Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain

Data Requirement:	Not applicable
Author:	Mark Bednarcik
Study Completion Date:	June 14, 2010
Syngenta Study No.:	TKRS0000035
Report No.:	SSB-004-10
Sponsor and Testing Facility:	Syngenta Biotechnology, Inc. Regulatory Science and Trait Safety PO Box 12257 3054 East Cornwallis Road Research Triangle Park, NC 27709-2257, USA
Additional Performing Laboratory:	Food Protein R & D Center Building 8525 Texas A&M University 3100 Highway 47 Bryan, TX, 77807 USA

STATEMENTS OF DATA CONFIDENTIALITY CLAIMS

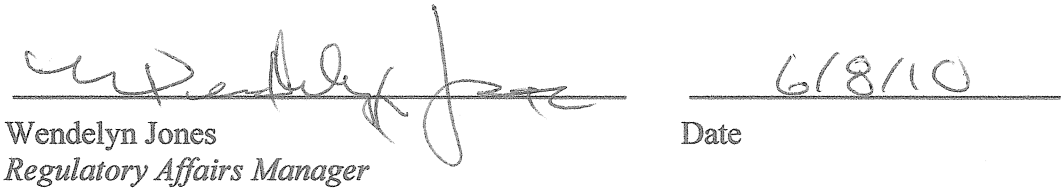
The following statement applies to submissions to the United States Environmental Protection Agency (US EPA).

Statement of No Data Confidentiality Claim

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:



Wendelyn Jones — Date
Regulatory Affairs Manager

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

STATEMENT CONCERNING GOOD LABORATORY PRACTICES STANDARDS

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

- Characterization of the test and control substances was not conducted according to GLPS

Study Director:

Mark J. Bednarcik — 6/14/10

Mark Bednarcik
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Catherine Kramer — 6/14/10

Catherine Kramer
Product Safety
Syngenta Biotechnology, Inc.

Date

QUALITY ASSURANCE STATEMENT

Study Title: Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions from Event 5307 Maize Grain

Study Director: Mark Bednarcik

Study Number: TKRS0000035

Report Number: SSB-004-10

Pursuant to Good Laboratory Practices 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 Date
Audit Protocol	12-MAR-2009 - 12-MAR-2009	12-MAR-2009
Inspect Analytical	16-NOV-2009 - 16-NOV-2009	16-NOV-2009
Audit Final Report (1st audit)	21-MAY-2010 - 27-MAY-2010	27-MAY-2010
Audit Final Report (2nd audit)	02-JUN-2010 - 02-JUN-2010	02-JUN-2010

See Appendix C, Processing Phase Report, for additional audits/inspections dates.

Prepared by: Connie L. Connor **Date:** June 14, 2010

Connie L. Connor
Senior Quality Assurance Auditor
Quality Assurance Unit
Syngenta Biotechnology, Inc.

syngenta

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LIST OF ACRONYMS AND ABBREVIATIONS

ELISA	enzyme-linked immunosorbent assay
GLPS	Good Laboratory Practices Standards
LOD	limit of detection
LOQ	limit of quantitation
PBS	phosphate-buffered saline
PMI	phosphomannose isomerase
PVP	polyvinylpyrrolidone
RBDD	refined, bleached, deodorized, winterized
SBI	Syngenta Biotechnology, Inc.
TMB	3,3′,5,5′-tetramethylbenzidine
Tris	2-amino-2(hydroxymethyl)-1,3-propanediol
Tween® 20	polyoxyethylene sorbitan monolaurate

SUMMARY

The purpose of this study is to determine the concentrations of eCry3.1Ab and phosphomannose isomerase (PMI) proteins in key wet- and dry-milled fractions produced from maize grain derived from transformation Event 5307 maize.

Using laboratory scale milling methodology, Event 5307 maize grain and nontransgenic, near-isogenic control grain were processed into commercially representative food and feed fractions: gluten, starch, and germ fractions were produced from wet-mill processing procedures, and flour and germ fractions were produced from dry-mill processing procedures.

Enzyme-linked immunosorbent assays were used to quantify eCry3.1Ab and PMI in the wet- and dry-milled fractions and in the grain used to produce those fractions.

The results of this study indicate that eCry3.1Ab and PMI proteins were present at quantifiable levels in flour and germ when produced by standard dry-mill processing of 5307 maize grain. In contrast, the eCry3.1Ab and PMI proteins were below the limit of detection in the dried germ, starch, and gluten when produced by standard wet-mill processing of 5307 maize grain.

