There were moderate alignments to proteins identified as being from toxic organisms, but only as common homologues. The identity to the homologue of *Saccharomyces cerevisiae* was much was much higher. Thus the likely risk of toxicity is extremely low.	

Table 6. Bioinformatics Summary Results of Protein Band #3. The 679 AA Transketolase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	No matches to proteins in AllergenOnline version 16 with an *E* score <1. There were >20 matches with E score between 1 and 10. But low identities of <35% and generally short, 49 to 134 AA. These matches are not significant.
AllergenOnline 80mer FASTA3	No identity match of >35% over any segment of 80 amino acids compared to any protein in AllergenOnline version 16.
AllergenOnline Exact 8 AA	No exact 8 AA matches with any protein in AllergenOnline version 16.
NCBI BLASTP Allergen	No significant alignment with any protein in NCBI with using the keyword allergen.
NCBI BLASTP Toxin or Toxic	Many small *E* score identity matches of just under 50% identity to transketolases of bacteria (e.g. *Bacillus cereus*) or cyanobacteria that are known to be toxic organisms. These are homologues of the enzyme involved in sugar catabolism from photosynthesis. No references were obvious showing any toxic effects of these enzymes on consumers.
NCBI BLASTP No keyword	Many high scoring identity matches with protein homologous proteins from other yeasts and molds. A transketolase from the commonly used food yeast, *Saccharomyces cerevisiae* has an *E* score of 0 and 70% overall identity to the transketolase of *P. pastoris*.
Conclusion: The data indicate there is no obvious risk of allergy or toxicity from the transketolase protein. The higher scoring alignment with transketolase of *Saccharomyces cerevisiae* provides additional assurance of safety.	

Table 7. Bioinformatics Summary Results of Protein Band #4. The 621 AA Glycerol kinase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	No matches to proteins in AllergenOnline version 16 with an *E* score <1. There were >20 matches with E score between 1 and 10. But low identities of <35% and generally short, 49 to 134 AA. These matches are not significant.
AllergenOnline 80mer FASTA3	No matches of >35% identity over 80 AA were found compared to any protein in AllergenOnline version 16.
AllergenOnline Exact 8 AA	No exact 8 AA matches were identified to any protein in AllergenOnline version 16.
NCBI BLASTP Allergen	No significant alignments were identified.
NCBI BLASTP Toxin or Toxic	A number of low scoring identity matches to homologous proteins (small E scores e.g. 6e-118) that are proteins from organisms that are classified as toxic to insects (e.g. *Bacillus thuringiensis*), or mammals (e.g. *Mycobacterium tuberculosis*), with up to 41% identity matches and many gaps over 550 AA, but no significant alignment to an obvious toxin or toxic proteins.
NCBI BLASTP No keyword	Many higher scoring identity matches with protein homologous proteins from other yeasts and molds were found. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 0.0 and identity of 53% over 664 AA.
Conclusion: There does not seem to be a risk of allergy based on the lack of alignment to allergens. There are modest alignments to proteins that have not been demonstrated to be toxins, but are from organisms that are known to be toxic. However, with higher identities to proteins from other yeasts, including *Saccaromyces sp.*, it is very unlikely that this protein is a toxin.	

Table 8. Bioinformatics Summary Results of Protein Band #5a. The 510 AA Catalase A protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	One highly significant match with 2.6 e-058 but identity match with only 37% identity over 475 AA was identified to catalase of *Penicillium citrinum*, a putative allergen called Pen c 30, based on IgE binding to mold allergic subjects in Taiwan. There are two Asn-linked glycans on the natural *Penicillium citrinum* protein and the deglycosylated form had reduced binding, but still some IgE binding. One subject had a positive SPT to Pen c 30. Other alignments had large E scores near or above 1 and short low identity matches.
AllergenOnline 80mer FASTA3	The only alignment with >35% identity over 80 AA was to the *Penicillium citrinum* catalase and the best aligned 80 AA segment was 60% identity, indicating restricted conservation of sequence to a subsection of the protein.
AllergenOnline Exact 8 AA	No exact 8 AA matches were identified to any protein in AllergenOnline version 16, not even to the catalase of *Penicillium citrinum.*
NCBI BLASTP Allergen	Only the single alignment of *Penicillium citrinum* was identified, with an *E* score of 9e-93 and identity score of 38% over 424 AA.
NCBI BLASTP Toxin or Toxic	Many significant (very small *E* score) alignments were identified (as low as 5e-149 for toxin, 0.0 for toxic) to homologues from organisms noted for toxins or toxicity (e.g. *Bacillus sp., Enterococcus faecalis, Streptomyces sp., Clostridium sp.*). The alignments are approximately 50% for proteins identified with "toxin", and 71% as the highest for those identified with "toxic" organisms (*Candida boidinii*). The enzyme is common to all organisms living in oxygen containing environments as it detoxifies hydrogen peroxides by converting them to water and oxygen. It is not a toxin and is not toxic to mammalian cells. No published evidence was found that catalase A is a toxin.
NCBI BLASTP No keyword	Many high scoring identity matches were found with homologous proteins from other yeasts and molds. The full-length catalase A of *Pichia pastoris* represents a conserved catalase A common to most yeast and fungi. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 0.0 and identity of 66% over 494 AA.

Conclusion: There does not seem to be a risk of allergy based on the lack of alignment to allergens. Catalase from ***Penicillium citrinum*** has been identified as an inhaled spore allergen, with no described allergic reactions to ingestion. ***Penicillium camembertii*** is widely consumed with brie and other soft-ripened cheeses, which have versions of catalase that are closer to the allergenic ones than is the ***Pichia*** protein. ***Penicillium*** is a member of the fungal subdivision ***Pezizomycetes***, which is distinct from the subdivision ***Saccharomycotina***, which contains ***Pichia***. Several members of ***Pezizomycetes*** are widely eaten (e.g. morels) and have versions of catalase that are closer to the allergenic ones than is the ***Pichia*** protein. The lack of cross-reactivity upon consumption of morels and soft ripened cheeses establishes it as highly unlikely that the ***Pichia*** catalase will elicit cross-reactivity. Similarly, several members of the ***Saccharomycotina*** (including ***S. cerevisiae***), whose catalases are equally closely related to the allergenic ones as is ***Pichia*** are widely eaten without cross-reactivity. There are modest alignments to proteins that have not been demonstrated to be toxins, but are from organisms that are known to be toxic. Many homologues were identified to proteins from yeasts, including *Saccharomyces sp.*, thus it is unlikely that this protein is a toxin.

Table 9. Bioinformatics Summary Results of Protein Band #5b. The 504 AA glucose 6 phosphate dehydrogenase protein (G6PD) was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Low scoring matches were identified with E scores ranging from 0.91 to 9.4 for 6 proteins that had identity matches of 24% to 47% over short alignments of 88 down to 30 AA. These proteins were not homologues and the identity matches appear random.
AllergenOnline 80mer FASTA3	The only alignment with >35% identity over 80 AA was to the putative allergen, *Blattella germanica*, German cockroach, Bla g 3 (hemocyanin) with 37% identity as the best aligned 80 AA segment.
AllergenOnline Exact 8 AA	No exact 8 AA matches were identified to any protein in AllergenOnline version 16.
NCBI BLASTP Allergen	No significant identity match was identified in NCBI with keyword allergen.
NCBI BLASTP Toxin or Toxic	Many significant (very small *E* score) alignments were identified (as low as 6e-93 for toxin, 6e-95 for toxic) to homologues from organisms noted for toxins or toxicity (e.g. *Bacillus sp., Enterococcus faecalis, Streptomyces sp., Clostridium sp.*). The alignments are approximately 35% for proteins identified with "toxin", and 35% as the highest for those identified with "toxic" organisms (*Fictibacillus phosphorivorans*). The enzyme is common to all organisms. It is not a toxin and is not toxic to mammalian cells.
NCBI BLASTP No keyword	Many high scoring identity matches were found with homologous proteins from other yeasts and molds. The full-length G6PD alignment to Saccharomyces sp. was with an *E* score of 0.0 and identity of 64% over 495 AA.
Conclusion: There does not seem to be a risk of allergy based on the lack of alignment to allergens. There are modest alignments to proteins that have not been demonstrated to be toxins, but are from organisms that are known to be toxic. Many homologues were identified to proteins from yeasts, including *Saccharomyces sp.*, thus it is unlikely that this protein is a toxin.	

Table 10. Bioinformatics Summary Results of Protein Band #5c. The 525AA hypothetical protein (PAS) was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Two very significant scoring alignments were identified, one with an *E* score of 5e-126 and 58% identity (*Davidiella sp.* aldehyde dehydrogenase) and one with 1e-124 *E* score and 58% identity to *Alternaria alternata* aldehyde dehydrogenase. The next highest alignments were to diverse species and of much greater *E* scores and lower identities. These are minor hypothetical allergens.
AllergenOnline 80mer FASTA3	Three alignments were found with >35% identity over 80 AA. The highest scoring ones were to the same *Davidiella sp.*, and *Alternaria sp.* as the full-length FASTA, with highest scoring 80mers of 72% identity. The third was to a storage mite, *Lepidoglyphus destructor*, with 35% identity.
AllergenOnline Exact 8 AA	A number of exact 8 AA matches were identified to the *Davidiella sp.* and *Alternaria sp.* proteins in AllergenOnline version 16.
NCBI BLASTP Allergen	There were a few significant alignments using NCBI with keyword allergen. The best scoring ones were to *Davidiella sp.* (*Cladosporium sp.*) and *Aureobasidium namibiae*.
NCBI BLASTP Toxin or Toxic	Some significant (very small *E* score) alignments were identified (as low as 2e-171 for toxin, 2e-177 for toxic) to homologues from organisms noted for toxins or toxicity (e.g. *Bacillus sp.)*. The alignments are approximately 53% for proteins identified with "toxin", and "toxic" organisms. The enzyme is common to all organisms. It is not a toxin and is not toxic to mammalian cells.
NCBI BLASTP No keyword	Many high scoring identity matches were found with homologous proteins from other yeasts and molds. The full-length PAS alignment to *Saccharomyces sp.* was with an *E* score of 0.0 and identity of 69% over 512 AA.
Conclusion: There does not seem to be a risk of allergy based on the lack of alignment to allergens. The two hits are to members of the fungal subdivision *Pezizomycetes*, which is distinct from the subdivision *Saccharomycotina*, which contains *Pichia*. Several members of *Pezizomycetes* are widely eaten (e.g. morels) and have versions of ALDH that are closer to the allergenic ones than is the *Pichia* protein. The lack of cross-reactivity upon consumption of morels establishes it as highly unlikely that the *Pichia* ALDH will elicit cross-reactivity. Similarly, several members of the *Saccharomycotina* (including *S. cerevisiae*), whose ALDH's are equally closely related to the allergenic ones as is *Pichia* are widely eaten without cross-reactivity. There are modest alignments to proteins that have not been demonstrated to be toxins, but are from organisms that are known to be toxic. Many homologues were identified to proteins from yeasts, including *Saccharomyces sp.*, thus it is unlikely that this protein is a toxin.	

Table 11. Bioinformatics Summary Results of Protein Band #6. The 501 AA mitochondrial aldehyde dehydrogenase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Two high scoring matches with 2.4e-138 and 1.2e-131 with identity matches of 60% and 57% were identified to aldehyde dehydrogenases. One was *Davidiella tassiana* (*Cladosporium herbarum*) and one to *Alternaria alternata* with nearly 60% identities over nearly full length alignments. These are minor airway allergens identified only by low-level IgE binding in the studies used to identify them as putative allergens.
AllergenOnline 80mer FASTA3	The same two mold allergens were identified in alignments with best-scoring identity matches of 76% and 74% identities.
AllergenOnline Exact 8 AA	The same two mold allergens had a large number of 8 AA matches as expected based on high identity matches in AllergenOnline version 16.
NCBI BLASTP Allergen	High scoring mitochondrial aldehyde dehydrogenase fungal proteins were identified in NCBI as the clear alignments using keyword allergen. *Cladosporium* (*Davidiella sp.*) and *Aureobasidium namibiae* were highest scoring with *E* scores of 0.0 and identities of 61% and 60%. These proteins are putative allergens, IgE binding, no biological activity.
NCBI BLASTP Toxin or Toxic	Slightly lower scoring alignments (compared to the fungal mitochondrial aldehyde dehydrogenases) or 6e-170 and 4e-165 and identity scores of 53% and 51% respectively were found for toxin. Interestingly, a human mitochondrial aldehyde dehydrogenase was the highest scoring protein with "toxic" as a search term, having an E score of 6e-173 and 52% identity. The next scoring match was to aldehyde dehydrogenase of *Bacillus thuringiensis* were identified using toxin and toxic keywords. The matches were to homologous proteins from bacteria (e.g. *Bacillus thuringiensis*) that do not have mitochondria, but the function is still needed in these bacteria. There is no published evidence was found that any aldehyde dehydrogenase is a toxin.
NCBI BLASTP No keyword	Many high scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 0.0 and identity of 62% over 488 AA.
Conclusion: There is a very minor potential risk of allergy based on the identity alignments to two putative mold airway allergens however, the enzyme is ubiquitous and there has not been proof that these proteins cause allergic disease. Only that they can bind IgE from some airway sensitized individuals. The two hits are to members of the fungal subdivision *Pezizomycetes*, which is distinct from the subdivision *Saccharomycotina*, which contains *Pichia*.	

