syngenta

Quantification of *p*-Hydroxyphenylpyruvate Dioxygenase and Phosphinothricin Acetyltransferase in Processed Food/Feed Fractions Prepared from Event SYHT0H2 Soybean Seed

Final Report

DATA REQUIREMENT(S): Not Applicable

AUTHOR(S): [redacted]

STUDY COMPLETION DATE: May 25, 2012

PERFORMING LABORATORY:
Syngenta Crop Protection, LLC
Product Safety
3054 East Cornwallis Road
Research Triangle Park, NC 27709-2257 USA

LABORATORY PROJECT ID:
Report Number: TK0059702
Task Number: TK0059702

SUBMITTER:
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SPONSOR:
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STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

The following statement applies to submissions to the United States Environmental Protection Agency (US EPA).

No Claim of Confidentiality

No claim of confidentiality is made for any information contained in this report on the basis of its falling within the scope of Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Section 10 (d) (1) (A), (B), or (C).

Company: *Syngenta Seeds, Inc.*

Company Representative:

[redacted]

May 25, 2012

Date

Manager, Regulatory Affairs

These data are the property of Syngenta Seeds, Inc. and, as such, are considered to be confidential for all purposes other than compliance with the regulations implementing FIFRA Section 10. Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality that may exist under any other provision of common law or statute or in any other country.

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Its submission does not constitute a waiver of any right to confidentiality that may exist in any other country.

GOOD LABORATORY PRACTICECOMPLIANCE STATEMENT

This study was conducted in compliance with the relevant provisions of Good Laboratory Practice Standards (GLPS) (40 CFR Part 160, US EPA 1989) pursuant to the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) with the following exceptions:

1. Thermometer used to record inlet temperature on the Anhydro spray dryer used during the Processing Phase was not calibrated.

Study Director:

[redacted] May 25, 2012

Date

Technical Expert, Product Safety
Syngenta Crop Protection, LLC

Submitted by:

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Date

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QUALITY ASSURANCE STATEMENT

Study Title: Quantification of *p*-Hydroxyphenylpyruvate Dioxygenase and Phosphinothricin Acetyltransferase in Processed Food/Feed Fractions Prepared from Event SYHT0H2 Soybean Seed

Study Director: [redacted]

Study Number: TK0059702

Pursuant to Good Laboratory Practice Regulations (40 CFR Part 160), this statement verifies that the aforementioned study was inspected and/or audited and the findings reported to Management and to the Study Director by the Quality Assurance Unit on the dates listed below.

Inspection/Audit Type	Inspection/Audit Dates	Reporting Dates
Audit Protocol	12-APR-2011 - 12-APR-2011	12-APR-2011
Inspect Analytical	19-DEC-2011 - 19-DEC-2011	20-DEC-2011
Audit Study Data	18-JAN-2012 - 23-JAN-2012	25-JAN-2012
Audit Study Data	07-MAR-2012 - 08-MAR-2012	08-MAR-2012
Audit Final Report, 1st audit	30-APR-2012 - 01-MAY-2012	02-MAY-2012
Inspect Sample Verification	08-MAY-2012 - 08-MAY-2012	14-MAY-2012
Audit Final Report, 2nd audit	18-MAY-2012 - 18-MAY-2012	18-MAY-2012
Audit Final Report, 3rd audit	21-MAY-2012 - 21-MAY-2012	21-MAY-2012

Prepared By: [redacted] Date: May 25, 2012

Staff Quality Assurance Auditor
Syngenta Crop Protection, LLC

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
Allison Horner	Study Director, Syngenta Crop Protection, LLC Effective September 12, 2011
Justin McDonald	Study Director, Syngenta Crop Protection, LLC Effective April 27, 2011 – September 11, 2011
Carl Vavra	Processing Principal Investigator, Texas A&M University
Jani Mudrak	Processing, Data Recording and Shipping Technician
Emmanuel Ferew	Sample Preparation Technician, Syngenta Crop Protection, LLC

Study Dates

Study initiation date:	April 27, 2011
Experimental start date:	May 16, 2011
Experimental end date:	February 23, 2012

Records Retention

Raw data, the original copy of this report, and other relevant records are archived at Syngenta, 3054 East Cornwallis Road, Research Triangle Park, NC 27709-2257, USA.

Additional Testing Site

Food Protein R & D Center
Building 8525
Texas A&M University
Riverside Campus
3100 Highway 47 South
Bryan, TX 77807, USA

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LIST OF ACRONYMS AND ABBREVIATIONS

avhppd-03	*p*-hydroxyphenylpyruvate dioxygenase gene derived from oat
AvHPPD-03	*p*-hydroxyphenylpyruvate dioxygenase enzyme encoded by *avhppd-03*
DW	dry weight
ELISA	enzyme-linked immunosorbent assay
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
FW	fresh weight
g	gram
GLPS	Good Laboratory Practice Standards
HPPD	*p*-hydroxyphenylpyruvate dioxygenase enzyme
LOD	limit of detection
ml	milliliter
ng	nanogram
PAT	phosphinothricin acetyltransferase
SD	standard deviation
US EPA	United States Environmental Protection Agency
µg	microgram

1.0 EXECUTIVE SUMMARY

The purpose of this study was to determine the concentrations of the proteins *p*-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) and phosphinothricin acetyltransferase (PAT) in food and feed fractions processed from soybean seed derived from transformation Event SYHT0H2.

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the genes *avhppd-03* derived from oat *(Avena sativa* L.*)* and *pat* from *Streptomyces viridochromogenes*. The gene *avhppd-03* encodes a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, designated AvHPPD-03, that catalyzes the formation of homogentisic acid, the aromatic precursor in plastoquinone and vitamin E biosynthesis. In comparison with the native soybean HPPD, the AvHPPD-03 isozyme from oat has lower binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in the transgenic Event SYHT0H2 soybean plants confers a mesotrione-tolerance phenotype. The gene *pat* encodes the enzyme phosphinothricin acetyltransferase (PAT), which inactivates the herbicide glufosinate, an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway. Expression of *pat* confers a glufosinate-tolerance phenotype, which was used as a selectable marker in the development of Event SYHT0H2 soybeans.

Laboratory-scale milling methodology equivalent to industry-standard processing was used to process the seed of SYHT0H2 soybean and of a nontransgenic, near-isogenic control soybean into the following commercially representative food and feed fractions: milk, tofu, hulls, full-fat flour, flakes, white flakes, defatted toasted meal, protein concentrate, and protein isolate. Enzyme-linked immunosorbent assay was used to quantify AvHPPD-03 and PAT in the processed soybean fractions and in the seed from which they were produced.

AvHPPD-03 and PAT were detected in SYHT0H2 soybean seed and in the hulls, full-fat flour, flakes, white flakes, and defatted toasted meal processed from SYHT0H2 soybean seed. AvHPPD-03 and PAT were not detected in the protein concentrate, protein isolate, milk, or tofu processed from SYHT0H2 soybean seed. AvHPPD-03 and PAT were not detected in any samples processed from nontransgenic soybean seed.