INTRODUCTION

The purpose of this study is to determine the concentrations of eCry3.1Ab and phosphomannose isomerase (PMI) proteins in key wet- and dry-milled fractions produced from maize grain derived from transformation Event 5307 maize grain.

Using the techniques of modern molecular biology, Syngenta has transformed maize (*Zea mays*) to produce Event 5307 maize, a new cultivar that has insecticidal activity against certain corn rootworm (*Diabrotica*) species. Maize plants derived from transformation Event 5307 ("5307 maize") contain the gene *ecry3.1Ab*, encoding an eCry3.1Ab protein, and the gene *pmi* (also known as *manA*), encoding the enzyme phosphomannose isomerase (PMI). The eCry3.1Ab protein is an engineered chimera of modified Cry3A (mCry3A) and Cry1Ab proteins. The gene *pmi* was obtained from *Escherichia coli* strain K-12; the protein it encodes was utilized as a plant selectable marker during development of 5307 maize.

Using laboratory scale milling methodology, fractions from wet-mill and dry-mill processing procedures were produced from 5307 maize grain and from nontransgenic, near-isogenic maize grain. Key fractions most likely to enter the food and feed chain were analyzed for eCry3.1Ab and PMI by enzyme-linked immunosorbent assays (ELISA). Data from this study provides an estimate of the amount of eCry3.1Ab and PMI present in food and feed fractions produced from industry standards related to the processing of 5307 maize grain.

MATERIALS AND METHODS

Test, Control, and Reference Substances

The test substance for this study is Event 5307 maize grain and the control substance is nontransgenic, near-isogenic maize grain. Table 1 depicts the pedigree codes for the maize hybrids used to produce the test and control substances.

Table 1. Test and control substances

Substance description	Pedigree code
5307 maize grain (test)	NP2171 × NP2460 (5307)
Nontransgenic, near-isogenic maize grain (control)	NP2171 × NP2460

Test and control substances underwent the appropriate Stewardship Quality Control (SQC) testing.

Table 2 lists the protein reference substances used to produce standard curves for each ELISA.

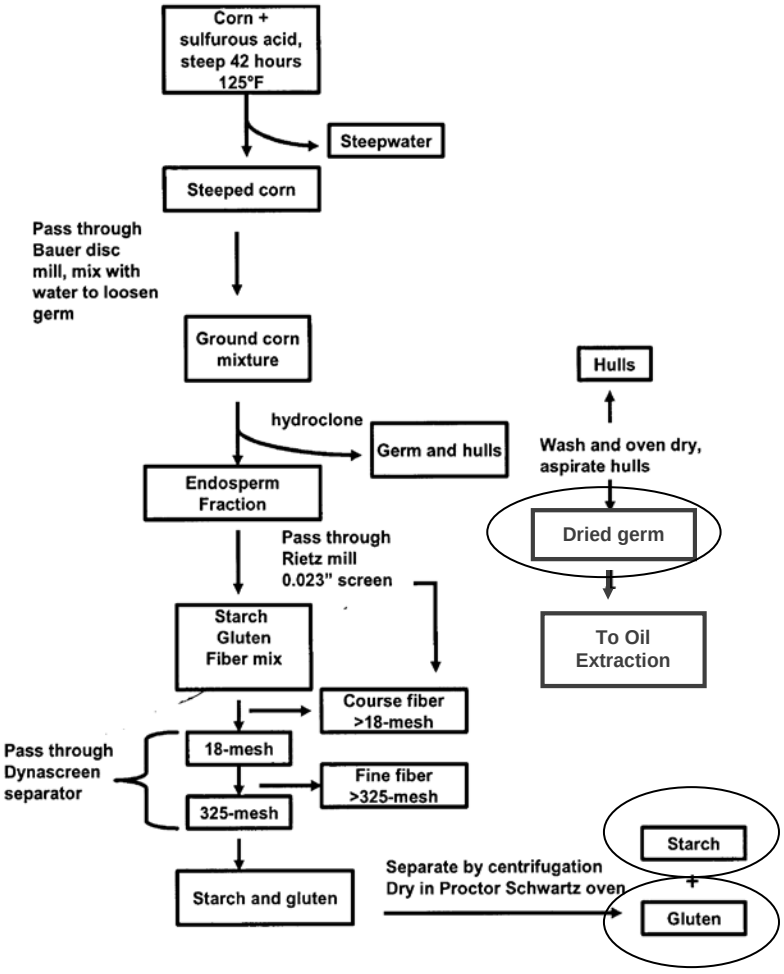
Table 2. Reference substances for ELISA analyses

Protein	Reference substance ID	Characterization report
eCry3.1Ab	ECRY3.1AB-0208	Nelson 2008a
PMI	PMI-0105	Nelson 2008b