Several members of *Pezizomycetes* are widely eaten (e.g. morels) and have versions of ALDH that are closer to the allergenic ones than is the *Pichia* protein. The lack of cross-reactivity upon consumption of morels establishes it as highly unlikely that the *Pichia* ALDH will elicit cross-reactivity. Similarly, several members of the *Saccharomycotina* (including *S. cerevisiae*), whose ALDH's are equally closely related to the allergenic ones as is *Pichia* are widely eaten without cross-reactivity. It is very unlikely that this protein is an allergen or a toxin.

Table 12. Bioinformatics Summary Results of Protein Band #7a. The 341 AA Delta-aminolevulinate dehydratase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Very low-scoring alignments with *E* scores of 4 to 10 were identified to glucanase enzymes from diverse sources (e.g. rubber tree, grass pollen, cockroach and wheat). These alignments are unlikely to represent a risk of possible cross-reactivity.
AllergenOnline 80mer FASTA3	No matches were identified with >35% identity in any 80 AA match with AllergenOnline version 16.
AllergenOnline Exact 8 AA	No exact 8 AA matches were identified with AllergenOnline version 16.
NCBI BLASTP Allergen	Two high identity matches were found to a dehydrogenase of *Candida albicans* as a putative allergen. However, there is no published proof that this protein is an allergen. One of the entries was from genomic cloning. The other was describing the protein with monoclonal antibody recognition. No connection to allergy.
NCBI BLASTP Toxin or Toxic	Modest scoring alignments were identified using toxin and toxic keywords. The matches of approximately 40% identity were to homologous proteins from bacteria. No published evidence was found that these proteins are toxins.
NCBI BLASTP No keyword	Many high scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 0.0 and identity of 76% over 340 AA.
Conclusion: It is unlikely that there is any risk of allergy for this protein. The same is true for toxicity. In addition, there are very good alignments to homologous proteins from many molds including *Saccaromyces sp.*, with 76% identity over 340 AA. Thus it is very unlikely that this protein is an allergen or a toxin.	

Table 13. Bioinformatics Summary Results of Protein Band #7b. The 350 AA mitochondrial alcohol dehydrogenase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	One significant-scoring alignment was identified with an *E* score of 1.2e-108 and 76% identity over the full-length (350AA) to *Candida albicans* Can f 1.0101. This protein should be considered a putative allergen as the publication demonstrating IgE binding to the apparent MW of a partially purified (Shen et al., 1989) and was also expressed as a recombinant-with very light IgE binding (compared to the native) by a pool of 4 sera (Shen et al., 1991) have not absolutely demonstrated that the large number of subjects bound IgE to this specific protein. However the natural protein is glycosylated that might impact IgE binding. There was no demonstration of biological activity.
AllergenOnline 80mer FASTA3	Only the single protein Can f 1.0101 identified by full-FASTA was identified with >35% identity in any 80 AA match with AllergenOnline version 16.
AllergenOnline Exact 8 AA	Only Can f 1.0101 contained exact 8 AA matches compared to protein band #7b with AllergenOnline version 16.
NCBI BLASTP Allergen	Two high identity matches (*E* scores 0.0, identities of 80% and 76%) were found to a dehydrogenase of *Candida albicans* as a putative allergen. A third alignment was identified with an E score of 0.001 and identity of 28% over 120 AA to *Salmonella enterica* putative oxidoreductase.
NCBI BLASTP Toxin or Toxic	Modest scoring alignments were identified using toxin and toxic keywords to alcohol dehydrogenase enzymes of bacteria that are associated with toxicity (*Corynebacterium ulcerans, Bacillus thuringiensis, B. cereus, Escherichia coli and Streptococcus sp.*). The E scores were small 1e-87, 2e-55 and percent identities 36% to 42%. However, no published evidence was found that these proteins are toxins.
NCBI BLASTP No keyword	Many high scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a Saccharomyces sp. was with an *E* score of 0.0 and identity of 74% over 347 AA.
Conclusion: It is unlikely that there is any risk of allergy for this protein. The same is true for toxicity. In addition, there are very good alignments to homologous proteins from many molds including *Saccaromyces sp.* Thus it is very unlikely that this protein is an allergen or a toxin.	

Table 14. Bioinformatics Summary Results of Protein Band #7c. The 342 AA malate dehydrogenase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	One significant scoring alignment was identified with an *E* score of 4.2e-54 and 51% identity over the full-length (341AA) to *Malassezia furfur* Mala f 4.0101. This protein should be considered a putative allergen as the publication demonstrating IgE binding to a natural, semi-purified protein, but there was another isoform isolated and two other proteins from the partially pure one called Mala f 4 (Onishi et al., 1999). However the natural protein is glycosylated that might impact IgE binding. There was no demonstration of biological activity.
AllergenOnline 80mer FASTA3	Two proteins were identified with >35% identity in any 80 AA match with AllergenOnline version 16. One was as expected, Malassezia furfur Mala f 4.0101, with a highest identity score of 70%. The second was to convicilin of *Pisum sativum* with 36.2% identity as the highest scoring 80mer. The overall alignment to that protein was poor, *E* score of 45, 31% identity in an 84 AA overlap.
AllergenOnline Exact 8 AA	Only Mala f 4.0101 contained exact 8 AA matches compared to protein band #7c with AllergenOnline version 16.
NCBI BLASTP Allergen	The only significant match by BLASTP was to *Malassezia sympodialis* Mala f 4.0101with an *E* score of 6e-71 and an identity of 45%.
NCBI BLASTP Toxin or Toxic	Significant scoring alignments were identified using toxin and toxic keywords to the malate dehydrogenase enzyme. The highest scoring hit was to a rat (*Rattus norvegicus*) with E score 2e-94 and 47% identity. Then a number of toxic organisms (*Vibrio cholera* and *E. coli*) with slightly lower identities and slightly higher *E* scores.
NCBI BLASTP No keyword	Many high scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a Saccharomyces sp. was with an *E* score of 4e-109 and identity of 53% over 339 AA.
Conclusion: The *C. albicans* ADH has been identified as an allergen linked (weakly) to *Candida*-related asthma. But the similar degree of identity to *S. cerevisiae* ADH, which is known to be highly expressed in many *S. cerevisiae* linked foods/beverages, make this a low concern. Thus, it is unlikely that there is any risk of allergy for this protein as only one putative dermal allergen had a significant match. The risk of toxicity is similarly low. In addition, there are very good alignments to homologous proteins from many molds including *Saccaromyces sp.* Thus it is very unlikely that this protein is an allergen or a toxin.	

Table 15. Bioinformatics Summary Results of Protein Band #7d. The 328 AA putative protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	One low-scoring alignment was identified with an *E* score of 0.23 and 24.6% identity over the full-length (328AA) to *Juniperus occidentalis* Jun o 4.0101. This alignment is very low scoring and unlikely to be relevant to risks. There was no demonstration of biological activity.
AllergenOnline 80mer FASTA3	No alignments with >35% identity over any 80 AA match with AllergenOnline version 16 were identified.
AllergenOnline Exact 8 AA	No exact 8 AA matches compared to protein band #7c with AllergenOnline version 16.
NCBI BLASTP Allergen	No significant match was identified using BLASTP and keyword Allergen.
NCBI BLASTP Toxin or Toxic	Modest scoring alignments were identified using toxin and toxic keywords to the malate dehydrogenase enzyme. The highest scoring hit was to a bacterial protein (*Escherichia coli* aldehyde oxidase) with E score 2e-39 and 32% identity. Then a number of toxic organisms (*E. coli, Microcystis aeruginosa, Raphidiopsis brookii*) with slightly lower identities and slightly higher *E* scores.
NCBI BLASTP No keyword	Many high scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 3e-68 and identity of 40% over 336 AA.
Conclusion: It is unlikely that there is any risk of allergy for this protein as matches were only to a putative allergen had a significant match. The risk of toxicity is similarly low. In addition, there are very good alignments to homologous proteins from many molds including *Saccharomyces sp.* Thus it is very unlikely that this protein is an allergen or a toxin.	

Table 16. Bioinformatics Summary Results of Protein Band #8. The 248 AA Triose Phosphate isomerase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Four high-scoring alignments were identified with *E* scores smaller than 9.3e-51 and 5-53% identities over nearly the full length of four triosephasphate-isomerase proteins of diverse sources (wheat, two from house dust mite and one from shrimp). Yet triosephosphate isomerases are ubiquitous and there is no published evidence for such wide-spread cross-reactivity. Thus these alignments are unlikely to represent a risk of possible cross-reactivity.
AllergenOnline 80mer FASTA3	The same four proteins were identified as having alignments with >35% identity in any 80 AA match with AllergenOnline version 16. The highest alignment was 62% identity.
AllergenOnline Exact 8 AA	A number of exact 8 AA matches were identified with the same four proteins in AllergenOnline version 16.
NCBI BLASTP Allergen	Similar identity matches were found by BLASTP to triosephosphate isomerase of house dust mites and scabies. However, there is not clear published proof that this protein is cross-reactive.
NCBI BLASTP Toxin or Toxic	Moderately high scoring alignments were identified using toxin and toxic keywords. The matches with *E* scores of 2e-55 to 7e-65 and identities of 40% to 50% were found to homologous proteins from bacteria (e.g. *Escherichia coli, Bordetella sp., Clostridium sp.).* No published evidence was found that these proteins are toxins.
NCBI BLASTP No keyword	Many very high scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 1e-128 and identity of 71% over 248 AA.
Conclusion: It is unlikely that there is any risk of allergy for this protein. The same is true for toxicity. In addition, there are very good alignments to homologous proteins from many molds including *Saccaromyces sp.* Thus it is very unlikely that this protein is an allergen or a toxin.	

Table 17. Bioinformatics Summary Results of Protein Band #9a. The 161 AA hypothetical protein (cyclophilin superfamily) was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Seven relatively high-scoring alignments were identified with *E* scores smaller than 1e-46 to cyclophilin proteins of diverse fungal, house dust mite and plant sources (*Aspergillus sp, Dermatophagoides sp, Caharanthus, Daucus sp.*). Yet cyclophilins are ubiquitous and while there is evidence of *in vitro* cross-reactivity, there is no published evidence for such wide-spread clinical cross-reactivity. Thus these alignments are unlikely to represent a risk of possible clinical cross-reactivity.
AllergenOnline 80mer FASTA3	Seven proteins were identified as having alignments with >35% identity in any 80 AA match with AllergenOnline version 16. The highest alignment was 87% and all had 80 identities greater than 78%.
AllergenOnline Exact 8 AA	All of the same proteins had 8 AA matches in AllergenOnline version 16.
NCBI BLASTP Allergen	A few cyclophilins were identified with high matches of diverse sources (e.g. mold, *Aspergillus fumigatus* and *Malazessia furfur*; mites, *Suidasia medanensis* and *Dermatophagoides farina;* liver fluke, *Clonorchis sinensis*, . However, there is not clear published proof that these proteins are clinically cross-reactive from such diverse sources.
NCBI BLASTP Toxin or Toxic	Modest scoring alignments were identified using toxin and toxic keywords. The matches with *E* scores of 3e-32 or larger and identity scores of approximately 40% to 50% identities to homologous proteins from bacteria (e.g. *Enterococcus sp., Legionella sp., Corynebacterium sp.*, also with some unusual species in terms of toxicity, such as *Danio rerio).* No published evidence was found that these proteins are toxins.
NCBI BLASTP No keyword	Many higher scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 9e-88 and identity of 75% over 162 AA.
Conclusion: Cyclophilins are unlikely to pose a risk of allergy. While a few cyclophilins have been identified as putative allergens, and they have been demonstrated to bind IgE from some subjects, the proteins are intracellular in function and ubiquitous. The closest hit is to *Aspergillus* allergen. *Aspergillus oyzae* is widely consumed in miso, tamari, and soy sauce and have versions of cyclophilins that are closer to the allergenic ones than is the *Pichia* protein. *Aspergillus* is in the fungal subdivision *Pezizomycetes*, which is distinct from the subdivision *Saccharomycotina*, which contains *Pichia*. Several members	

of *Pezizomycetes* are widely eaten (e.g. morels) and have versions of cyclophilins that are closer to the allergenic ones than is the *Pichia* protein. The lack of cross-reactivity upon consumption of morels, miso, tamari, and soy sauce establishes it as highly unlikely that the *Pichia* cyclophilin will elicit cross-reactivity. Similarly, several members of the *Saccharomycotina* (including *S. cerevisiae*), whose cyclophilin's are more closely related to the allergenic ones as is *Pichia* are widely eaten without cross-reactivity. The same is true for toxicity. In addition, there are very good alignments to homologous proteins from many molds including *Saccharomyces sp.* Thus it is very unlikely that this protein is an allergen or a toxin.