2.0 INTRODUCTION

The purpose of this study was to determine the concentrations of the proteins p-hydroxyphenylpyruvate dioxygenase (AvHPPD-03) and phosphinothricin acetyltransferase (PAT) in food and feed fractions processed from soybean seed derived from transformation Event SYHT0H2.

Soybean (*Glycine max* [L.] Merrill) has been genetically modified to express the genes *avhppd-03* derived from oat *(Avena sativa* L.*)* and *pat* from *Streptomyces viridochromogenes*. The gene *avhppd-03* encodes a *p*-hydroxyphenylpyruvate dioxygenase (HPPD) enzyme, designated AvHPPD-03, that catalyzes the formation of homogentisic acid, the aromatic precursor in plastoquinone and vitamin E biosynthesis. In comparison with the native soybean HPPD, the AvHPPD-03 isozyme from oat has lower binding affinity for mesotrione, an herbicide that inhibits HPPD. Expression of *avhppd-03* in the transgenic Event SYHT0H2 soybean plants confers a mesotrione-tolerance phenotype. The gene *pat* encodes the enzyme phosphinothricin acetyltransferase (PAT), which inactivates the herbicide glufosinate, an inhibitor of glutamine synthetase, an enzyme in the nitrogen assimilation pathway. Expression of *pat* confers a glufosinate-tolerance phenotype, which was used as a selectable marker in the development of Event SYHT0H2 soybeans.

The concentrations of AvHPPD-03 and PAT in samples of SYHT0H2 soybean seed and the processed fractions were quantified by enzyme-linked immunosorbent assay (ELISA).

3.0 MATERIALS AND METHODS

3.1 Test, Control, and Reference Substances

The test substance for this study was SYHT0H2 soybean seed in the genetic background 'Jack' (Nickell *et al.* 1990). The control substance was nontransgenic, near-isogenic soybean seed of the same genetic background as the test substance. Table 1 shows the descriptions and material identification codes for the test and control substances.

TABLE 1 Test and control substances

Seed Identification	Material identification
Nontransgenic soybean (Control)	11RE000070
SYHT0H2 soybean (Test)	11RE000064

Seed lots of the test and control substances were characterized by real-time polymerase chain reaction testing (Ingham *et al*. 2001) to confirm identity and purity.

Table 2 shows the protein reference substance used to produce the standard curve for each ELISA.

TABLE 2 Protein reference substance for ELISA analyses

Protein	Reference substance ID	Characterization report
AvHPPD-03	AvHPPD-03-0209	Winslow 2009
PAT	PAT-0109	Seastrum 2009

3.2 Preparation of the Processed Fractions

The seed was processed into food and feed fractions at the Food Protein Research and Development Center, Texas A&M University, Bryan TX, USA, through the use of laboratory-scale milling methodology equivalent to industry-standard processing of soybean seed. The details of the processing methodology are described in the processing phase report, provided as Appendix A.

OECD guidelines were consulted to choose the most appropriate fractions for testing that were most related to human and animal consumption (OECD 2001). The following fractions of soybean seed were selected and processed for analysis:

milk
tofu
hulls
full-fat flour
flakes
refined oil (not analyzed)
white flakes
defatted toasted meal
protein concentrate
protein isolate

Samples of the processed fractions and of the SYHT0H2 seed were shipped on dry ice to Syngenta Crop Protection, LLC, Research Triangle Park NC, USA. Upon receipt, the samples were stored at -80°C ± 10°C.

3.3 Sample Preparation

The soybean seed, milk, tofu, hulls, full-fat flour, flakes, white flakes, defatted toasted meal, protein concentrate, and protein isolate samples were ground to a fine powder in the presence of dry ice. Nontransgenic samples were processed first to prevent possible contamination. Each powdered sample was mixed thoroughly to ensure homogeneity. The full-fat flour, protein concentrate, and protein isolate samples were received as a fine powder and did not require further grinding. A subsample from each homogenous powdered sample was lyophilized for protein extraction and analysis. The soybean milk samples were received as a frozen liquid and were lyophilized as received. The refined oil samples did not require grinding or lyophilization. All samples were stored at -80°C ± 10°C except for the refined oil samples which were stored at -20°C ± 5°C.The percent dry weight (DW) of each sample was determined from the fresh weight (FW) of the sample before lyophilization and the DW of the sample after lyophilization by the following formula:

$$\% \text{ DW} = \left(\frac{\text{DW (g)}}{\text{FW (g)}}\right) \times 100$$

3.4 Protein Extraction and ELISA Analysis

Protein extractions were performed on three representative aliquots of each sample corresponding to SYHT0H2 soybean, except for the refined oil sample. ELISA methodology was used to quantify AvHPPD-03 and PAT in each extract. Refined oil samples were not analyzed by ELISA as the non-aqueous characteristics of oil may inhibit accurate detection of the proteins. Nontransgenic sample extracts were analyzed to confirm the absence of sample-matrix effects and the specificity of the ELISA methods.

For each ELISA, a standard curve was generated with known amounts of the corresponding reference protein. The mean absorbance for each sample extract was plotted against the appropriate standard curve to obtain the amount of the protein as nanograms per milliliter of extract. The concentrations were converted to represent the amount of protein as micrograms per gram of tissue by the following formula:

$$\frac{(\text{ng/ml}) \times (\text{dilution factor}) \times (\text{volume of buffer [ml]})}{(\text{amount of tissue [g]}) \times 1000}$$

Descriptions of the AvHPPD-03 and PAT quantification procedures, including validation of ELISA sensitivity and extraction efficiency, can be found in Appendices B and C.

Protein concentrations were converted from a DW basis to a FW basis by the following formula:

$$\mu\text{g/g FW} = \mu\text{g/g DW} \times (\%\ \text{DW} \div 100)$$

3.5 Adjustments for Extraction Efficiency

All AvHPPD-03 and PAT concentrations measured from a single extraction were adjusted to the estimated absolute AvHPPD-03 and PAT concentration in each sample through the use of the determined extraction efficiencies (provided in Appendices B and C) in the following formula:

$$\left(\frac{\text{amount of protein measured from a single extraction } (\mu\text{g/g})}{\text{extraction efficiency } (\%)}\right)$$

3.6 Control of Bias Statement

Protein extractions were performed on representative aliquots of homogeneous samples, and each extract was analyzed in triplicate. Any rejected data, and the documented reasons for the rejection of those data, are retained in the study file.

3.7 Statistical Analysis Statement

All calculations, including means and standard deviations (SD), were performed with Microsoft Excel® 2007 spreadsheet software. All decimal places associated with the concentrations determined for each replicate sample were used in calculation of the means, and were then rounded to two decimal places for reporting consistency.

4.0 RESULTS AND DISCUSSION

Table 3 shows the AvHPPD-03 and PAT concentrations in the SYHT0H2 soybean seed and in each fraction processed from the SYHT0H2 soybean seed.