Preparation of the Processed Fractions

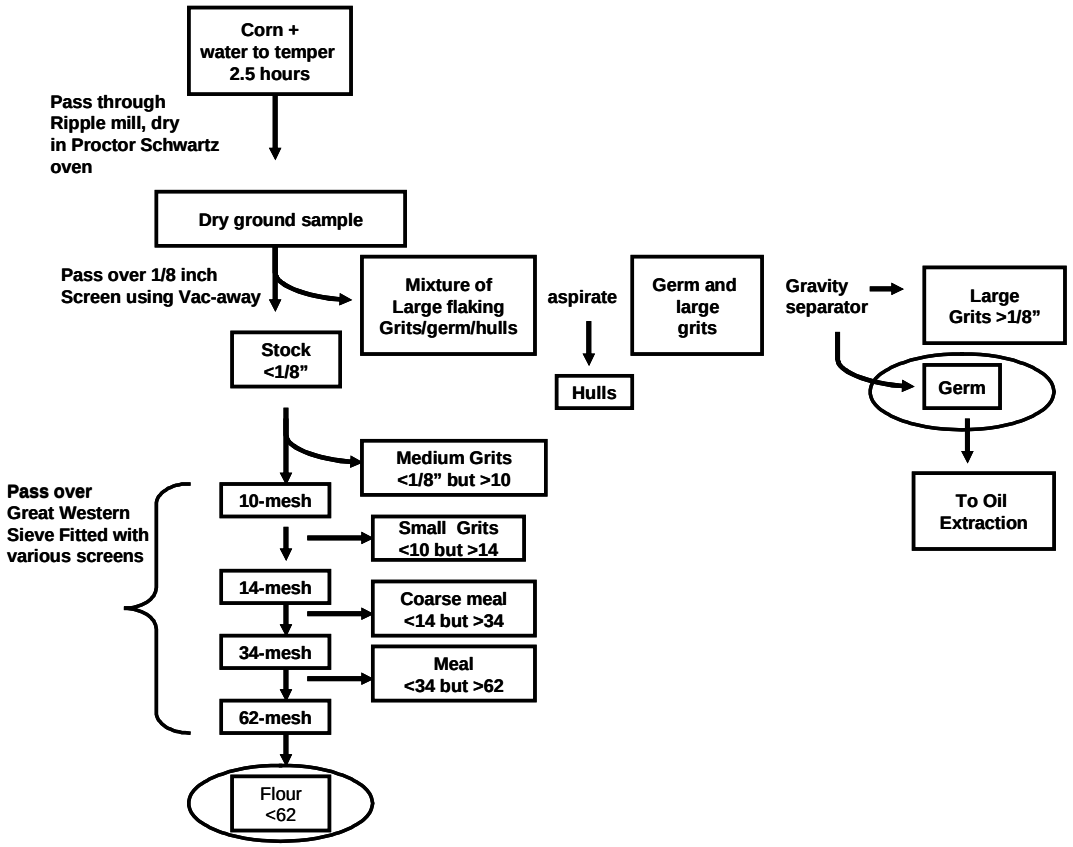
The test and control grains were processed into food and feed fractions at the Food Protein Research and Development Center, Texas A&M University, Bryan, Texas, USA, using standard wet- and dry-milling processes (Figures 1 and 2). Appendix C describes the details of the processing methodology.

Figure 1. Wet-milling flowchart



Vavra 2009; Appendix C

Figure 2. Dry-milling flowchart



Vavra 2009; Appendix C

The key end products of the wet- and dry-milling processes are circled in Figures 1 and 2 and listed in Table 3. These fractions are those most likely to enter the food and feed chain, and were selected for analysis in this study.

Table 3. Key processed fractions produced for this study

Wet-mill processing	Dry-mill processing
Dried germ Starch Gluten	Flour Germ

The processed fractions were shipped on ice packs to the Regulatory Science and Trait Safety Laboratory, Syngenta Biotechnology, Inc. (SBI), Research Triangle Park, NC, USA. Upon receipt, the wet- and dry-milled fractions and samples of the Event 5307 maize grain and the nontransgenic, negative control grain were stored at -80˚C ± 10˚C.

Sample Preparation

Table 3 shows the grain samples and the processed fractions; these samples were ground to a fine powder in the presence of dry ice. Nontransgenic samples were ground first to

prevent possible contamination by the transgenic samples. Each powdered sample was mixed thoroughly to ensure homogeneity and stored at -80ºC ± 10ºC.