Table 18. Bioinformatics Summary Results of Protein Band #9b. The 154 AA cytosolic superoxide dismutase protein was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Twenty three isoforms of olive pollen superoxide dismutase in AllergenOnline version 16 were aligned with nearly identical scores relative to this *P. pastoris* protein. They ranged from E score of 3.9e-30 to 8.6e-35 and identity scores of 53% to 57%. The next highest scoring superoxide dismutases were *E* scores of 3.5 to 6.5 and identities of 27% to 45% over 30 to 58 AA. Butteroni et al. (2005) demonstrated IgE binding using sera from a large number of olive pollen allergic subjects, but there are no publications demonstrating biological activity (e.g. BHR, SPT).
AllergenOnline 80mer FASTA3	Twenty three isoforms of olive pollen superoxide dismutase were identified as having matches with >35% identity over 80 or more amino acids. The olive pollen proteins are almost identical and the identities range from 55% to 60% over the best 80 aa alignment.
AllergenOnline Exact 8 AA	All of the same 23 proteins had 8 AA matches in AllergenOnline version 16.
NCBI BLASTP Allergen	Multiple isoforms of olive pollen (Ole e 5) were identified as having identity matches with *E* scores ranging from 3e-55 to 9e-55 and approximately 57% identity over 152 AA. As noted above, there is evidence of IgE binding to the olive pollen MnSOD protein using olive-pollen allergic subjects, but no demonstration of direct biological reactivity.
NCBI BLASTP Toxin or Toxic	Modest scoring alignments were identified using toxin and toxic keywords. The matches with *E* scores of 2e-8 or larger and identity scores of approximately 30-34% identities to homologous proteins from bacteria (e.g. *Clostridium sp., Corynebacterium sp.*, using the keyword toxin. High sequence identities (E scores smaller than 1e-80 and >70% identity) to a wide variety of fungal MnSOD proteins were identified using the term "toxic". However the linkage appears to be due to the ability of the MnSODs in a wide array of organisms to de-toxify free radicals. No published evidence was found that these proteins are toxins.
NCBI BLASTP No keyword	High scoring identity matches with homologous proteins from other yeasts and molds were identified. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 5e-81 and identity of 76% over 152 AA.

Conclusion: Superoxide dismutase of *Pichia pastoris* is unlikely to pose a risk of allergy. While a few superoxide dismutase proteins (MnSOD) have been identified as putative allergens, and they have been demonstrated to bind IgE from some subjects, the proteins are intracellular in function and ubiquitous. There is not evidence of wide-spread cross-reactivity shared by MnSOD related proteins that corresponds to clinical reactivity. The same is true for toxicity, no evidence of toxicity associated with MnSODs. In addition, there are very good alignments to homologous proteins from many molds including *Saccharomyces sp.* Thus it is very unlikely that this protein is an allergen or a toxin.

Table 19. Bioinformatics Summary Results of Protein Band #10. The 84 AA hypothetical protein (mitochondrial ATPase inhibitor) was compared with AllergenOnline.org version 16 and with NCBI Protein on 17 May, 2016.

AllergenOnline overall FASTA3	Relatively low-scoring alignments were identified with *E* scores between 0.1 and 10 and identities of 19-30% that were primarily identified to the muscle protein, tropomyosins. The alignments are clearly poor and do not represent conservation of homology. Thus these alignments are unlikely to represent a risk of possible clinical cross-reactivity.
AllergenOnline 80mer FASTA3	No matches were identified with >35% identity in any 80 AA match with AllergenOnline version 16.
AllergenOnline Exact 8 AA	All of the same proteins had 8 AA matches in AllergenOnline version 16.
NCBI BLASTP Allergen	No significant alignments were identified.
NCBI BLASTP Toxin or Toxic	No significant alignments were identified.
NCBI BLASTP No keyword	A few significant alignments were found for yeasts and molds. The highest scoring alignment with a *Saccharomyces sp.* was with an *E* score of 3e-20 and identity of 49% over 74 AA.
Conclusion: The ATPase inhibitors do not appear to be allergens or toxins. The highest identity score to *Saccharomyces sp.*, was with an E score of 3e-20 and 49% identity over 69 AA. Thus it is very unlikely that this protein is an allergen or a toxin.	

4.3 Bioinformatics summary for the seventeen proteins from *Pichia pastoris*. None of the results from the bioinformatics searches of the *Pichia pastoris* (*Komagataella phaffii*) proteins identified by Impossible Foods from their production batch of Soy Leghemoglobin Preparation suggest that these proteins would present an important risk of allergy or toxicity to consumers. At least not beyond the potential risks of inhalation allergy for those with allergies to a variety of yeasts or molds. The sequence identities are quite similar to proteins from *Saccharomyces cerevisiae, Candida albicans* and other common molds or other organisms commonly encountered in the environment or in foods. Some of the common intracellular enzymes identified as putative allergens from a few organisms are highly conserved across broad taxonomic categories without evidence of shared allergic reactivity. The evidence that the specific sequence matched "allergens" cause allergies is quite weak. The identity matches suggested as significant in this study based on small *E* score and moderate to high percent identities to allergens are to proteins with limited demonstration of risk of allergy based on referenced publications in AllergenOnline.org, version 16. It is important to note that any further testing to evaluate potential allergenicity would require the identification of subjects who are specifically allergic to sources of sequence matched proteins, and have IgE that binds specifically to the matched allergen from the source. That is quite challenging, especially for minor allergens. And even if willing serum donors can be identified, few are mono-sensitized to a given allergenic source, and few IgE binding tests are 100% definitive. What is clear is that no unique risks of allergy were identified. In addition, the identity matches of significance to proteins using keywords toxin and toxic are primarily because the proteins are from toxic sources. There was no clear evidence that any of the bioinformatics matches were to proteins that are demonstrated to be toxic to humans or other mammals.

5.0 Conclusions

Bioinformatics analyses were performed on proteins from *Pichia pastoris* that were identified as residual proteins from the heterologous expression system using this yeast to express the LegHb protein from soybean (*Glycine max*) for food use. The purpose of this evaluation was to determine whether there might be some safety concerns for foods produced with these proteins included as ingredients.

Based on the evidence and my knowledge of cross-reactive IgE binding, there is not a scientifically justifiable reason to perform serum IgE binding studies with the *Pichia pastoris* proteins that were identified. There is no "at-risk" population of

allergic subjects that could be identified to evaluate potential cross-reactivity and risks of allergy or allergenic cross-reactivity are low (Goodman, 2008). And there are no other tests such as animal model tests that would be predictive of the risks of food allergy to any consumed protein. The science supports the introduction of food products made with the heterologously expressed LegHb, including minor components from *Pichia pastoris*, to be used as food.

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APPENDIX 1. AllergenOnline.org Database version 16, January 2016 (105 pages)

Annex 10

STUDY TITLE

Soy Leghemoglobin Preparation: *in vitro* digestibility study in human simulated gastric fluid (pH 2.0) at two different pepsin-protein ratios, 10 units per µg and 1 unit per µg

AUTHORS

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STUDY COMPLETED ON

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REG 2016-Pepsin LegHb

Statement of No Data Confidentiality

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA Section 10(d)(l)(A), (B), or (C) and which pertains to a registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA section 10(g).

Company

Company Agent:

(b) (6)

Rachel Fraser, PhD
Impossible Foods, Inc.

10/13/16
Date

These Data May Be Considered Confidential In Countries Outside The United States.

Good Laboratory Practice Compliance Statement

This study was not conducted and reported in compliance with the requirements of the Good Laboratory Practice Standards (40 CFR Part 160) of the Code of Federal Regulations of the United States of America. This is a characterization assessment of the similarity of the introduced proteins to known and putative allergens based on source of the genes and the sequences of the proteins. There is no test system. However, raw data including PubMed searches and bioinformatics comparisons were archived in PDF format in the Authors laboratory with a copy given to the sponsor.

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Study Number:	REG - 2016 – Pepsin LegHb
Title:	Leghemoglobin: *in vitro* digestibility study in human simulated gastric fluid (pH 2.0) at two different pepsin-protein ratios, 10 units per µg and 1 unit per µg
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Study Start Date:	1 March, 2016
Study Completion Date:	10 October, 2016
Records Retention:	All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.

Signature of Final Report Approval:

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10 October, 2016

Principal Investigator: Richard E. Goodman — Date

SUMMARY

The Soy Leghemoglobin (LegHb) protein used in this study was produced in *Pichia pastoris* and supplied by Impossible Foods, Inc. of Redwood City, CA. The gene was originally derived from soybean (*Glycine max*) and encodes the 145 amino acid protein sequence listed as Accession number P02236 in the UniProt protein database. The Soy Leghemoglobin Preparation test material was supplied as a liquid protein solution by the study sponsor (Impossible Foods, Inc.) with assurance of the soy leghemoglobin protein identity and purity being approximately 66%, with the remaining 34% of proteins from the host, *Pichia pastoris* (synonym *Komagataella pastoris*).

The Soy Leghemoglobin Preparation was supplied as a concentrated aqueous solution and was subjected to digestion in pepsin based on the protocol in Thomas *et al.* (2004), as refined by Ofori-Anti et al., 2008 with minor modifications. The time to reach 90% digestion of the protein by pepsin was estimated as the first sample time having less than 10% residual protein primary protein compared to diluted non-digested sample protein. The ability of the assay to detect 10% residual protein was determined prior to the digestion tests using serial dilutions of the test protein in a similar SDS-PAGE, Coomassie blue staining to ensure that a residual of 10% undigested control sample detectable under the conditions used for the study. The primary LegHb band migrated at ~ 13 kDa in SDS-PAGE. Pepsin was diluted in simulated gastric fluid (SGF) with the pH adjusted to 2.0. The pepsin solution was tested for proteolytic activity by digestion of hemoglobin within 24 hours of each assay day. The mass ratio of pepsin to LegHb preparation was adjusted to achieve ~ 10 units of pepsin activity per microgram of total protein in solution. An additional assay was performed with the ratio of 1 unit of pepsin activity per microgram of test proteins. Digestions were performed at 37°C under timed conditions. Samples of the digestion mixtures were removed and neutralized at various time points from 30 seconds to 60 minutes and samples of each were electrophoresed in SDS-PAGE and stained with Coomassie blue to evaluate digestion completeness.

The results of this study demonstrated that the *P. pastoris*-produced LegHb protein and the *Pichia* host proteins within the Soy Leghemoglobin Preparation were rapidly digested in pepsin at pH 2.0 at both ratio of 1 µg in 10 units (as per standard protocol) and 1 µg in 1 unit pepsin activity (as an experimental protocol). The SDS-PAGE Coomassie blue gel staining method demonstrated that more than 90% of the *P. pastoris*-produced LegHb protein and the *Pichia* host proteins were digested in less than 2 minutes in replicate assays. No degradation bands were found to result from digestion of the LegHb protein or the *Pichia* proteins. Therefore, our conclusion is that the *P. pastoris* produced LegHb and the *Pichia* host proteins are rapidly digested at both ratio of 1 µg in 10 units and 1 µg in 1 unit activity of pepsin at pH 2 and that no pepsin-stable fragments were identified in the assay. Based on Codex (2003) guidelines for the allergenicity assessment, there is no added concern of risk based on stability of this LegHb and *Pichia* protein preparation in pepsin.