TABLE 3 Concentrations of AvHPPD-03 and PAT in SYHT0H2 soybean seed and in food and feed fractions processed from SYHT0H2 soybean seed

Sample	AvHPPD-03		PAT	
	Mean ± SD µg/g DW	**Mean ± SD µg/g FW**	**Mean ± SD µg/g DW**	**Mean ± SD µg/g FW**
Seed	20.36 ± 1.76	18.91 ± 1.63	9.25 ± 0.45	8.59 ± 0.42
Milk	<LOD[a]	—	<LOD[b]	—
Tofu	<LOD[a]	—	<LOD[b]	—
Hulls	2.53 ± 1.14	2.27 ± 1.02	1.10 ± 0.45	0.99 ± 0.40
Full-fat flour	21.03 ± 5.61	20.34 ± 5.43	6.74 ± 3.32	6.51 ± 3.21
Flakes	14.68 ± 4.48	13.84 ± 4.23	3.18 ± 0.28	3.00 ± 0.26
White flakes	17.36± 5.46	16.55 ± 5.20	5.82 ± 1.79	5.55 ± 1.71
Defatted toasted meal	1.42 ± 0.09	1.41 ± 0.09	0.09 ± 0.02	0.09 ± 0.02
Protein concentrate	<LOD[a]	—	<LOD[b]	—
Protein isolate	<LOD[a]	—	<LOD[b]	—

N = 3 replicate analyses for each sample.
The concentrations were adjusted for extraction efficiency.
— = Because lyophilized samples were analyzed, LOD values were not determined on a FW basis.
[a]LOD = 0.0313 µg/g of sample.
[b]LOD = 0.025 µg/g of sample.

The low AvHPPD-03 and PAT concentrations in the SYHT0H2 soybean hulls were expected, as soybean hulls are characteristically low in total protein content (Ludden *et al.* 1995) and are used primarily as a source of fiber.

It has been demonstrated that AvHPPD-03 is readily degraded at high temperatures. In a study of AvHPPD-03 prepared from recombinant *Escherichia coli,* its immunoreactivity decreased by 96.9% after incubation at 65°C for 30 minutes and was below the limit of detection after incubation at 95°C for 30 minutes (Moore and Winslow 2011). Therefore, lower concentrations of AvHPPD-03 in the soybean fractions compared with seed are consistent with the expected effect on AvHPPD-03 after high heat during processing (as described in Appendix A).

PAT concentrations diminished in all of the fractions and therefore PAT appears to be labile at the applied temperatures as part of the processing of the soybean seed. Although Herouet et al., (2005) concluded that immunoreactivity of PAT would be stable throughout typical processing of soybean seed containing PAT, those experiments included temperatures no greater than 90°C. Conformational changes to the PAT protein, higher temperatures in this study and the inherent complexity of the seed matrix likely account for the observed sensitivity to processing conditions in this study.

The concentration of AvHPPD-03 was higher in the full-fat flour processed from SYHT0H2 soybean seed than in the whole SYHT0H2 soybean seed. This result is consistent with the documented higher percentage of total protein in full-fat flour (50% total protein; Lusas and Riaz 1995) than in intact soybean seed (40% total protein; Maughan *et al.* 2000). Because the full-fat flour was produced by pulverization of the dehulled kernel without high heat or the use of solvents, the potential for protein degradation was minimal, and degradation of AvHPPD-03 was not apparent from the results.

AvHPPD-03 and PAT were not detected in any samples processed from nontransgenic soybean seed. Analysis of the nontransgenic soybean sample extracts confirmed the absence of performance-inhibiting sample-matrix effects on the ELISA methods, and the specificity of the ELISA methods.

4.1 Data Quality and Integrity

No circumstances occurred during the conduct of this study that would have adversely affected the quality or integrity of the data generated.

5.0 CONCLUSION

AvHPPD-03 and PAT were detected in SYHT0H2 soybean seed and in hulls, full-fat flour, flakes, white flakes, and defatted toasted meal processed from SYHT0H2 soybean seed. AvHPPD-03 and PAT were not detected in the protein concentrate, protein isolate, milk, or tofu processed from SYHT0H2 soybean seed.

6.0 REFERENCES

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Winslow, S. 2009. *Characterization of Microbially Produced Test Substance Containing p-Hydroxyphenylpyruvate Dioxygenase Protein (AvHPPD-03) and Certificate of Analysis*. Report No. SSB-041-09 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology, Inc.

APPENDICES SECTION

APPENDIX A Processing Phase Report

SPONSOR:

Syngenta Crop Protection, LLC

Greensboro, North Carolina

STUDY DIRECTOR:

[redacted]

PHASE REPORT:

Soybean Processing

STUDY TITLE:

Quantification of p-hydroxyphenylpyruvate dioxygenase and phosphinothricin acetyltransferase in Processed Food/Feed Fractions Prepared from Event SYHT0H2 Soybean Seed

AUTHOR:

[redacted]

10-24-11
Date

PROCESSING FACILITY:

GLP Program
Texas A & M University
Food Protein Research and Development Center
3100 Highway 47, Building 8525
Bryan, TX 77807

STUDY IDENTIFICATION:

Study Number: TK0059702

GLP COMPLIANCE STATEMENT

PROJECT TITLE: **Processing Phase for Study: Quantification of p-hydroxyphenylpyruvate dioxygenase and phosphinothricin acetyltransferase in Processed Food/Feed Fractions Prepared from Event SYHT0H2 Soybean Seed**

This processing study was conducted and reported in accordance with the Environmental Protection Agency's Good Laboratory Practices Standards, 40 CFR 160, Federal Register, effective date October 16, 1989, with the following exceptions:

1. Thermometer used to record inlet temperature on the Anhydro spray drier was not calibrated.

[redacted]

Process Principal Investigator

10-24-11
Date

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: **Quantification of p-hydroxyphenylpyruvate dioxygenase and phosphinothricin acetyltransferase in Processed Food/Feed Fractions Prepared from Event SYHTOH2 Soybean Seed**

In compliance with the Good Laboratory Practice regulations, an inspector with the Quality Assurance Unit has inspected at least one phase of this study. Inspection findings were reported to GLP Program management, the study director, and the study director's management. The Quality Assurance Unit has reviewed the processing report and certifies that it accurately describes the methods and standard operating procedures used and the reported results accurately reflect the raw data generated during this processing phase.