Protein Extraction and ELISA Analysis

Protein extractions were performed on three representative aliquots of each fraction: three Event 5307 maize grain aliquots were extracted and three nontransgenic maize grain aliquots were extracted. For each sample, the insoluble material was subjected to iterative extractions, retaining the supernatants for analysis until the recovery of transgenic protein was either (1) not more than 5% of the total transgenic protein recovered from all extractions combined, (2) non-quantifiable, or (3) there was evidence that a subsequent extraction would produce a non-quantifiable result.

The extracts were analyzed by ELISA to quantify the amount of eCry3.1Ab and PMI in each sample (Tijssen 1985). Sample extracts were assayed in triplicate and standard curves were generated with known amounts of the corresponding protein reference substance for each ELISA plate. The mean absorbance levels of the extracts were plotted against the standard curves to obtain the concentrations of eCry3.1Ab and PMI in each sample extract (ng/ml). The amount of eCry3.1Ab and PMI protein in each sample was reported as the sum of the recovery from all quantifiable extractions.

The concentrations of the analyzed proteins in each sample (µg/g) were calculated as follows:

$$\frac{(\text{ng/ml}) \times (\text{dilution factor}) \times (\text{ml buffer})}{(\text{g sample}) \times 1000}$$

Appendices A and B describe the protein extraction and ELISA procedures.

LOQ and LOD Determination

The limit of quantitation (LOQ) for eCry3.1Ab and PMI is estimated based on acceptable recovery of a known concentration of reference protein added to nontransgenic sample extracts assayed at the minimal acceptable dilution factor.

The limit of detection (LOD) for eCry3.1Ab and PMI is estimated based on the lowest concentration of reference protein that can be distinguished from the background signal of nontransgenic sample extracts.

Control of Bias

Representative aliquots were analyzed from homogeneous samples. Any rejected data, and the documented reasons for the rejection of those data, are retained in the study file.

Statistical Analysis

All calculations, including means and standard deviations, were performed using Microsoft Office Excel® 2007 software.

RESULTS AND DISCUSSION

Table 4 shows the results of the analysis of the fractions produced by wet- and dry-milling of 5307 maize grain.

Table 4. Concentrations of eCry3.1Ab and PMI in 5307 maize grain, and key processed fractions derived from 5307 maize grain

Sample	Mean ± SD	
	eCry3.1Ab µg/g	PMI µg/g
Grain	4.98 ± 0.36	1.31 ± 0.05
Wet-milled fractions		
Gluten	< LOD [a]	< LOD [d]
Starch	< LOD [b]	< LOD [e]
Dried germ	< LOD [c]	< LOD [f]
Dry-milled fractions		
Flour	1.06 ± 0.03	0.20 ± 0.01
Germ	19.33 ± 2.08	3.97 ± 0.32

n = 3 replicate analyses for each sample
[a] LOD = 0.048 µg/g sample
[b] LOD = 0.048 µg/g sample
[c] LOD = 0.024 µg/g sample
[d] LOD = 0.003 µg/g sample
[e] LOD = 0.002 µg/g sample
[f] LOD = 0.003 µg/g sample

Analysis of nontransgenic sample extracts confirmed the absence of matrix effects for extracts of each sample type.

The concentrations of eCry3.1Ab and PMI are higher in the dry-mill corn germ than in the grain; this is consistent with the distribution of total protein within the kernel. Corn germ, which only accounts for 10% of the total dry weight of mature kernel, is 18% protein whereas the intact kernel is 8% to 10% protein (Boyer and Hannah 2001). More protein is extracted during dry-milling than wet-milling (Parris *et al*. 2006), and is likely to account for the differences in concentrations of eCry3.1Ab and PMI proteins in the dry-mill germ compared to the wet-mill germ.

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.

CONCLUSIONS

The results of this study indicate that eCry3.1Ab and PMI proteins were present at quantifiable levels in flour and germ when produced by standard dry-mill processing of 5307 maize grain. In contrast, the eCry3.1Ab and PMI proteins were below the limit of detection in the dried germ, starch, and gluten when produced by standard wet-mill processing of 5307 maize grain.

RECORDS RETENTION

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

CONTRIBUTING SCIENTISTS

Processing of grain into key fractions was performed under the supervision of Carl Vavra, Texas A & M University. Analytical work at SBI was conducted by Misti Patton, B.S., Jim Branson, and Emmanuel Ferew at the Regulatory Science and Trait Safety Laboratory, Research Triangle Park, NC, USA.

Reported by: Mark J. Bednarcik 6/14/10

Mark Bednarcik — Date
Regulatory Scientist, Protein Analysis Team
Product Safety
Syngenta Biotechnology, Inc.