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Figures

Abbreviations

aa	amino acid
$A_{280\ nm}$	Absorbance of light at a wavelength of 280 nm
BSA	Bovine serum albumin
CFR	Code of Federal Regulations
D0-60	Digestion samples (hemoglobin plus pepsin) from time 0 (quenched prior to digestion) to time 60 min
E0	Experimental control pepsin without the hemoglobin, time 0
E60	Experimental control pepsin without the hemoglobin, 60 min
Hb	Bovine blood hemoglobin
kDa	kilodalton
LegHb	Leghemoglobin from *Glycine max* (Soy), *Pichia pastoris* produced
LOD	Lower limit of detection
ma	milliampere
mg	milligram
ml	milliliter
mM	millimolar
µl	microliter
na	Not applicable
ng	nanogram
OVA	Ovalbumin
P0	Experimental control protein without pepsin, time 0
P60	Experimental control protein without pepsin, 60 min
PAGE	Polyacrylamide gel electrophoresis
P1/10	Experimental control protein at 10% loading
R^2	Square of the correlation coefficient
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid (without pepsin)
SOP	Standard operating procedure
T	Time point
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
v/v	solute volume to solution volume
w/v	solute weight to solution volume

1. Introduction

Impossible Foods, Inc. of Redwood City, CA is developing a potential food product that contains a hemoglobin protein from soybean (*Glycine max*), called Soy Leghemoglobin (LegHb), as the primary protein ingredient. Impossible Foods, Inc. sponsored tests and an evaluation of the potential allergenicity of the Soy Leghemoglobin preparation, which contains Soy Leghemoglobin protein and proteins from the *Pichia pastoris* production host, in order to consider whether there is a risk of food allergy associated with consumption of the proteins. This report describes the rationale, test methods for testing the protein and results from an *in vitro* digestion assay intended to provide data relative to potential risks of food safety.

The *Codex Alimentarius* Commission guidelines for assessing the allergenicity of GM plants (2003) recommends assessing the introduced protein for stability in pepsin at acidic pH using standard conditions as an assay to help evaluate whether the introduced protein is likely to either increase the rate of sensitization to the host crop, or increase the likelihood of eliciting an allergic response in food allergic consumers. The pepsin stability assay is one study in a weight of evidence approach intended to assess the potential allergenicity of genetically modified crops (Codex, 2003). The test method for the assessment was first described by Astwood *et al.* (1996). The assay is not meant to predict whether a given protein will always be digested in the stomach of the human consumer, but the assay does provide a simple *in vitro* correlation to evaluate protein digestibility. Investigation of proteins that have been tested suggest a marked positive predictive value that food allergens causing systemic reactions are relatively stable in the assay, while non-allergenic food proteins are typically digested relatively quickly (Bannon *et al.*, 2002). Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in nine laboratories (Thomas *et al.*, 2004). Porcine pepsin is an aspartic endopeptidase with broad substrate specificity. Pepsin is optimally active between pH 1.2 and 2.0, but markedly less active at pH 3.5 and irreversibly denatured at pH 7.0 (Collins and Fine, 1981; Crevieu-Gabriel *et al.*, 1999). The assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. An additional assay was performed using 1 unit of activity per microgram, which is one-tenth the published standard activity ratio. A relatively pure form of pepsin was used for this assay from Worthington Biochemical Co., pepsin A, product LS003319.

The original assay described by Astwood *et al.* (1996) recommended performing the digestion at pH 1.2, however, the FAO/WHO (2001) suggested using two pH conditions (pH 1.2 and pH 2.0). In comparing pH 2.0 vs. pH 1.2, Thomas *et al.* (2004) showed that protein digestion at pH 2.0 resulted in slightly slower rates of full-length protein and fragment degradation, but did not alter the overall sensitivity of a protein to digestion. Results at pH 1.2 were more consistent than at pH 2.0, with 91% and 77% agreement between laboratories, respectively. However, more recently, we have digested a number of proteins at both pH 1.2 and 2.0 and have did not

demonstrate significant differences (Ofori –Anti et al., 2008). Therefore in this study we only evaluated stability of the protein at pH 2.0.

The digestion was performed at 37°C and samples are removed at specific times and the activity of pepsin is quenched by neutralization with carbonate buffer and Laemmli loading buffer, then heating to more than 85°C for 10 minutes. The timed digestion samples are separated by SDS-PAGE and stained with Coomassie blue to evaluate the extent of digestion. A review of the digestibility assay by Bannon *et al.* (2002) and by Thomas *et al.* (2004) indicates that most of the non-allergenic food proteins that have been tested are digested in around 30 seconds, while many major food allergens are stable, or produce pepsin-stable fragments that are visible for eight to 60 minutes in this assay.

Assay parameters used in this study included verification of pepsin activity, established limit of detection of the protein in the stained gel (at 10% total stainable protein) and use of an objective measurement of the time of digestion required to reach 90% digestion as described by Ofori-Anti et al. (2008). The activity of the pepsin in SGF was tested on each day of assay based on digestion of bovine hemoglobin, as described by Worthington, to ensure that it is within a tolerance interval reported by Worthington for that lot of enzyme. The results of our activity assay did not exactly duplicate the labeled activity determined by Worthington for the lot, but did fall within the acceptance criterion of the Worthington certified activity, plus or minus 1,000 activity units per mg of pepsin. A second important criterion included in our standard operating procedure (SOP) is an objective measured level of residual test protein (LegHb in this case) that must be reached in determining the time of digestion. We defined the time of digestion required to achieve 90% reduction in stained band intensity as the time-point when the residual is less than or equal to 10% of the amount of test protein in the initial sample. To accomplish that a dilution series of test protein is tested in the same SDS-PAGE and colloidal blue staining system as the digests are analyzed with to evaluate a limit of detection (LOD). The LOD must be lower than 10% to perform this assay. The analytical gel for the pepsin digests includes a 10% test protein sample mixed with quenched pepsin (high pH, to avoid digestion). Details and results of the study are reported here.

2. Materials

2.1 Test Substance

The test substance for this study was Soy Leghemoglobin Preparation, which contains leghemoglobin from soybean (*Glycine max)* expressed recombinantly in *Pichia pastoris* (strain MXY0291). The sample was provided by Impossible Foods' from the production run PP-PGM2-15-320-101. The protein sample was in solution in a 50 ml screw cap disposable polypropylene centrifuge tube, shipped on ice packs. The total protein was labeled as 79 mg/ml and the Soy Leghemoglobin composed 66% of total protein calculated by HPLC, according to the certificate of analysis. The buffer indicated by Impossible Foods was 200 mM NaCl. The concentration was evaluated in our lab

using GE 2D Quant assay and determined to be 79.94 mg/ml. Although the predicted molecular weight of LegHb is 15.5 kDa, LegHb migrates at ~13 kDa on SDS-PAGE. Impossible Foods has measured the intact mass of LegHb using mass spectrometry and confirmed that *Pichia pastoris* expresses the full-length form of the protein excluding the N-terminal methionine. Exclusion of the N-terminal methionine is common in microbial protein expression and does not affect protein function. The solution was aliquoted and stored at -20 °C.

2.2 Control Substance

The control substances for this study were bovine hemoglobin, bovine serum albumin (BSA) and chicken ovalbumin (OVA). Each was tested in separate digestion assays to demonstrate the validity based on previous tests and results. The control substance tests were performed prior to the testing the samples.

2.3 Reference Substance

There was no reference substance for this study. Analytical reference standards (e.g., molecular weight markers) used in this study were documented in the data and are described in this report.

2.4 Characterization of Test, Control, and Reference Substances

Characterization of the Soy Leghemoglobin Preparation PP-PGM2-15-320-101 was the responsibility of Impossible Foods, Inc. Impossible Foods shared the molecular weight and the protein (amino acid sequence) with us prior to the study, which was important in analyzing results.

2.5 Critical Analytical Reagents

- Pepsin A, Worthington Biochemical Corporation, product #3319, lot #35B15585, certified as having 2,810 activity units per mg solid
- SGF without pepsin: A 35 mM NaCl solution is adjusted in pH to 2.0 as measured with a calibrated pH meter, using 6.1 N HCl.
- SGF plus pepsin 4000 U: Dissolved the mass of powdered pepsin in SGF to achieve a final activity of 4,000 units per 1.52 mL of SGF, based on the activity units from Worthington, which is 10 units activity per 1 µg of tested protein.
- SGF plus pepsin 400 U: Dissolved the mass of powdered pepsin in SGF to achieve a final activity of 400 units per 1.52 mL of SGF, based on the activity units from Worthington, which is 1 unit activity per 1 µg of tested protein.
- Bovine Serum Albumin (BSA) from Sigma Chemical Co., product #A9647-100G, lot #SLBP1123V.
- Ovalbumin (OVA), from Worthington Biochemical Corporation, product #3054, lot #52P13864.

- Hemoglobin (Hb) from bovine blood, Sigma Chemical Co., product #H2625 -25 G, lot #SLBD9300V is used to test protein pepsin activity and pepsin digestibility.
- Limit of detection determination diluent: Mixed 40 ml of SGF, pH 2.0 with 14.7 ml of carbonate buffer, pH 11.0. $NaHCO_3$, Fisher Scientific, cat #S78284, lot #AD-10033-32.
- Pepsin quenching solution: 200 mM $NaHCO_3$, pH 11
- 6X Laemmli buffer, Boston BioProducts, CAS #BP-111NB, lot #J20Z4R.
- β-mercaptoethanol, BioRad #161-0710, lot #210009868
- Precision Plus Protein™ Dual Xtra Standards from BIO-RAD, product #161-0377, control #64046347
- Novex 10-20% tris-glycine polyacrylamide gels, 1.5 mm thick, 15 wells (Invitrogen EC61385BOX), lot #16022941.
- Tris-Glycine-SDS 10 x running buffer, cat #BP1341-4L, lot #153375.
- BIO-RAD Coomassie Brilliant Blue R-250 staining solution, cat #161-0436, control #200005684.
- BIO-RAD Coomassie Brilliant Blue R-250 destaining solution, cat #161-0438, control #210012192.

3. Test System.

The test system for this study was an *in vitro* digestion model using pepsin in simulated gastric fluid (SGF). Standard Operating Procedures (SOPs) for preparation of the SGF, determination of the detection limit assay, pepsin activity assay, digestion assay, SDS-PAGE and gel staining are on record in the laboratory. The SGF preparation and digestion procedures were based on the methods described by Thomas *et al.* (2004) as modified by Ofori-Anti et al., (2008).

The pepsin activity assay was based on the method described by Worthington for determining the activity of pepsin. An appropriate mass of pepsin powder was dissolved in prepared SGF, pH 2.0 to provide 0.9 mg/ml as a 30 x stock, which was then diluted to 1 x with SGF. Acidified bovine hemoglobin (2% mass to volume) was prepared and digestions to evaluate the labeled pepsin activity were performed in triplicate (1.25 ml per tube).

The amount of pepsin powder used to prepare SGF was calculated from the specific activity labeled on the product as 2,810 units /mg solid pepsin product. One unit activity is defined as a change in $A_{280\,nm}$ of 0.001 at 37 °C, measured as trichloracetic acid (TCA)-soluble products using bovine hemoglobin as the substrate. The assay was designed for fixed volumes and a fixed amount of test protein so the amount of pepsin diluted in SGF is adjusted to provide the appropriate ratio of 10 units of pepsin activity per microgram of test protein in the digestion mixture. The appropriate amount of solid pepsin was added to SGF to provide 4,000 units (for 10 units per microgram test protein) and 400 units (for 1 unit per microgram test protein) per

1.52 mL of SGF, respectively. The pepsin/SGF reaction mixture was preheated to 37°C in a water bath before adding 80 microliters of test protein (5 mg/mL) for a total volume of 1.6 mL, providing 10 units and 1 unit per microgram test protein, respectively.

Once the pre-heated (37°C) test protein solution was mixed with pre-heated pepsin-SGF, equal volume samples were withdrawn at predetermined times (between 0.5 and 60 minutes) and added to sample tubes containing neutralization (carbonate buffer, pH 11) and denaturing reagents (reducing Laemmli buffer) and immediately heated to 95°C, which stopped the digestion. Samples were then cooled in an ice-bath and then heated to > 85°C before running in SDS-PAGE. All samples from a single digestion were applied to wells of the same SDS-PAGE gel along with molecular weight markers, undigested test protein equivalent to the initial undigested test protein sample and a 10% test protein sample and pepsin alone (to assess pepsin stainable protein bands). Samples were separated by electrophoresis, stained with Coomassie Brilliant Blue R-250 solution (at least 6 hours), destained in R-250 destaining solution and water, and the stained gels captured using a Kodak Gel Logic 440 system (Carestream, Rochester, NY). The stability of the protein was defined as the time required to achieve 90% digestion, which was estimated based on the shortest time-digested sample with a band intensity equal to, or less than the 10% undigested standard well (P1/10). Any new bands above approximately 3,000 MW, which were generated as intermediate products of digestion, were noted as stable (or partially stable) intermediate proteolytic fragments and were considered based on stability. If those bands were also in the pepsin only controls (time 0 and time 60 mins), they were discounted as being from pepsin. Otherwise they would be analyzed by proteomic methods to determine whether they were fragments of the test protein.