Signed: [redacted] Date: October 24, 2011

Quality Assurance Coordinator
Food Protein Research and Development Center

INSPECTION		DATES REPORTED TO:	
TYPE	DATE	GLP PROGRAM MANAGEMENT	STUDY DIRECTOR & STUDY DIRECTOR'S MANAGEMENT
1) Processing Inspection: - SOP 8.3 R14 Sec.5 "Solvent Extraction"	May 18 & 25 , 2011	June 7, 2011	June 7, 2011
2) Process Study Report Audit	July 21-22, 2011	July 22, 2011	July 22, 2011

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STUDY TITLE: **Processing Phase for Study: Quantification of p-hydroxyphenylpyruvate dioxygenase and phosphinothricin acetyltransferase in Processed Food/Feed Fractions Prepared from Event SYHT0H2 Soybean Seed**

SPONSOR: Syngenta Crop Protection, LLC
410 Swing Road
P.O. Box 18300
Greensboro, NC 27419-8300 USA

STUDY DIRECTOR: [redacted]

SPONSOR MANAGEMENT: [redacted]

PROCESS PRINCIPAL INVESTIGATOR: [redacted]

PROCESSING, DATA RECORDING & SHIPPING TECHNICIANS: [redacted]

SAMPLE (SOYBEAN SEED) RECEIPT DATE: May 12, 2011

PROCESSING START DATE: May 16, 2011

PROCESSING TERMINATION DATE: June 15, 2011

FRACTION SHIPMENT DATE:

June 21, 2011
Whole Beans from Control and SYHT0H2
Full-Fat Flour from Control and SYHT0H2
Flakes from Control and SYHT0H2
Hulls from Control and SYHT0H2
White flakes from Control and SYHT0H2
Defatted Toasted Meal from Control and SYHT0H2
Refined Oil from Control and SYHT0H2
Soymilk from Control and SYHT0H2
Tofu from Control and SYHT0H2
Soy Protein Isolate from Control and SYHT0H2
Soy Protein Concentrate from Control and SYHT0H2

INTRODUCTION:

Control and test soybean seed were received from the sponsor and were processed into commercially representative fractions. These fractions were sent to Syngenta Crop Protection, LLC, Product Safety Testing Facility in Research Triangle Park, North Carolina.

TEST SUBSTANCE: [From protocol and/or MSDS]

Product: SYHT0H2 SOYBEAN SEED

OBJECTIVE:

The objective of the processing phase was to generate commercially representative processed fractions from soybean seed.

METHODS & MATERIALS:

Sample Receipt:

Two soybean seed samples (RAC/seed) were received at the Food Protein Research and Development Center in Bryan, Texas on May 12, 2011. The samples were shipped on May 11, 2011 by Syngenta Crop Protection, LLC, Product Safety Testing Facility in Research Triangle Park, North Carolina via Federal Express. The samples were for study number TK0059702. The samples were identified and processed in the following order: Control soybean and SYHT0H2 (Test) soybean.

Storage Conditions:

All samples were stored at 1°C to 8°C upon receipt. Recorded in the data are the times and dates for removal or placement of samples/fractions in freezers or coolers, and the freezer temperature records.

Sample/Fraction Handling:

Samples were handled in a manner that minimizes the possibility of contamination. Containers and utensils were washed with detergent and rinsed with water. The control substance was processed first and the test substance was processed afterward to minimize potential contamination.

Processing Methods:

The whole soybean sample moisture content was 7-10%. The light impurities were separated using a Kice aspirator. After aspiration, the sample was screened in a Vac-Away two screen cleaner. Large and small foreign particles (screenings) were separated from the soybean.

The whole soybean was fed into a Bauer disc mill to crack the hull and liberate the kernel. After hulling, the material was passed through the Kice aspirator to separate the hull and kernel material. Hulls were additionally aspirated and screened to yield the hull fraction. A portion of kernel material was processed through a pulverizer to produce full-fat flour. A portion of whole soybean was processed in a Soy Quick soy maker to produce soymilk and tofu (described below).

The kernel material was heated to 160-175°F (71-80°C). The heated kernel material was flaked in a Ferrell-Ross flaking roll with a gap setting of 0.008-0.012". This step yielded a flake fraction. The remaining flakes were taken directly to solvent extraction.

The flakes were placed in stainless steel batch extractors and submerged in 120-140°F (49-60°C) solvent (hexane). After 30 minutes, the hexane was drained and fresh hexane was added to repeat the cycle two more times. The final two washings were for 15 to 30 minutes each. Following the final draining, warm air was forced through the marc to remove residual hexane to yield solvent extracted flakes (or white flakes). White flakes were used to produce defatted toasted meal, soy protein isolate, and soy protein concentrate (see methods below).

The miscella (crude oil and hexane) was passed through a Precision Scientific Recovery unit to separate the crude oil and hexane. Crude oil was then heated to 163-194°F (75-90°C) for hexane removal.

The crude oil recovered from solvent extraction was refined as follows. After determining the percent of free fatty acid in the crude oil, a weighed sample was placed in a Laboratory Oil Refining Machine. A weighed amount of 14 degree Baume (NaOH) was added to the crude oil, as calculated on the basis of percent free fatty acid present. The solution was mixed for 90 minutes (± 1 minute) at 250 RPM (± 10 RPM) and a temperature of 68-75°F (20-24°C) and then for an additional 20 minutes (± 1 minute) at 70 RPM (± 10 RPM) at a temperature of 145-153°F (63-67°C). Neutralized oil was allowed to settle for one hour at 140-149°F (60-65°C). The oil solution was refrigerated overnight. After refrigeration, refined oil was decanted and filtered. The fraction settling to the bottom of the refrigerated container was soapstock.

After refining, the refined oil and soapstock were separated. The refined oil fraction was stored frozen.

To produce defatted toasted meal, the moisture content of the white flake was determined, and then adjusted to 20%. The moisture was allowed to equilibrate in the meal for 15-30 minutes. Meal was then placed into an electric heated cooker and heated to 220-235°F (104-113°C). The meal was held in this temperature range for 15-30 minutes to yield defatted toasted meal.

To prepare for making soy protein isolate and soy protein concentrate, white flake was ground in a coffee bean grinder to produce white flake flour.

To make soy protein isolate, flour was added to reverse osmosis (RO) water (15:1 ratio by weight, water:flour) and mixed for a time period (suggested 15 minutes or less) and at a RPM rate sufficient enough to provide proper dispersion.

The solution temperature was increased to 55 °C ± 5°C (122°F, range:119-140°F) and 25% sodium hydroxide (NaOH) was added until solution maintained a pH of 8.5 pH ± 0.1.

Solution was allowed to mix for 45 minutes (± 2 minutes). After mixing, solution was centrifuged to yield a clear supernatant (liquid fraction).

At a low mixing RPM, the pH of the supernatant was adjusted to 4.5 ± 0.1 with 25% hydrochloric acid (HCl). The solution temperature was allowed to decrease with no artificial cooling applied. When the desired pH was achieved, it was centrifuged and the liquid (whey) and solids were recovered. The solid fraction was suspended in RO water (5:1 ratio by weight, water to starting weight of flour) to allow a second centrifugation step. The whey fraction was discarded. After centrifugation, enough RO water was added to the solid fraction to form a slurry.

The pH of this slurry was adjusted to 6.8 ± 0.1 with 25% NaOH. The slurry was dried in a spray dryer. The spray dryer was set with an air entry temperature of 375 °F (± 50°F)(191°C, range:163-218°C) and an air exit temperature of 200 °F (± 50°F)(93°C, range: 66-121°C). The thermometer monitoring inlet temperature could not be removed for calibration. Operating temperature of the spray dryer was effective for the production of dried product. This yielded soy protein isolate.