Approved by: Catherine Kramer 6/14/10

Catherine Kramer — Date
Product Safety
Syngenta Biotechnology, Inc.

CRITICAL DATES

Study initiation date:	March 19, 2009
Experimental start date:	April 6, 2009
Experimental end date:	November 23, 2009

REFERENCES

Published

Boyer CD, Hannah LC. 2001. Kernel mutants of corn. In *Specialty Corns*. Hallauer A.R., ed. Boca Raton, FLA: CRC Press. p 1.

Parris N, Moreau RA, Johnston DB, Singh V, Dickey LC. 2006. Protein Distribution in commercial wet- and dry-milled corn germ. *J. Agric. Food Chem*. **54**: 4868-4872.

Tijssen P. 1985. Processing of data and reporting of results of enzyme immunoassays. In *Practice and Theory of Enzyme Immunoassays*. *Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 15.* Amsterdam, Netherlands: Elsevier Science Publishers. pp. 385–421.

US EPA. 1989. Good Laboratory Practices Standards. 40 CFR Part 160.

Unpublished

Nelson A. 2008a. *Characterization of Test Substance ECRY3.1AB-0208 and Certificate of Analysis.* Report No. SSB-010-08 (unpublished). Research Triangle Park, NC. Syngenta Biotechnology.

Nelson A. 2008b. *Characterization of Phosphomannose Isomerase Test Substance PMI-0105 and Certificate of Analysis.* Report No. SSB-025-07 (unpublished). Research Triangle Park, NC: Syngenta Biotechnology.

APPENDICES

Appendix A: eCry3.1Ab Quantification Procedure

Buffers

The buffers used for extraction and enzyme-linked immunosorbent assay (ELISA) analysis of eCry3.1Ab are listed in the following table.

Name of buffer	Constituents
Phosphate-buffered saline (PBS)	140 mM sodium chloride, 8.24 mM sodium phosphate dibasic, 1.81mM sodium phosphate monobasic, pH 6.75
Borate extraction buffer	0.1 M sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 0.5% Tween® 20; titrated to pH 10.0. Complete™ Protease Inhibitor Cocktail Tablet (added on day of extraction)
Dilution buffer	PBS, 0.05% Tween® 20, 1% BSA, 0.02% sodium azide
Wash buffer	10 mM Tris, 0.05% Tween® 20, 0.02% sodium azide

eCry3.1Ab Extraction

For each sample, 6 ml of borate extraction buffer was added to ~ 500 mg of sample. The samples were mixed, placed on wet ice for at least 30 minutes, homogenized, and centrifuged at 2°C to 8°C to form a pellet. The supernatants were transferred to a fresh tube and the pellets underwent additional protein extraction procedures until quantifiable levels of protein were no longer recovered. Supernatants were diluted 1:2 in dilution buffer and stored at -20°C ± 5°C until analysis.

eCry3.1Ab Quantification

The eCry3.1Ab ELISA kit was manufactured at Beacon Analytical Systems (BAS), Portland, ME. The assay is a double-antibody sandwich assay in which the eCry3.1Ab protein is affixed to the wells of a microtiter plate using a monoclonal, anti-mCry3A antibody that binds to the mCry3A domains of the eCry3.1Ab protein. The primary antibody was diluted and added to each well of a 96-well microtiter plate. The plate was then blocked using a proprietary method. Dilutions of each tissue extract and the appropriate serial dilutions of the protein reference substance, ECRY3.1AB-0208 (Nelson 2008a), were prepared in dilution buffer and applied to the pre-coated plates at a total volume of 100 µl/well. The plates were incubated at room temperature on a titre plate shaker at 400 rpm for one hour. The plates were washed five times with wash buffer in a microplate washer. After washing the plates, a secondary polyclonal rabbit anti-Cry1Ab antibody (provided in the kit) was then used to bind the Cry1Ab domain of the eCry3.1Ab protein at 100 µl/well. The plates were incubated at room temperature on a titre plate shaker at 400 rpm for one hour and washed five times.

After the plates were washed, a tertiary donkey anti-rabbit antibody conjugated with alkaline phosphatase was diluted in dilution buffer and added to each of the wells (100 µl/well). The plates were then incubated at room temperature on a titer plate shaker at 400 rpm for one hour, and then washed five times (described above). After the plates were washed, an alkaline phosphatase substrate solution (provided in the kit) was added at a volume of 100 µl/well. The plates were incubated for 30 minutes at room

temperature on a titer plate shaker at 400 rpm. The reaction was stopped by the addition of 3N sodium hydroxide (100 µl/well), and the colorimetric reaction was measured at a dual wavelength (405-492 nm) with an absorbance reader. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2, using a four-parameter algorithm.