Proteins with more than 10% stainable full-length protein band remaining at 60 minutes were considered stable. Proteins reduced to < 10% stainable band at 5 to 30 minutes were considered of intermediate stability. Proteins reduced to < 10% stainable band by 2 minutes were considered labile (rapidly digested).

3.1 Justification for Selection of the Test System

In vitro digestion models are used commonly to assess the digestibility of ingested substances. Previous studies have used this simple, *in vitro* assay to evaluate potential risk of food allergy, and demonstrated that stability in pepsin is a risk factor for food allergy, which might be related to initial sensitization or to elicitation once the individual is sensitized (Astwood *et al.*, 1996 and del Val *et al.*, 1999). The FAO/WHO (2001) suggested conducting the pepsin digestion assay at pH 1.2 and pH 2.0. We have performed additional independent tests showing results were quite similar for most test proteins using pH 1.2 or 2.0 (Ofori-Ant *et al.*, 2008). In this analysis, digestion was performed at pH 2.0 as a conservative approach as some authors have claimed a lack of predictive value for the digestion assay in pepsin at pH 1.2 (Fu *et al.*, 2002; Yagami *et al.*, 2000). However, Bannon *et al.* (2002) reviewed a broad range of published representative

pepsin digestion studies and found a strong positive predictive value when comparing the stability of allergenic and non-allergenic dietary proteins. As defined by Codex (2003), this assay, which measures the resistance of a test protein to proteolysis in a test tube assay. It is not meant to be a stand-alone determinant in evaluating the potential allergenicity of proteins introduced into GM crops and is not intended to predict the fate of proteins in the digestive tract of consumers. The results are to be judged in a weight of evidence approach which should also include history of safe use, sequence identity matches to known allergens and abundance of the protein in food material.

3.2 Experimental Controls

Controls in this study were meant to ensure assay reliability and include:

- Measurement of the activity of pepsin in SGF.
- Evaluation of the sensitivity of the staining properties of the test protein from serially diluted samples, in a separate, but similar SDS-PAGE gel.
- Inclusion of samples of pepsin without test protein at times zero and 60 minutes to determine whether any stainable protein bands observed in digestion samples with test protein are from the test protein, contaminants in pepsin or from pepsin autocatalysis.
- Inclusion of protein in SGF without pepsin at times zero and over 60 minutes to evaluate the effect of acid and heat alone.

3.3 Sample Retention

Samples of test protein and digested samples were numbered to distinguish assay time points and assay replicates by date. Residual samples were stored at -20°C and will be discarded approximately six months after the completion of the study.

4. Detailed Study Methods. This study evaluated the stability of recombinant leghemoglobin from Glycine *max* in pepsin at pH 2.0. A number of control steps were performed to ensure study validity. A detailed description of the study is presented here. Laboratory records and protocols are on file in the Goodman laboratory, Dept. of Food Science & Technology, University of Nebraska, Lincoln, USA.

4.1 Verification of Detection System Specificity and Sensitivity. A dilution series of sample was prepared with sample quantities loaded in SDS-PAGE gel using 1 x reducing Laemmli buffer, covering the range representing 200% total protein per well (296 µg/ml of total protein) down to 2.5 % (3.7 µg/ml of total protein. Bio-Rad precision plus protein MW markers were applied to separate lanes. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue for at least 2 hours. The gels were destained 3 times with destaining solution and water until the background was clear. The image was captured using the Kodak Gel Logic 440 Image Station.

4.2 **Preparation of SGF Plus Pepsin.** The simulated gastric fluid (SGF) reaction buffer was prepared by adding 122.8 mg of NaCl to 59.94 mL of distilled water. The pH of the solution was adjusted to pH 2.0 using approximately 60 µl of 6.1 N HCl and water. The HCl content was approximately 0.084 N, and the salt concentration was 35 mM NaCl. The certified activity of pepsin A from Worthington was used to calculate the amount of solid pepsin that was dissolved in 1.52 mL of SGF. For this lot, the certified value was 2810 units per mg of pepsin solid material. The first target was 4,000 units of activity per 1.52 ml solution which is 10 units pepsin activity per 1 µg tested protein. Based on the Worthington analysis, the concentration of pepsin A used in the assay was 0.93 mg/ml, which is 0.093 g of solid pepsin adding to 100 ml of SGF. The second target was 400 units of activity per 1.52 ml solution which is 1 unit activity per 1 µg tested protein. The concentration used was 0.093 mg/ml, which is 10 dilution of the previously made solution with SGF. After thoroughly dissolved and mixed, the pepsin solutions were stored at 4°C and assayed for activity and used within 24 hours.

4.3 **Pepsin Activity Assay.** Each time SGF plus pepsin was prepared for a digestion assay; the activity of the pepsin and the digestion assay were both completed within 24 hours. The purpose of performing the activity assay was to ensure that the pepsin was active within a pre-defined range around the certified claim of activity by Worthington. This product has a labeled activity of 2,810 units per mg of solid material. The activity assay we used was similar, but not identical to that used by Worthington. The tolerance was +/- 23% of the target units per mg compared to the Worthington certified claim. The SGF plus pepsin was freshly prepared and stored at 4°C just before use, and then warmed to 37°C before the addition of the target protein. The procedure was performed as follows:

4.3.1 A solution of 2% acidified bovine hemoglobin (Hb) was prepared by dissolving 0.5 g of hemoglobin (Sigma # H2625) in 20 mL of distilled water, then mixing with 5 mL of 300 mM HCl.

4.3.2 Three polypropylene screw-top centrifuge tubes were labeled as Test (#1-3), three were labeled as Blank (#1-3), each received 1.25 mL of 2% acidified Hb and all were preheated to 37°C for 10 min.

4.3.3 At a timed interval (~ 1 min.), each of the test tubes in turn received 0.25 mL of SGF plus pepsin, was mixed by gentle vortex and returned to the incubator. As each test tube reached 10 min. incubation time, 2.5 mL of 5% TCA (Sigma 6.1 N product T0699, diluted 1:20 with distilled water) was added to stop the reaction, the tube was mixed briefly by multiple inversion and then placed on ice to cool down. Then insoluble material (undigested hemoglobin) was removed using syringes (LuerLok BD 309646, 5 ml) and syringe filters (Corning Incorporated, 0.45 µm PTFE, product #431220).

4.3.4 Blank tubes were interspersed with the Test tubes. Blank tubes (with 1.25 mL of Hb) received 2.5 mL of 5% TCA, multiple inversion, then 0.25 mL of SGF plus

pepsin. After 10 min incubation time, these tubes were also placed on ice and then filtered to remove insoluble material.

4.3.5 The absorbance at 280 nm was measured on a spectrophotometer (Spectronic Genesys 5, MILTON ROY). The activity units of pepsin per mL were calculated as the mean net absorbance (A280 nm Hb – A280 controls) multiplied by a conversion factor of 1,000 to yield units of activity per mg of solid pepsin.

4.4 Control Protein Digestions (Hemoglobin, BSA and OVA). Bovine hemoglobin, bovine serum albumin (BSA) and chicken ovalbumin (OVA) digestion assays were tested as control proteins to verify the appropriate activity of the test system.

4.5 Test Protein Digestion. The Soy Leghemoglobin (LegHb) concentration within the Soy Leghemoglobin Preparation was estimated by Impossible Foods as 79 mg/ml of total protein containing 66% LegHb, and was measured as 79.94 mg/ml of total protein in our laboratory. We have used 79.94 mg/ml value for calculating concentrations. Protein solutions were aliquoted and kept at −20 °C until immediately before use.

4.5.1 Sample tube preparation. 1.5 mL centrifuge tubes were labeled as P1/10, P0, P60, D0, D0.5, D2, D5, D10, D20, D30, D60, E0, E60.

4.5.2 70 µL of pepsin quenching solution (carbonate buffer) and 70 µL of 5X Laemmli, reducing buffer were added to each tube in 4.4.1.

4.5.3 An aliquot of hemoglobin in a tube labeled as P, was prepared.

4.5.3 $P_{1/10}$: 190 µL of SGF plus pepsin was added, quick heated at 85°C, then 10 µL 1/10 diluted hemoglobin solution was added. Solution was vortexed and then heated at 85°C for 10 min.

4.5.4 Label a tube P_{mx} (no pepsin, protein control): 80 µL out of tube P and then 1.52 mL SGF were added and mixed.

4.5.4.1 Immediately 200 µL into the P0 tube were removed, mixed and heated at 85°C for 10 min.

4.5.4.2 After 60 minutes at 37°C water bath, 200 µL into the P60 tube were removed, mixed and heated at 85°C for 10min.

4.5.5 Label a tube E_{mx} (pepsin enzyme, no protein control): 80 µL distilled water were added to 1.52 mL SGF plus pepsin, and then were mixed.

4.5.5.1 Immediately 200 µL into the E0 tube were removed, mixed and heated at 85°C for 10 min.

4.4.5.2 After 60 minutes at 37°C water bath, 200 µL into the E60 tube were removed, mixed and heated at 85°C for 10 min.

4.5.6 Label a tube D_{mx} (digestion mixture): 80 µL out of tube P was added to 1.52 mL SGF plus pepsin and mixed, then placed in 37°C water bath.

4.5.6.1 At 0.5, 2, 5, 10, 20, 30, 60 min internals, 200 µL of digestion mixture were withdrawn into D0.5, D2, D5, D10, D20, D30, D60 quenching tubes. (e.g.

D0.5 at 30 sec., D2 at 2 min), each sample tube was heated to 85°C for 10 min.

4.5.7 P_0: 190 µL of SGF plus pepsin was added, quick heated at 85°C, then 10 µL out of tube P was added. Solution was vortexed and then heated at 85°C for 10 min.

4.6 SDS-PAGE Gel. All samples on any one gel were from a single digestion experiment. Novex 10-20% tris-glycine gels were used with SDS-PAGE buffer.

4.6.1 10 µL of each sample tube was loaded per well, containing 1.47 µg of starting LegHb per well except in wells for $P_{1/10}$ tube which was 0.147 µg.

4.6.2 4 µL of pre-stained precision plus protein™ Dual Xtra Standards molecular weight marker proteins were loaded in the outer two wells.

4.6.3 Electrophoresis was accomplished at a constant 150 voltage.

4.6.4 Gels for staining were stained for a minimum of 6 hours in Coomassie Brilliant Blue as detailed by Bio-Rad, then destained for at least 30 min in destaining solution and water.

4.7 Image Analysis. The destained gels were visualized in a Gel Logic 440 Image Station under white light trans-illumination. The image was captured and the image intensity adjusted to optimum background and band intensities. The raw image was saved as an archival file.

4.7.1 The molecular weight of the hemoglobin, BSA, ovalbumin, LegHb and any resulting degradation band that was not in the pepsin only lane was noted.

4.7.2 The 10% control band (P1/10) was used as the standard for comparison of all digested samples on a given gel.

4.7.3 The first time point the digested band appeared to be less than the10% concentrated sample was used to estimate the time to achieve 90% digestion.

4.8 Proteomic LC-MS Identity of Bands from *Pichia pastoris*. Impossible Foods performed LC-MS/MS analysis of 10 faint bands that were visible in Figure 10 a and b, which corresponded to proteins from *Pichia pastoris* that were ≥1% of the Soy Leghemoglobin Preparation total protein fraction. All of the *Pichia* protein bands were digested and no longer visible in Fig. 9 and 10a), demonstrating they are rapidly digested.

5. Results & Discussion

5.1 Limit of Detection. The stained gel of the dilution series of total protein (Figure 1) demonstrated a clear pattern of reduced intensity of stained bands with each step in the dilution series. The minimum amount of protein that was detected was 0.075 µg of total protein which contains 0.049 µg of LegHb. Based on these data, the limit of detection was approximately 5% of total protein which contains 3.3% as LegHb at 100% loaded in the digestion samples. This level of sensitivity was clearly sufficient to detect 10% residual of hemoglobin or any other protein in the digest.

5.2 Pepsin Activity. The certified activity of the lot of pepsin from Worthington used in this study was labeled as 2,810 units per mg of solid and was tested 2826 units per mg right before the digestion assays were performed.