To create soy protein concentrate, flour was added to aqueous ethanol (65 to 70% ethanol) (10:1 ratio by weight, aqueous ethanol:flour) and mixed for 15 minutes or less and at a RPM rate sufficient enough to provide proper dispersion. The solution was mixed for 45 minutes (± 2) minutes at a temperature of 45 °C (± 5°C)(113°F, range: 104-122°F).

The solution was centrifuged to separate liquid (contains sugars, ash and other minor constituents) and solid (concentrated protein curd) fractions. The solid fraction was suspended in reverse osmosis (RO) water (5:1 ratio by weight, water to starting weight of flour) and soon after 25% hydrochloric acid (HCl) was added until the pH of the solution was 4.5 (± 0.1).

After pH adjustment, solution was allowed to mix at 45 °C (113°F) for 10 minutes (± 2 minutes). At the end of this period the solution was centrifuged. Enough RO water was added to the solid fraction to form a slurry.

The pH of the slurry was adjusted to 6.8 (± 0.1) with 25% NaOH, then homogenized to form the feed for the spray dryer. The slurry was then dried using a spray dryer. The spray dryer was set with an air entry temperature of 400 °F (± 50°F)(204°C, range:

177-232°C) and an air exit temperature of 200°F (± 50°F)(93°C, range: 66-121°C). The thermometer monitoring inlet temperature could not be removed for calibration. Operating temperature of the spray dryer was effective for the production of dried product. This yielded soy protein concentrate.

To prepare soymilk, whole soybean was washed two times with reverse osmosis water. The wash water was discarded.

Note: All water used to make soymilk was RO water.

Water washed beans were soaked for a minimum of 12 hours in water at ambient temperature (3.5 to 4.0 gallons of water per pound of soybean). After the soaking period, water was drained and the beans were rinsed with fresh water. Water was discarded.

To prepare soymilk samples, an all-in-one soymilk/tofu machine was used. The sample was heated to a minimum of 200-212°F ± 5°F (93-100°C, range 91-103°C). Each sample reached a minimum temperature of at least 206.1°F (98°C) for 7 minutes (entire process takes about 13-15 minutes according to machine manufacturer). The resulting liquid after the cooking period is soymilk. The insoluble residue by-product (Okara) was discarded.

Tofu was then produced from a fraction of the soymilk. Soymilk was mixed and heated to a temperature of 75-85°C (167-185°F). After this temperature was achieved, mixing was continued and a calcium sulfate solution (0.5 grams ± 0.1 grams calcium sulfate per 2.5 ml ± 0.1 ml water) was slowly added. As the curd formed, calcium sulfate solution was added until whey (liquid) became transparent. The whey was removed. The resulting curd is tofu.

Comparison to Industrial Practice:

The soybeans were processed in a way that simulates industrial practice as closely as possible. Because of compliance monitoring requirements and sample size, the samples were processed by batch rather than continuous, as in commercial operation.

Processing Results:

Soybean samples were processed into hulls, flakes, white flakes, refined oil, soymilk, tofu, full-fat flour, defatted toasted meal, soy protein concentrate and soy protein isolate. An unprocessed sample (RAC) was taken before processing. All fractions collected during this study are listed in the original raw data.

Other Circumstances Pertaining to Study:

As written in the compliance statement, the thermometer on the spray drier measuring air inlet temperature was not calibrated. The thermometer could not be removed for calibration.

Fraction Shipment:

Processed soybean fractions were shipped priority overnight, packed in dry ice to Syngenta Crop Protection, LLC, Product Safety Testing Facility in Research Triangle Park, North Carolina on June 21, 2011 by Federal Express. A Chain of Custody accompanied the sample shipment.

CONCLUSIONS:

Control and test soybean seed samples were successfully processed into commercially representative fractions requested under the processing phase for Study #TK0059702. A summary is shown in Figure 1.

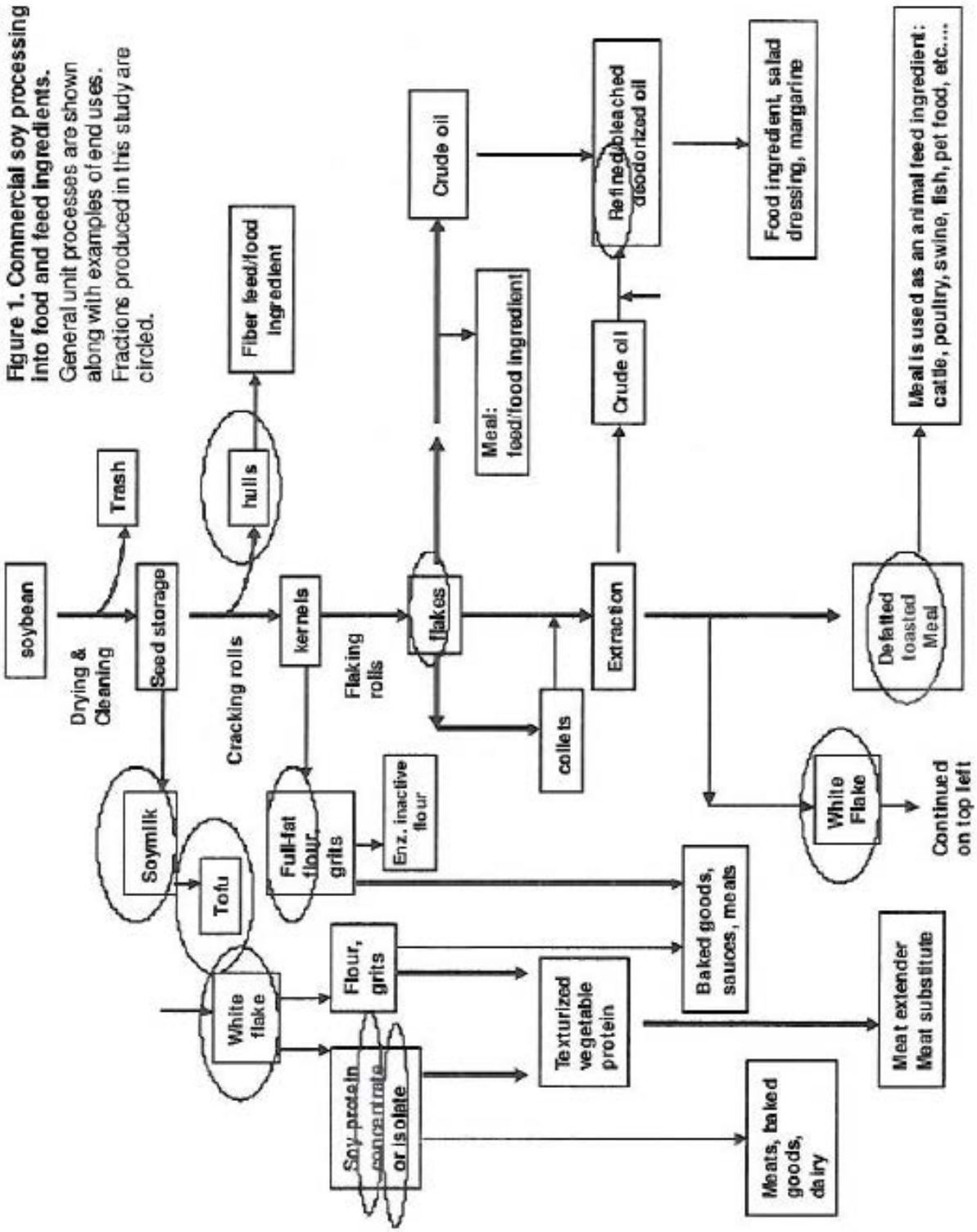


Figure 1. Commercial soy processing into food and feed ingredients.
General unit processes are shown along with examples of end uses. Fractions produced in this study are circled.