Appendix B: PMI Quantification Procedures

Buffers

The buffers used for extraction and enzyme-linked immunosorbent assay (ELISA) analysis of PMI are listed in the following table.

Name of buffer	Constituents
Borate extraction buffer	0.1 M sodium tetraborate decahydrate, 0.2% PVP-360, 7.69 mM sodium azide, 1.2% concentrated hydrochloric acid, 0.5% Tween® 20; pH approximately 7.5. Complete™ Protease Inhibitor Cocktail Tablet added on day of extraction
Carbonate-bicarbonate buffer	34.9 mM sodium bicarbonate, 15.0 mM sodium carbonate, pH 9.5
Citrate-phosphate buffer	23.8 mM citric acid, 59.9 mM disodium phosphate, pH 5.0
Super Block® T20 (PBS) Blocking Buffer	A protein based blocker formulation in phosphate buffered saline containing 0.05% Tween® 20
1X Tris wash buffer	10mM Tris, 0.05% Tween® 20, pH 8.0

PMI Extraction

For each sample, 6 ml of borate extraction buffer was added to ~ 500 mg of sample. The samples were mixed, placed on wet ice for at least 30 minutes, homogenized, and centrifuged at 2°C to 8°C to form a pellet. The supernatants were transferred to a fresh tube and the pellets underwent additional protein extraction procedures until quantifiable levels of protein were no longer recovered. Supernatants were stored at 2°C to 8°C until analysis.

PMI Quantification

A polyclonal rabbit anti-PMI antibody was diluted in carbonate-bicarbonate buffer and added to each well of a 96-well microtiter plate at a volume of 100 µl/well. The plates were stored overnight in a refrigerator set at 2°C to 8°C. The plates were washed three times with 1X Tris wash buffer. As a blocking step, Super Block® T20 (PBS) Blocking Buffer was added to the plates at a volume of 300 µl/well. Blocking step was repeated two more times. After blocking incubation, the plates were washed three times as described above and dilutions of each tissue extract and appropriate serial dilutions of the protein reference substance PMI-0105 (Nelson 2008b) prepared in Super Block® T20 (PBS) Blocking Buffer were applied to the plates at a total volume of 100 µl/well. The plates were incubated at ambient temperature for 1 hour while shaking at approximately 400 rpm. After incubation, plates were washed five times as described above and a monoclonal anti-PMI antibody diluted in Super Block® T20 (PBS) Blocking Buffer was added to the plate at a volume of 100 µl/well and incubated at ambient temperature for one hour while shaking at approximately 400 rpm.

The plates were washed three times after incubation and a horseradish-peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody diluted in Super Block® T20 (PBS) Blocking Buffer was added at a volume of 100 µl/well and incubated at ambient temperature for one hour while shaking at approximately 400 rpm. After incubation, the plates were washed three times, and 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution (was added at a volume of 100 µl/well (one tablet per 10 ml of citrate-phosphate

buffer) and incubated at room temperature in the dark for 15 minutes while shaking at approximately 400 rpm. The reaction was stopped by addition of 3 M sulfuric acid at a volume of 50 µl/well, and the absorbance of the reaction was read at 450 nm with an absorbance reader. The results were analyzed with BioMetallics DeltaSoft PC Microplate Analysis Software, v. 1.71.2, using a four-parameter algorithm.

Appendix C: Processing Phase Report

Corn: dry-milling, wet -milling, and oil production

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Syngenta Biotechnology, Inc.
Regulatory Science
Research Triangle Park, North Carolina

STUDY DIRECTOR:
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Regulatory Science
P.O. Box 12257
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Research Triangle Park, NC 27709

REPORT:

Corn: Dry Milling, Wet Milling, and Oil Production

STUDY TITLE:

Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain

AUTHOR:

Carl Vavra

Carl Vavra
Signature

11-16-09
Date

Testing Facility:

GLP Program
Texas A & M University
Food Protein Research and Development Center
3100 Highway 47, Building 8525
Bryan, TX 77801

GLP COMPLIANCE STATEMENT

Project Title: **Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain**

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

Carl Vavra
Processing Principal Investigator

11-16-09
Date

QUALITY ASSURANCE STATEMENT

PROJECT TITLE: Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain

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: Chm G. Mm Date: 11/18/2009

Christopher A. Mack
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) Process Phase - WET MILL SOP 8.5 R16 Sec. 2 "Conditioning Grain by Steeping"	April 28-30 & May 3-5, 2009	May 19, 2009	May 20, 2009
2) Process Phase - DRY MILL SOP 5.7 R08 "Shipment of Process Samples"	June 9, 2009	June 23, 2009	July 1, 2009
3) Process Report	October 27-30, 2009	November 3, 2009	November 3, 2009