5.3 Control Substance Digestion Results. Stained gels of digestion tests of control substance hemoglobin, BSA and ovalbumin (Figure 2 - 7) demonstrated that at both ratio of 10 units and 1 unit of pepsin activity per 1 μg of test protein, BSA and hemoglobin were digested rapidly within the SGF plus pepsin test system and that ovalbumin was stable with more than 10% visually stainable full-length protein band remaining at 60 minutes. However, BSA left residual pepsin resistant fragments at a low MW, which was more pronounced with a ratio of 1 unit activity per microgram of protein, compared to 10 units per microgram. At the ratio of 10 units of pepsin activity per 1 μg of test protein, OVA was mostly digested to a stable fragment just below the MW of pepsin, between 20 and 60 minutes. However, when the activity ratio was reduced to 1 unit of pepsin activity per 1 μg of test protein, OVA was markedly more stable, with little digestion. These results with 10 units with OVA and BSA are consistent with results from previous tests (Ofori-Anti, A.O. 2008), which demonstrates the reproducibility of this SGF plus pepsin test system.

5.4 Leghemoglobin Protein Digestion Results. Two representative stained gels of digestion experiments of LegHb at pH 2.0 at the ratio of 10 units and 1 unit of pepsin activity per 1 μg of test protein (Figure 8-9) demonstrated that at both ratios, the LegHb protein was stable in acid alone for 60 minutes (lane 3), but rapidly digested by pepsin in 2 minutes (lane 5) to below the visible band intensity of the quenched pepsin 10% LegHb control (P1/10 control in lane 13).

5.5 Pichia pastoris proteins. Gels in Figures 8, 9 and 10, lanes 1-3 have several faint bands showing between 10 kDa, and 250 kDa, which were identified through mass spectrometry by Impossible Foods as proteins from the host, *Pichia pastoris*. The sequences of the proteins have been evaluated by bioinformatics to evaluate sequence identity matches with known allergens and toxins. They include: alpha aminoadipate reductase (1400 aa), cobalamin-independent methionine synthase (768 aa), aconitase (780 aa), transketolase (679 aa), glycerol kinase (621 aa), catalase A (510 aa), GAPDH (504 aa), hypothetical protein PAS (525 aa), mitochondrial aldehyde dehydrogenase (501 aa), delta-aminevulinate dehydrogenase (341 aa), mitochondrial alcohol dehydrogenase III (350 aa), malate dehydrogenase (342 aa), putative protein of unknown function (328 aa), triose phosphate isomerase (248 aa), hypothetical protein-cyclophilin (161 aa), cytosolic superoxide dismutase (154 aa) and mitochondrial ATPase inhibitor (84 aa). These stained protein bands were rapidly digested to the point of being invisible at time 0.5 minutes or 2 minutes (lanes 4 and 5 in the gels shown in Figures 8, 9 and 10).

6. Conclusions

The results of this study demonstrated that the LegHb protein and the *Pichia pastoris* proteins within the Soy Leghemoglobin Preparation were rapidly digested after incubation in SGF plus pepsin at 37°C, at both ratio of 10 units and 1 unit of pepsin activity per 1 µg of total protein, both with more than 90% digested within 2 minutes based on Coomassie Blue staining detection.

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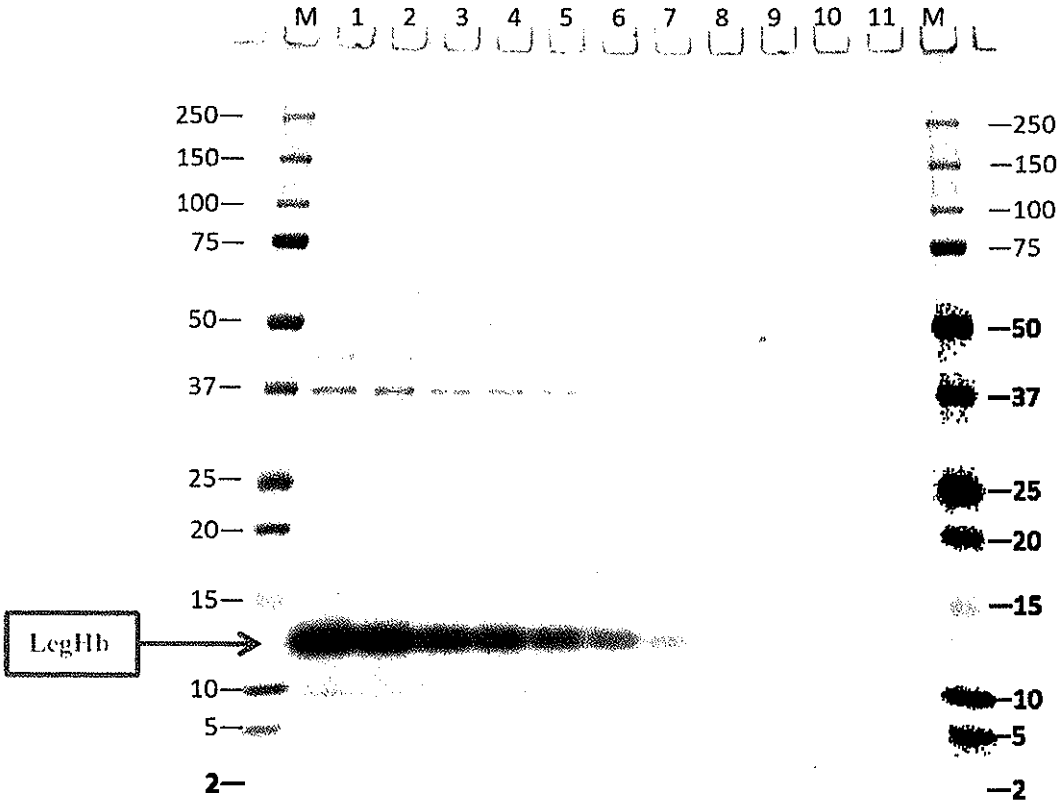

Figure 1. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Serial Dilution of Soy Leghemoglobin Preparation Starting from 200% of Total Protein. Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel.

Lane	Description	Protein Content
1	200% Total protein	2.96 µg (1.95 µg LegHb)
2	150% Total protein	2.21 µg (1.46 µg LegHb)
3	100% Total protein	1.47 µg (0.97 µg LegHb)
4	80% Total protein	1.18 µg (0.78 µg LegHb)
5	60% Total protein	0.88 µg (0.58 µg LegHb)
6	40% Total protein	0.59 µg (0.39 µg LegHb)
7	20% Total protein	0.29 µg (0.19 µg LegHb)
8	10% Total protein	0.15 µg (0.097 µg LegHb)
9	5% Total protein	0.075 µg (0.049 µg LegHb)
10	2.5% Total protein	0.037 µg (0.024 µg LegHb)
M	Molecular weight Marker	na

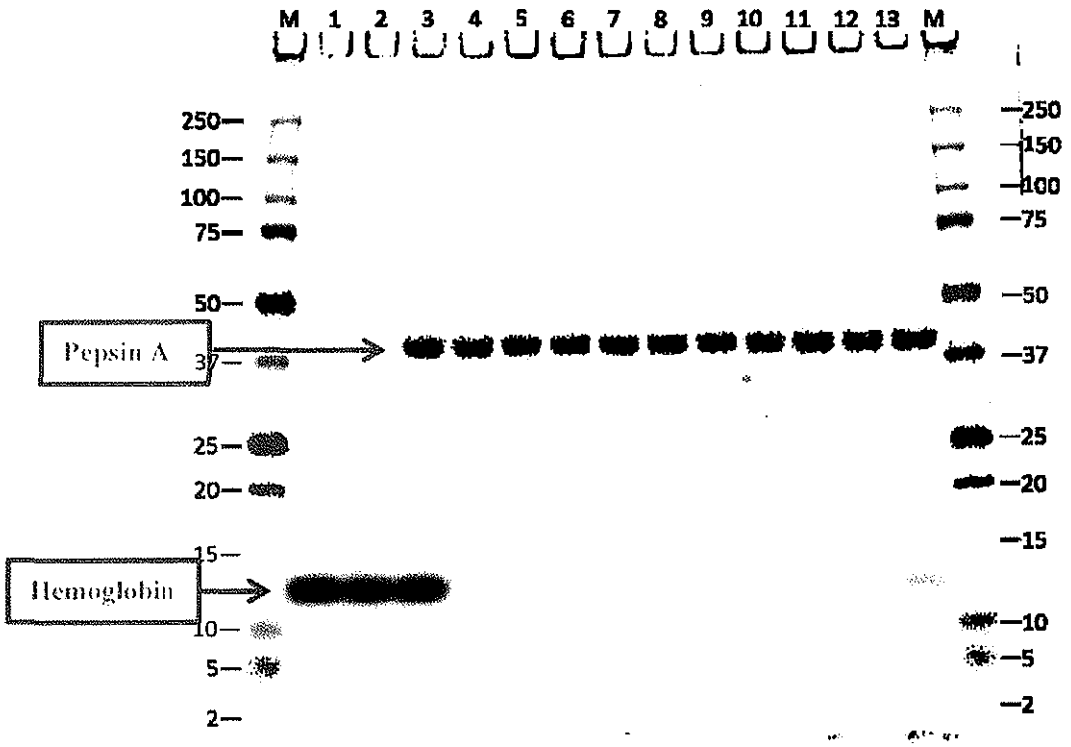

Figure 2. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Bovine Hemoglobin in Simulated Gastric Fluid at the ratio of 10 Units per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. Hemoglobin was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: Hemoglobin (P0)	0	min
2	Experimental control: Hemoglobin (P60)	60	min
3	Hemoglobin in SGF, (D0)	0	min
4	Hemoglobin in SGF, (D0.5)	0.5	min
5	Hemoglobin in SGF, (D2)	2	min
6	Hemoglobin in SGF, (D5)	5	min
7	Hemoglobin in SGF, (D10)	10	min
8	Hemoglobin in SGF, (D20)	20	min
9	Hemoglobin in SGF, (D30)	30	min
10	Hemoglobin in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% Hemoglobin with quenched pepsin (P1/10)	0	min

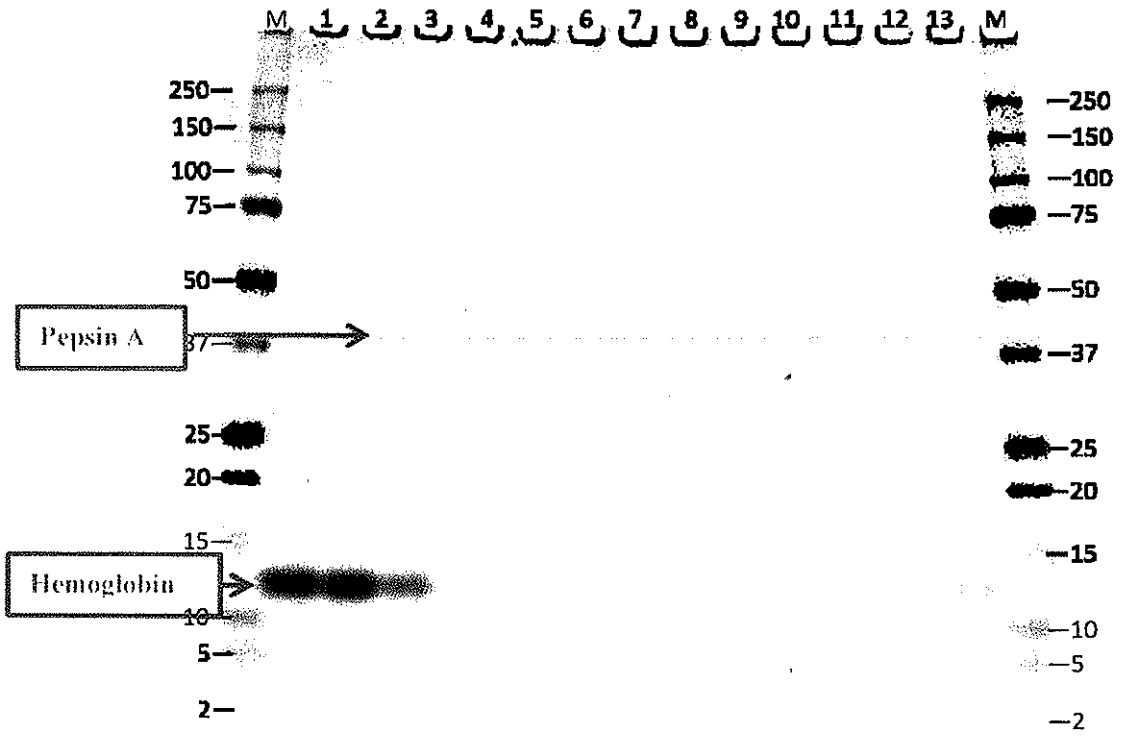

Figure 3. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Bovine Hemoglobin in Simulated Gastric Fluid at a Ratio of 1 units per µg (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. Hemoglobin was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: Hemoglobin (P0)	0	min
2	Experimental control: Hemoglobin (P60)	60	min
3	Hemoglobin in SGF, (D0)	0	min
4	Hemoglobin in SGF, (D0.5)	0.5	min
5	Hemoglobin in SGF, (D2)	2	min
6	Hemoglobin in SGF, (D5)	5	min
7	Hemoglobin in SGF, (D10)	10	min
8	Hemoglobin in SGF, (D20)	20	min
9	Hemoglobin in SGF, (D30)	30	min
10	Hemoglobin in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% Hemoglobin with quenched pepsin (P1/10)	0	min