DATA ARCHIVAL:

Record Transfer and Retention:

This processing report as listed in the table of contents has been sent via overnight letter or package to Justin McDonald at Syngenta Crop Protection, LLC Product Safety Testing Facility in Research Triangle Park, North Carolina for archiving.

The Food Protein Research and Development Center will archive the following study specific data:

-copy of the sponsor processing protocol
-exact copy of the processing report (main body)
-exact copy of the compliance statement
-exact copy of the sample material balance
-exact copy of the original raw processing data (includes communication logs, calculations, and deviation forms, when applicable)
-exact copy of personnel records (names and initials of personnel with processing study duties)
-exact copy of receiving record(s)
-exact copy of shipping record(s)
-exact copy of shipping bill of lading(s)

The Food Protein Research and Development Center will archive the following non-study specific data indefinitely:

-original freezer and/or cooler temperature records
-original equipment logs (includes scales, temperature recording devices, and processing equipment records)
-CVs of personnel and training records

REVISION# 06 FORM# 300.3

MATERIAL BALANCE of SOYBEAN

Sample # **1 (Control)** Code # **TK0059702**

WHOLE SOYBEAN 35.0 lbs.***

Drying N/A lbs. after drying

Aspiration 0.3 g **LIGHT IMPURITIES**

Screening 0.1 lbs. **SMALL SCREENINGS**

0.2 lbs. **LARGE SCREENINGS**

Hulling & Separation

KERNEL 31.0 lbs.** **HULL MATERIAL** 2.8 lbs.

CLEANED HULLS 1.6 lbs **WASTE** 1.0 lbs

30.0 lbs. heated and flaked

Solvent Extraction 29.4 lbs. extracted

CRUDE OIL 2821 g

***TOASTED MEAL** 1.6 lbs.

***SOLVENT EXT'D FLAKES (white flakes)** 23.1 lbs.

Refining

300.0 g refined

15.9 g NaOH added

REFINED OIL 272.0 g **SOAPSTOCK** 19.7 g

* 2.0 lbs of white flakes were used to produce 1.6 lbs. of defatted toasted meal. 1300 g of white flakes were used to produce 1289 g of flour for soy protein isolate and soy protein concentrate. 625 g of flour yielded 131.2 g of isolate or 241.1 g of concentrate.

** 1.0 lbs. of kernels were ground to produce 139.6 g of full-fat flour.

*** 200.0 g of whole seed after cleaning were used to make soymilk (1,457.8 g) and tofu (175.0 g).

Footnote: All weights measured in pounds are from scale E which has a readability of 0.1.
Conversion factor: 1 lb=453.6g

REVISION# 06 FORM# 300.3

MATERIAL BALANCE of SOYBEAN

Sample # **2 (Test (SYHT0H2))** Code # **TK0059702**

WHOLE SOYBEAN 35.0 lbs.***

Drying N/A lbs. after drying

Aspiration 0.6 g **LIGHT IMPURITIES**

Screening 0.1 lbs. **SMALL SCREENINGS**

0.1 lbs. **LARGE SCREENINGS**

Hulling & Separation

KERNEL 30.0 lbs.** **HULL MATERIAL** 3.9 lbs.

CLEANED HULLS 1.8 lbs **WASTE** 2.0 lbs

29.0 lbs. heated and flaked

Solvent Extraction 28.2 lbs. extracted

CRUDE OIL 2965 g ***TOASTED MEAL** 1.7 lbs.

***SOLVENT EXT'D FLAKES (white flakes)** 21.3 lbs.

Refining

300.0 g refined

15.9 g NaOH added

REFINED OIL 267.1 g **SOAPSTOCK** 20.5 g

* 2.0 lbs of white flakes were used to produce 1.7 lbs. of defatted toasted meal. 1300 g of white flakes were used to produce 1292g of flour for soy protein isolate and soy protein concentrate. 625 g of flour yielded 136.1 g of isolate or 214.5 g of concentrate.

** 1.0 lbs. of kernels were ground to produce 114.7 g of full-fat flour.

*** 200.0g of whole seed after cleaning were used to make soymilk (1,423.8 g) and tofu (193.4 g).

Footnote: All weights measured in pounds are from scale E which has a readability of 0.1.
Conversion factor: 1 lb=453.6g.

APPENDIX B AvHPPD-03 Quantification Procedure

Reagents and kits used for extraction and enzyme-linked immunosorbent assay (ELISA) analysis of *p*-hydroxyphenylpyruvate dioxygenase (AvHPPD-03)

Buffer/ Item	Constituents
Phosphate-buffered saline with 0.05% Tween 20 (PBST)	138 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4, 0.05% Tween 20
Qualiplate™ Kits for HPPD in Soy	96-well plate precoated with anti-AvHPPD-03 antibody, AvHPPD-03 enzyme conjugate, substrate solution

AvHPPD-03 Extraction

Soybean Seed, Hulls, Full-fat Flour, Flakes, White Flakes, Defatted Toasted Meal, Protein Concentrate, Protein Isolate, Milk, and Tofu

For all samples except defatted toasted soybean meal, PBST buffer was added to lyophilized sample at a ratio of 3 ml of buffer to 30 mg of sample. The samples were homogenized using an Omni Prep Multi-Sample Homogenizer set at 30,000 revolutions per minute for two 30 second bursts. Samples were centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at -20°C ± 5°C until analysis. For defatted toasted meal, the procedure was the same except that buffer was added at a ratio of 3 ml of buffer to 15 mg of sample, and the sample was placed on wet ice for at least one hour.

AvHPPD-03 Quantification

The appropriate number of 96-well plates pre-coated with the capture antibody, the appropriate amounts of antibody/enzyme conjugate, and substrate solution were removed from storage at 2°C to 8°C and allowed to equilibrate to room temperature (all aforementioned items are provided in the Qualiplate™ ELISA Kit for HPPD in Soy). The tube containing the substrate solution was covered to prevent exposure to light. Dilutions of each sample extract and the ELISA standard (prepared using protein reference substance AvHPPD-03-0209 [Winslow 2009]), prepared in PBST, were applied to the plates at a volume of 50 µl/well. The plates were incubated at room temperature for at least 30 minutes while shaking. The plates were then washed five times prior to addition of the AvHPPD-03 enzyme conjugate (50 µl/well) and incubated at room temperature for 30 minutes while shaking. The plates were then washed five times prior to addition of the substrate solution (100 µl/well). The plates were covered while shaking to prevent exposure to light during incubation at room temperature until appropriate color development was reached (approximately 5 to 10 minutes). The colorimetric reaction was stopped by the addition of 1 N hydrochloric acid (100 µl/well) and measured at 450 and 650 nm. The results were analyzed with Molecular Devices SoftMax Pro® GxP Microplate Data Compliance Software, v. 5.4.1. The 650-nm reference measurement was subtracted from the 450-nm measurement prior to further analysis. The sample results were interpolated from a standard curve generated through the use of a four-parameter algorithm.