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A. Original Raw Data
(includes communication logs, calculations, processing personnel id, and deviation forms, when applicable)

B. Freezer and Refrigerator Temperature Records
(exact copies)

C. Original Receiving (form #100.26) and Shipping (form #100.27) Records
(includes bill of lading(s), when applicable)

PROJECT Title: **Quantitation of eCry3.1Ab and Phosphomannose Isomerase in Key Processed Fractions Prepared from Event 5307 Maize Grain**

SPONSOR: Syngenta Biotechnology, Inc.
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P.O. Box 12257
3054 East Cornwallis Road
Research Triangle Park, NC 27709

STUDY DIRECTOR: Mark Bednarcik

STUDY MONITOR: David Patton

PROCESSING, DATA RECORDING & SHIPPING TECHNICIANS: Carl Vavra, George Sassano, Stacy Williams

SAMPLE RECEIPT DATE: April 03, 2009

PROCESSING START DATE: April 06, 2009

PROCESSING TERMINATION DATE: June 09, 2009

FRACTION SHIPMENT DATE:

April 21,2009 Dry Mill Flour from Sample A (5307 Negative Grain)
Dry Mill Flour from Sample B (5307 Positive Grain)

Dry Mill Germ from Sample A (5307 Negative Grain)
Dry Mill Germ from Sample B (5307 Positive Grain)
Whole Corn A&B Samples

May 19,2009 Wet Milled Starch from Sample A (5307 Negative Grain)
Wet Milled Starch from Sample B (5307 Positive Grain)

	Wet Milled Germ from Sample A (5307 Negative Grain)
	Wet Milled Germ from Sample B (5307 Positive Grain)
	Wet Milled Gluten from Sample A (5307 Negative Grain)
	Wet Milled Gluten from Sample B (5307 Positive Grain)
June 09, 2009	RBDD Oil Wet Milled from Sample A (5307 Negative Grain)
	RBDD Oil Wet Milled from Sample B (5307 Positive Grain)
	RBDD Oil Dry Milled from Sample A (5307 Negative Grain)
	RBDD Oil Dry Milled from Sample B (5307 Positive Grain)

INTRODUCTION:

Corn grain samples were received from Syngenta Biotechnology, Inc. and were processed into commercially representative fractions. Key fractions as requested by the Protocol were sent to Syngenta Biotechnology, Inc. in Research Triangle Park, North Carolina.

TEST SUBSTANCE: [From protocol and/or MSDS]

The test substance supplied by Syngenta was 5307 grain (Sample B).

CONTROL:

The control substance was the Nontransgenic grain (Sample A)

OBJECTIVE:

To produce key dry milling, wet milling and oil fractions from grains of transgenic field corn event 5307 and its non-transgenic counterpart for Quantitation of eCry3.1Ab and Phosphomannose Isomerase and PMI analysis.

Dry Milling Products
Flour
Germ

Wet Milling Products
Gluten
Starch
Dried Germ

Oil Products
Refined, bleached, dewaxed (winterized) oil from dry-milled germ
Refined, bleached, dewaxed (winterized) oil from wet-milled germ

METHODS & MATERIALS:

Sample Receipt:

Two corn grain samples were received at ambient temperature at the Food Protein Research and Development Center's GLP Program in Bryan, Texas on April 03, 2009. The samples were shipped April 02, 2009 from Research Triangle Park, North Carolina. The samples were identified and processed in the following order: Nontransgenic (Sample A) and 5307 Positive Grain (Sample B).

Storage Conditions:

GLP Program SOP 5.2 "Storage of Residue Samples in Walk-in Freezers" requires that freezer temperatures be maintained at or below 10 degrees Fahrenheit with the exception of the defrost cycle and removal and placement of samples in the freezers. However, according to Protocol samples received are to be stored at ambient temperature or 2-8°C. Samples received were actually stored in Cooler # 8525. However once the grain was removed from the cooler all fractions other than oil were stored at ambient temperature (Laboratory room 122, 74 °F) according to Protocol until shipment.

All Oil Samples (crude, refined, bleached, deodorized, dewaxed (winterized)) with the exception of partially refined oil samples (stored at 35-45°F) were stored at less than 10°F according to SOP 5.2.

Recorded in the data are the times and dates for removal or placement of samples/fractions in freezers, coolers or laboratory at room temperature. Included with this report are copies of temperature charts for applicable units.