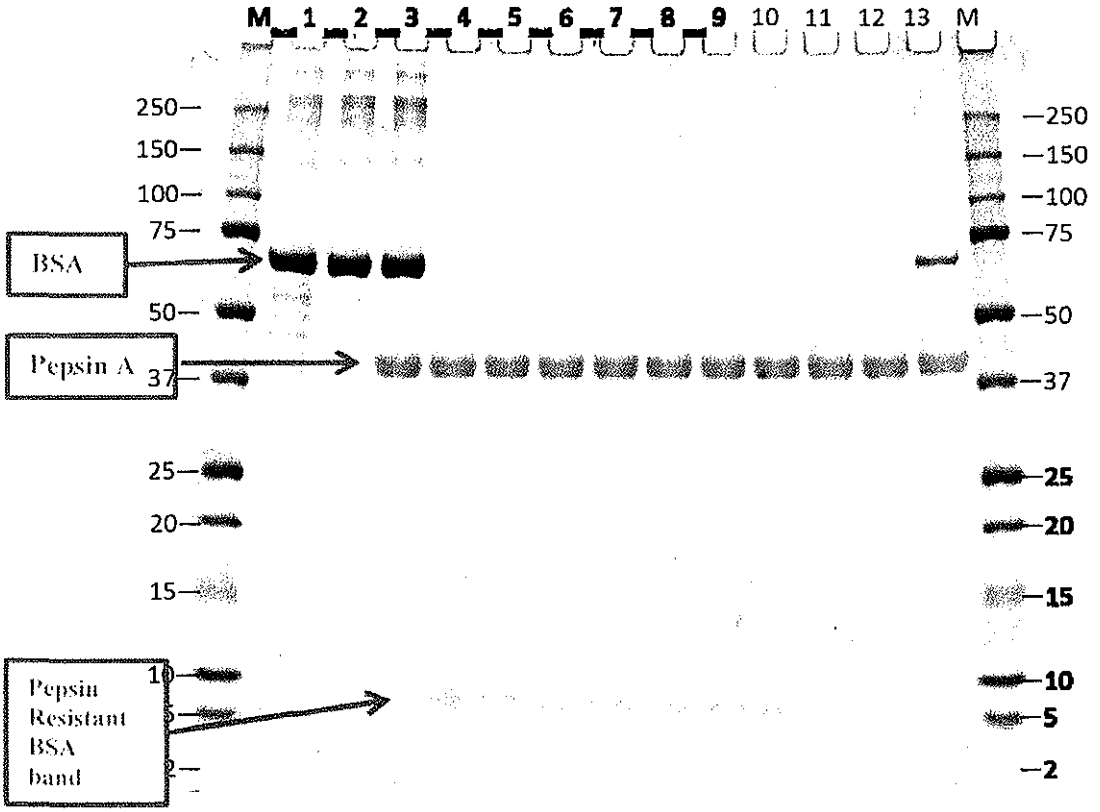

Figure 4. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of BSA in Simulated Gastric Fluid at the ratio of 10 units per µg protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. BSA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: BSA (P0)	0	min
2	Experimental control: BSA (P60)	60	min
3	BSA in SGF, (D0)	0	min
4	BSA in SGF, (D0.5)	0.5	min
5	BSA in SGF, (D2)	2	min
6	BSA in SGF, (D5)	5	min
7	BSA in SGF, (D10)	10	min
8	BSA in SGF, (D20)	20	min
9	BSA in SGF, (D30)	30	min
10	BSA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% BSA with quenched pepsin (P1/10)	0	min

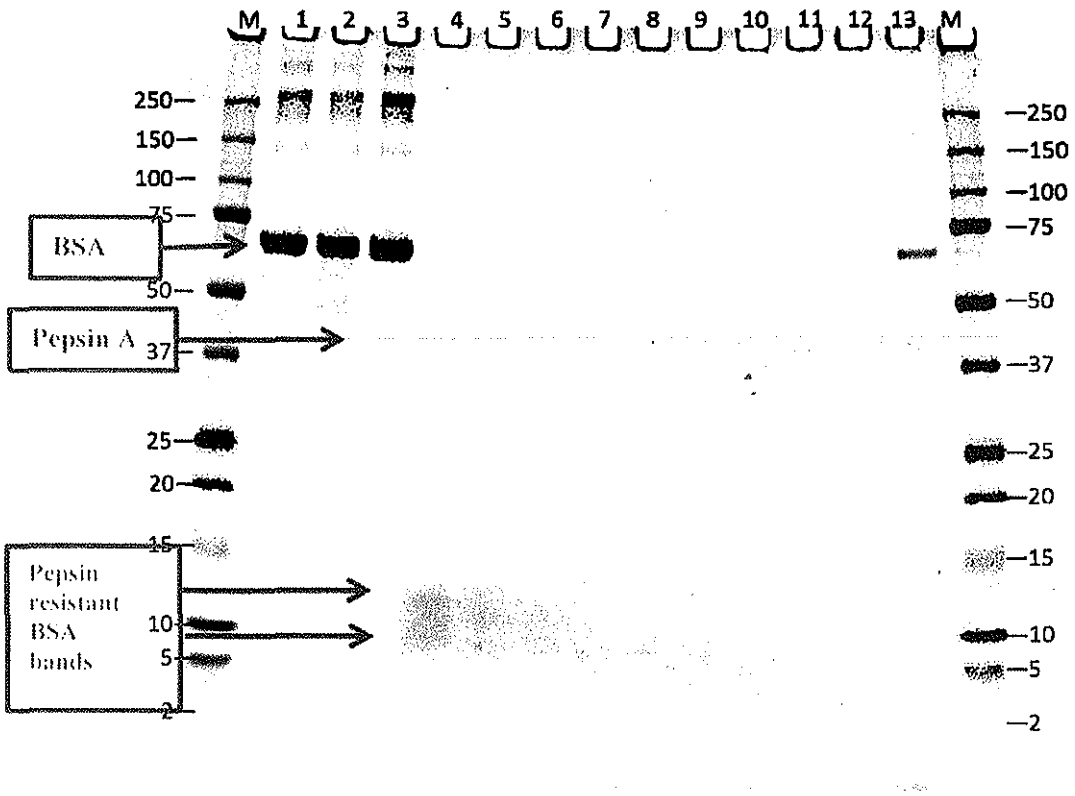

Figure 5. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of BSA in Simulated Gastric Fluid at the Ratio of 1 Unit per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. BSA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: BSA (P0)	0	min
2	Experimental control: BSA (P60)	60	min
3	BSA in SGF, (D0)	0	min
4	BSA in SGF, (D0.5)	0.5	min
5	BSA in SGF, (D2)	2	min
6	BSA in SGF, (D5)	5	min
7	BSA in SGF, (D10)	10	min
8	BSA in SGF, (D20)	20	min
9	BSA in SGF, (D30)	30	min
10	BSA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% BSA with quenched pepsin (P1/10)	0	min

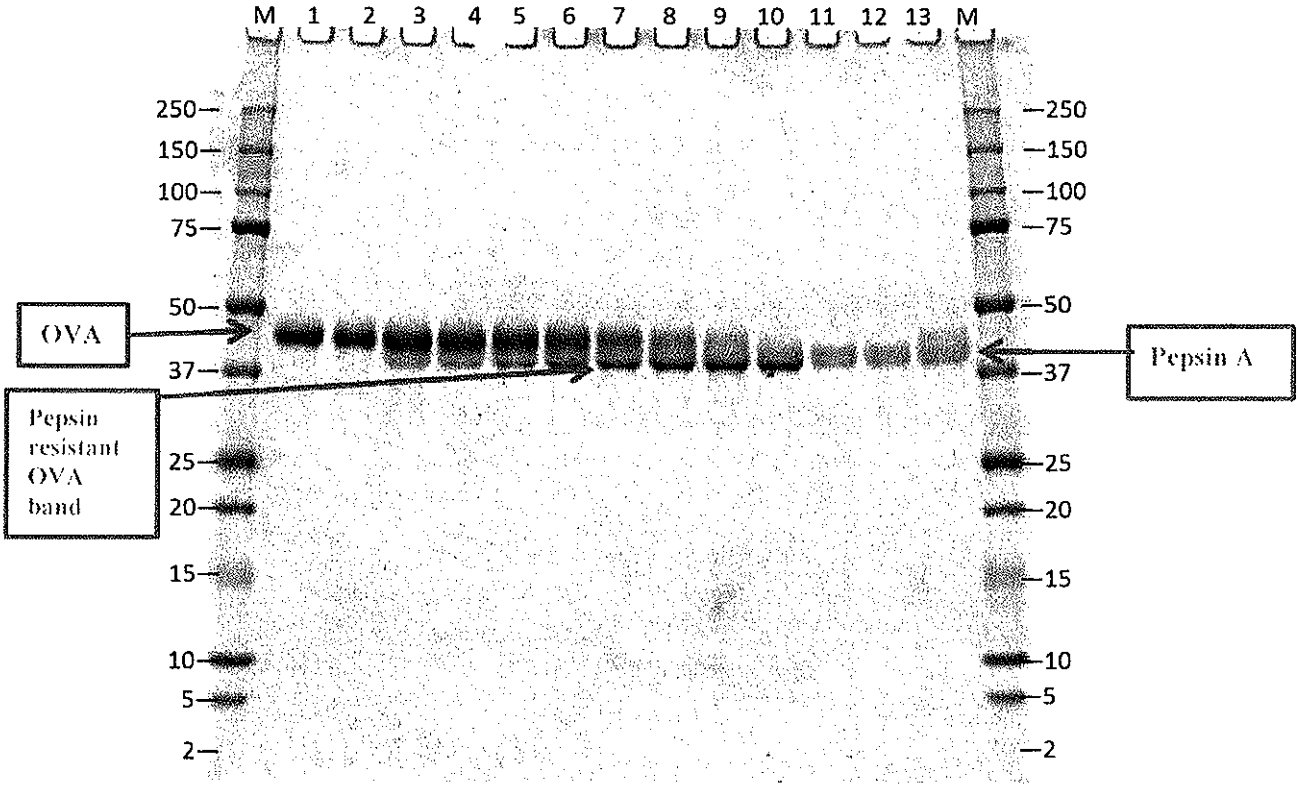

Figure 6. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of OVA in Simulated Gastric Fluid at the Ratio of 10 Units per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. OVA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: OVA (P0)	0	min
2	Experimental control: OVA (P60)	60	min
3	OVA in SGF, (D0)	0	min
4	OVA in SGF, (D0.5)	0.5	min
5	OVA in SGF, (D2)	2	min
6	OVA in SGF, (D5)	5	min
7	OVA in SGF, (D10)	10	min
8	OVA in SGF, (D20)	20	min
9	OVA in SGF, (D30)	30	min
10	OVA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% OVA with quenched pepsin (P1/10)	0	min