Validation of AvHPPD-03 Extraction Efficiency and ELISA Sensitivity

Protein extraction efficiency and method sensitivity (dilution factor, limit of detection [LOD], limit of quantitation [LOQ]) were determined for each sample type within this study. Method sensitivity data are summarized below.

Minimum dilution factor. The minimum dilution factor for each sample type was determined by analysis of a dilution series of nontransgenic extracts spiked with a known quantity of AvHPPD-03 reference protein. The most concentrated dilution of spiked sample extract that yielded a percent recovery between 70% and 120% and was followed by two subsequent dilutions with recoveries in the same range was selected as the minimum acceptable dilution factor.

The LOD for each sample type was evaluated by comparison of the mean optical density (OD) plus three standard deviations of the unspiked nontransgenic sample extract with the mean OD of the nontransgenic sample extract spiked with AvHPPD–03 reference protein. The measured LOD is the lowest spike concentration with an OD greater than the mean OD plus three standard deviations of the unspiked nontransgenic sample extract.

The LOD (micrograms per gram of sample) was calculated by the following formula:

$$\left(\frac{\text{LOD (ng/ml)} \times \text{dilution factor} \times \text{volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Limit of quantitation. The LOQ for each sample type was evaluated by spiking of nontransgenic sample extracts with known concentrations of AvHPPD-03 reference protein and measurement of the percent recovery of AvHPPD-03 protein. The LOQ was the lowest spike concentration of AvHPPD-03 that resulted in recovery of between 70% and 120% of nominal value and was greater than or equal to the LOD.

The percent recovery for each spiked sample was calculated by the following formula:

$$\left(\frac{\text{mean protein concentration of spiked extract } \left(\frac{\text{ng}}{\text{ml}}\right)}{\text{spiked protein concentration } \left(\frac{\text{ng}}{\text{ml}}\right)}\right) \times 100$$

The LOQ (micrograms per gram of tissue) was calculated by the following formula:

$$\left(\frac{\text{LOQ (ng/ml)} \times \text{dilution factor} \times \text{volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Extraction efficiency. The efficiency of the AvHPPD-03 extraction method was evaluated in each sample type through exhaustive protein extractions from transgenic samples. Each extraction was analyzed by ELISA to determine the concentration of AvHPPD-03 present.

The extraction efficiencies (percent) were calculated by the following formula:

$$\left(\frac{\text{Amount of AvHPPD-03 (ng/ml) from 1st extraction}}{\text{Total AvHPPD-03 (ng/ml) from all extractions}}\right) \times 100$$

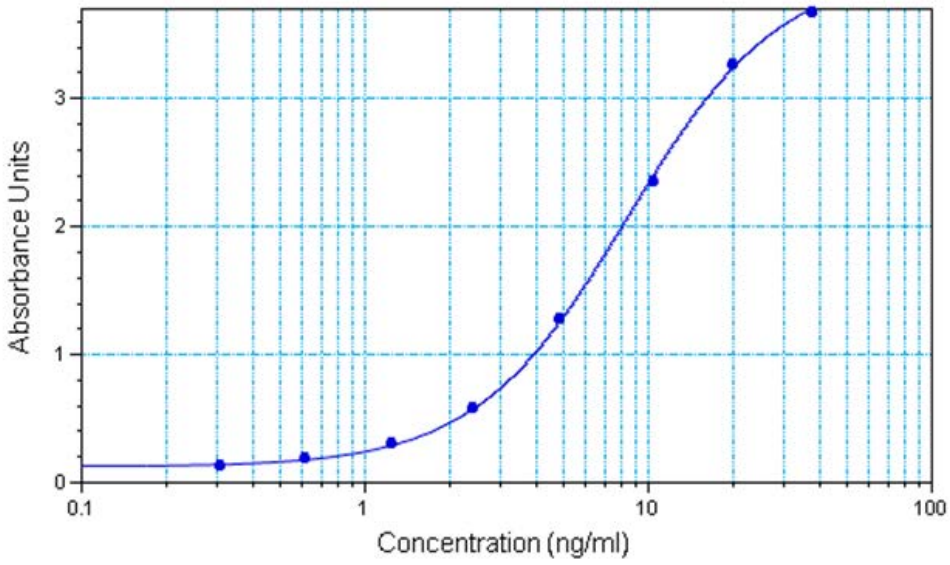
Extraction efficiency and method sensitivity data are summarized in the following table.

Protein extraction efficiency and method sensitivity for AvHPPD-03 quantitation in each matrix

Sample Type	Minimum Dilution Factor	Extraction Efficiency	LOD (µg/g[a])	LOQ (µg/g[a])
Soybean seed[b]	1	94%	0.0313	0.125
Hulls	1	79%	0.0313	0.0625
Full-fat flour	1	93%	0.0313	0.4
Flakes	2	88%	0.0625	0.8
White flakes	1	88%	0.0313	0.4
Defatted toasted meal	2	75%	0.0313	0.0625
Protein concentrate	1	—	0.0313	0.0625
Protein isolate	1	—	0.0313	0.0625
Milk	1	—	0.0313	0.0625
Tofu	1	—	0.0313	0.0625

[a] Sensitivity concentration limits for all matrices are reported as dry weight.
[b] The values for soybean seed are from Read (2011).
— = Extraction efficiency could not be determined because the levels of AvHPPD-03 were <LOD.

Representative Standard Curve. A representative standard curve for the AvHPPD-03 ELISA is depicted below. Concentrations used to generate this ELISA standard curve are: 40, 20, 10, 5, 2.5, 1.25, 0.625, and 0.313 ng/ml.



References

Winslow S. 2009. *Characterization of Microbially Produced Test Substance Containing p-Hydroxyphenylpyruvate Dioxygenase Protein (AvHPPD-03) and Certificate of Analysis.* Report No. SSB-041-09 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology, Inc.

APPENDIX C PAT Quantification Procedure

Reagents and buffers used for extraction and enzyme-linked immunosorbent assay (ELISA) analysis of PAT

Buffer/ Item	Constituents
Phosphate-buffered saline with 0.05% Tween® 20 (PBST)	138 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium phosphate, 1.8 mM potassium dihydrogen phosphate, pH 7.4, 0.05% Tween® 20
Qualiplate™ Kit for LibertyLink® PAT/*pat*	96-well plate precoated with anti-PAT antibody, PAT antibody/enzyme conjugate, substrate solution

PAT Extraction

Soybean Seed, Hulls, Full-fat Flour, Flakes, White Flakes, Defatted Toasted Meal, Protein Concentrate, Protein Isolate, Milk, and Tofu

PBST buffer was added to lyophilized sample at a ratio of 3 ml of buffer to 30 mg of tissue. The samples were homogenized using an Omni Prep Multi-Sample Homogenizer set at 30,000 revolutions per minute for two 30 second bursts. Samples were centrifuged at 2°C to 8°C to form a pellet. The supernatants were removed and stored at -20°C (±5°C) until analysis.