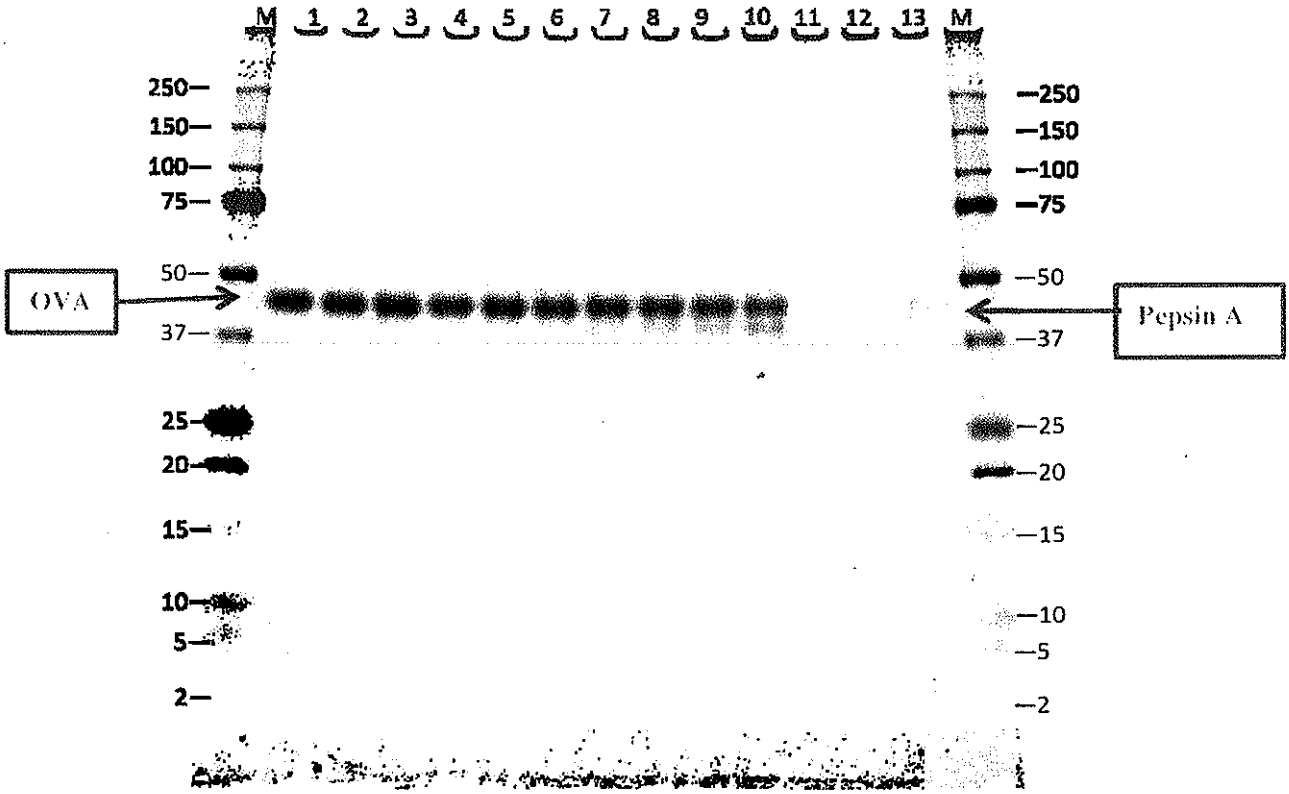

Figure 7. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of OVA in Simulated Gastric Fluid at the Ratio of 1 Unit per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. OVA was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: OVA (P0)	0	min
2	Experimental control: OVA (P60)	60	min
3	OVA in SGF, (D0)	0	min
4	OVA in SGF, (D0.5)	0.5	min
5	OVA in SGF, (D2)	2	min
6	OVA in SGF, (D5)	5	min
7	OVA in SGF, (D10)	10	min
8	OVA in SGF, (D20)	20	min
9	OVA in SGF, (D30)	30	min
10	OVA in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% OVA with quenched pepsin (P1/10)	0	min

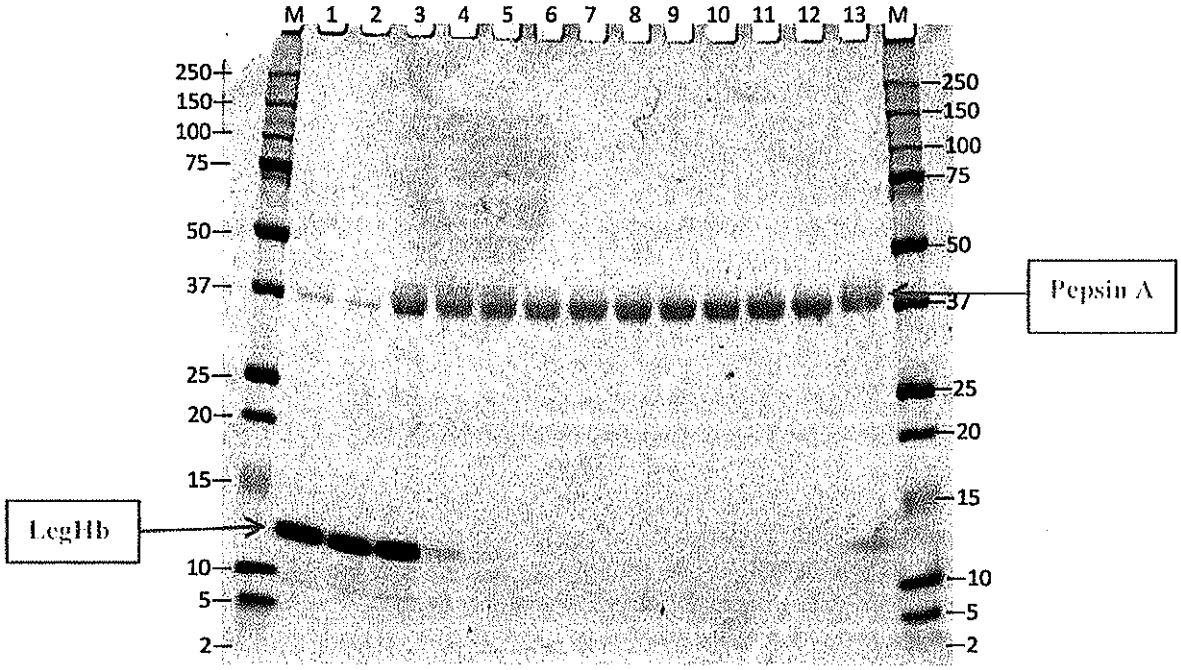

Figure 8. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Soy Leghemoglobin Preparation in Simulated Gastric Fluid at the Ratio of 10 Units per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. LegHb was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: LegHb (P0)	0	min
2	Experimental control: LegHb (P60)	60	min
3	LegHb in SGF, (D0)	0	min
4	LegHb in SGF, (D0.5)	0.5	min
5	LegHb in SGF, (D2)	2	min
6	LegHb in SGF, (D5)	5	min
7	LegHb in SGF, (D10)	10	min
8	LegHb in SGF, (D20)	20	min
9	LegHb in SGF, (D30)	30	min
10	LegHb in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% LegHb with quenched pepsin (P1/10)	0	min

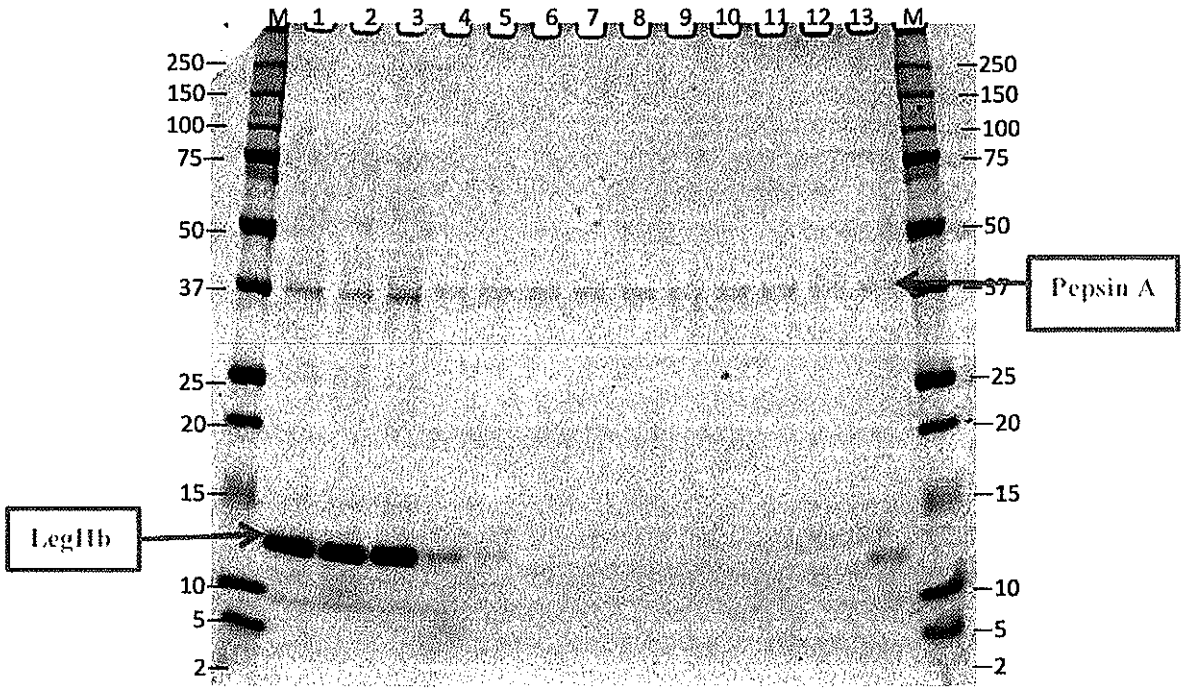

Figure 9. Coomassie Brilliant Blue Stained SDS-PAGE Gel Showing the Digestion of Soy Leghemoglobin Preparation in Simulated Gastric Fluid at the Ratio of 1 Unit per µg Protein (pH 2.0). Proteins were separated by SDS-PAGE using a 10→20% polyacrylamide gradient in a glycine buffered gel. LegHb was loaded 1.47 µg per lane based on pre-digestion concentration (pH 2.0).

Lane	Description	Incubation time	
M	Molecular weight Marker	na	
1	Experimental control: LegHb (P0)	0	min
2	Experimental control: LegHb (P60)	60	min
3	LegHb in SGF, (D0)	0	min
4	LegHb in SGF, (D0.5)	0.5	min
5	LegHb in SGF, (D2)	2	min
6	LegHb in SGF, (D5)	5	min
7	LegHb in SGF, (D10)	10	min
8	LegHb in SGF, (D20)	20	min
9	LegHb in SGF, (D30)	30	min
10	LegHb in SGF, (D60)	60	min
11	Experimental control: Pepsin (E0)	0	min
12	Experimental control: Pepsin (E60)	60	min
13	10% LegHb with quenched pepsin (P1/10)	0	min

Figure 10 a)

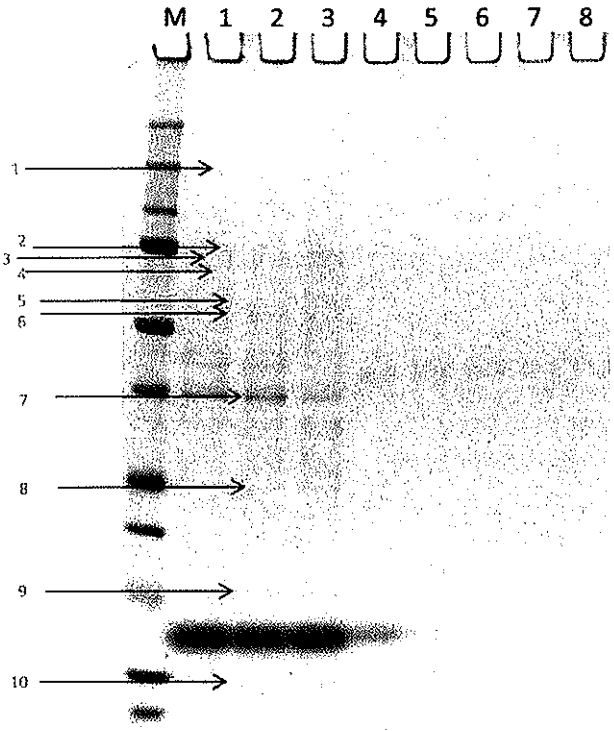

Figure 10 b)

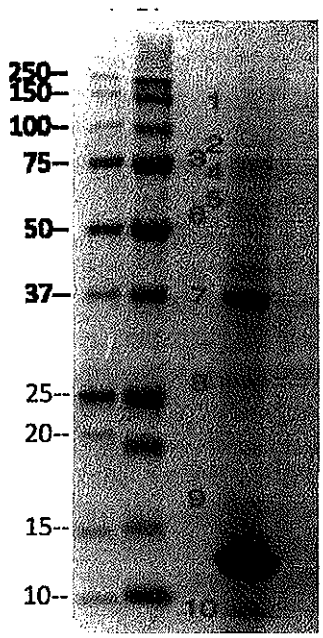

Figure 10. Coomassie Brilliant Blue Stained SDS-PAGE Gel of Soy Leghemoglobin Preparation with 1 Unit pepsin activity per µg Protein (pH 2.0) from Figure 9 (left panel, 10 a), and protein identity gel from Impossible Foods (right panel 10 b). Coomassie stained gels showing the 10 bands of *P. pastoris* proteins that were identified by LC-MS/MS. Note that all 10 bands of *Pichia* proteins are visible at time zero, but not at time 30 seconds of digestion in pepsin (lane 4 of figure 10 a).