PAT Quantification

The appropriate number of 96-well plates pre-coated with the capture antibody, the appropriate amounts of PAT antibody/enzyme conjugate, and substrate solution were removed from storage at 2°C to 8°C and allowed to equilibrate to room temperature (all aforementioned items are provided in the Qualiplate™ ELISA Kit for LibertyLink® PAT/*pat*). The tube containing the substrate solution was covered to prevent exposure to light. The PAT enzyme conjugate solution was applied to each well at a volume of 50 µl/well. Immediately following the addition of the PAT antibody/enzyme conjugate solution, dilutions of each sample extract and the ELISA standard (prepared using protein reference substance, PAT-0109 [Seastrum 2009]), prepared in PBST buffer, were added to the pre-coated plates (50 µl/well). The plates were mixed in a rapid circular motion on the benchtop for 10 seconds and incubated at room temperature for at least one hour. The plates were washed five times with PBST buffer and the substrate solution was applied (100 µl/well). The plates were incubated at room temperature in the dark until appropriate color development was reached (approximately 15 minutes). The colormetric reaction was stopped by the addition of 1N hydrochloric acid (100 µl/well) and measured at 450 nm and 650 nm with an absorbance reader. The results were analyzed with Molecular Devices SoftMax Pro® GxP Microplate Data Compliance Software, v. 5.4.1. The 650 nm reference measurement was subtracted from the 450 nm measurement prior to further analysis. Concentrations were interpolated from a standard curve generated using a quadratic curve fitting algorithm.

Validation of PAT Extraction Efficiency and ELISA Sensitivity

Protein extraction efficiency and method sensitivity (dilution factor, limit of detection [LOD], limit of quantitation [LOQ]) were determined for each sample type within this study. Method sensitivity data are summarized below.

Minimum dilution factor. The minimum dilution factor for each sample type was determined by analyzing a dilution series of nontransgenic extracts spiked with a known quantity of PAT reference protein. The most concentrated dilution of spiked sample extract that yielded a percent recovery between 70% and 120%, and was followed by two subsequent dilutions with recoveries in the same range was selected as the minimum acceptable dilution factor.

Limit of detection. The LOD for each sample type was evaluated by comparison of the mean optical density (OD) plus three standard deviations of the unspiked nontransgenic sample extract with the mean OD of the nontransgenic sample extract spiked with PAT reference protein. The measured LOD is the lowest spike concentration with an OD greater than the mean OD plus three standard deviations of the unspiked nontransgenic sample extract.

The LOD (micrograms per gram of sample) was calculated by the following formula:

$$\left(\frac{\text{LOD (ng/ml)} \times \text{dilution factor} \times \text{volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Limit of quantitation. The LOQ for each sample type was evaluated by spiking nontransgenic sample extracts with known concentrations of PAT reference protein, and measuring the percent recovery of PAT protein. The LOQ was the lowest spike concentration of PAT that recovered between 70% and 120% of nominal value and was greater than or equal to the LOD.

The percent recovery for each spiked sample was calculated by the following formula:

$$\left(\frac{\text{mean protein concentration of spiked extract (ng/ml)}}{\text{spiked protein concentration (ng/ml)}}\right) \times 100$$

The LOQ (micrograms per gram of sample) was calculated by the following formula:

$$\left(\frac{\text{LOQ (ng/ml)} \times \text{dilution factor} \times \text{volume of extraction buffer (ml)}}{\text{amount of tissue extracted (g)}}\right) \div 1000$$

Extraction efficiency. The efficiency of the PAT extraction method was evaluated in each sample type through exhaustive protein extractions from transgenic samples. Each extraction was analyzed by ELISA to determine the concentration of PAT protein present.

The extraction efficiencies (percent) were calculated by the following formula:

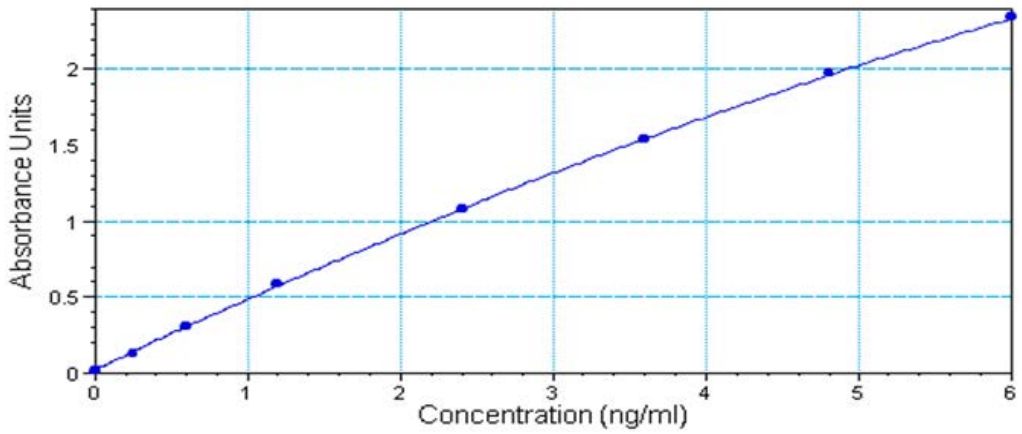
$$\left(\frac{\text{Amount of PAT (ng/ml) from 1st extraction}}{\text{Total PAT (ng/ml) from all extractions}}\right) \times 100$$

Protein extraction efficiency and method sensitivity for PAT quantitation in each matrix

Sample Type	Minimum Dilution Factor	Extraction Efficiency	LOD (µg/g[a])	LOQ (µg/g[a])
Soybean seed	1	88%	0.025	0.06
Hulls	1	77%	0.025	0.025
Full-fat flour	1	99%	0.025	0.06
Flakes	1	91%	0.025	0.06
White flakes	1	93%	0.025	0.06
Defatted toasted meal	1	70%	0.025	0.025
Protein concentrate	1	—	0.025	0.025
Protein isolate	1	—	0.025	0.025
Milk	1	—	0.025	0.025
Tofu	1	—	0.025	0.025

[a] Sensitivity concentration limits for all matrices are reported as dry weight.
— = Extraction efficiency could not be determined because the levels of PAT were <LOD.

Representative Standard Curve. A representative standard curve for the PAT ELISA is depicted below. Concentrations used to generate this ELISA standard curve are: 6.0, 4.8, 3.6, 2.4, 1.2, 0.60, 0.25, and 0.0 ng/ml.



References

Seastrum L. 2009. *Characterization of Microbially Produced Test Substance Containing Phosphinothricin Acetyltransferase (PAT) and Certificate of Analysis.* Report No. SSB-042-09 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology, Inc.