Sample/Fraction Handling:

Samples were handled in a manner that minimizes the possibility of contamination. It is this facility's policy to use only containers and utensils washed with detergent and rinsed with water.

Corn Cleaning:

Corn samples that were received were very clean with no visually detectable impurities, therefore no cleaning steps were performed.

Processing Methods

Dry Milling Process:

The whole corn grain was moisture conditioned to 20-22% and allowed to "temper" for 2-2.5 hours. After tempering, the corn was then impact milled in a Ripple mill. After milling, the cornstock was dried at 130-160°F for 30 minutes, allowed to cool to approximately 90°F after removal from the oven. The cornstock was passed over a 1/8" shaker screen. Material above the screen was further processed into large grits, germ, and hull (bran). Material through the screen was separated into medium and small grits, coarse meal, meal, and flour.

The material above the 1/8" screen was passed through a Kice aspirator to separate the hull material from the large grits and germ. Material was passed through a Ripple Mill and then the Vac-A-Way and finally the Kice aspirator. Large grits and germ from the aspiration were separated on an Oliver gravity separator. The germs were combined and dried at 130-160°F to 8-12% moisture.

The material passing through the 1/8" shaker screen was separated using a Great Western sample sifter. The sifter was fitted with the following screen sizes: 0.0800", 0.0540", 0.0204", and 0.0098". Material on top of the 0.0800" screen is medium grits; material on top of the 0.0540" screen is small grits; material on top of the 0.0204" screen is coarse meal; material on top of the 0.0098" screen is meal; and material through the 0.0098" screen is flour.

Wet Milling Process:

Corn was steeped in 120-130°F water containing 0.1-0.2% sulfur dioxide (sulfurous acid) for 22-48 hours. At the end of the steeping period, the whole corn was passed through a Bauer mill with devil toothed plates and a majority of the germ and hull were removed using a hydroclone. Germ and hull were dried at 165-195°F to obtain a final moisture between 5-10%. After drying, the germ and hull were separated using aspiration.

The cornstock (without germ and hull) was ground in a Rietz mill with a 0.023" screen. The material going through the 0.023" screen was passed through a Dynascreen equipped with a 43-micron screen. Material on top of the screen is a product of batch processing and is discarded. In commercial industry, only bran (hull material) remains on top of the screen. The process water (with starch and gluten) passing through the 43-micron screen was separated into component parts using batch centrifugation.

Oil Processing:

Both the dry and wet milled germ was heated to 160-175°F and held in this range for 10 minutes. Heated germ was flaked in a Ferrell Ross flaking roll with a gap setting of 0.008 to 0.012" and promptly taken to solvent extraction.

The flaked germ was placed in stainless steel batch extractors and submerged in 120-140°F solvent (hexane). After 30 minutes, the hexane was drained and fresh hexane added to repeat the cycle two more times. The final two washings were for 30 minutes each. After the final draining, meal was air desolventized overnight in stainless steel batch extractors with a cover (lid) to remove residual hexane.

Miscella (crude oil and hexane) was passed through a Precision Scientific Recovery unit to separate the crude oil and hexane. Crude oil is heated to 163-194°F for hexane removal. Miscella was further desoventized using a Buchi rotovap.

The crude oil recovered from solvent extraction was refined according to AOCS - American Oil Chemists Society method Ca9a52 as follows: After determining the percent free fatty acid in the crude oil, a weighed sample was placed in a Laboratory Oil Refining Machine. A weighed amount of 16 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 14-16 minutes at 250 RPM and a temperature of 68-75°F and then for an additional 11-13 minutes at 70 RPM and a temperature of 145-153°F. Neutralized oil is allowed to settle for one hour at 140-149°F. The oil solution is refrigerated overnight. After refrigeration, refined oil is decanted and filtered. The fraction settling to the bottom of the refrigerated container is soapstock.
After refining, the refined oil and soapstock are separated. The refined oil was **Bleached -** A weighed oil sample is mixed and heated to 40-50°C (104-122°F) prior to adding a specified amount of activated bleaching earth. As the solution is mixed, a vacuum of 24-30" Hg is applied, and the temperature is increased to a range of 85-100°C (185-212°F). Agitation is continued under vacuum at a rate to provide sufficient contact between adsorbent and oil (operator discretion).Maintain the agitation, vacuum, and temperature for 10 to 15 minutes. At the end of this period, reduce the temperature of the solution to 58-68°C (136-154°F) and break the vacuum. Bleached oil is promptly filtered.
Deodorization - A weighed oil sample is heated and steam bathed for 30 minutes ± 2 minutes under a vacuum of 26 to 30" Hg. The

temperature of the oil is held between 428-446°F (220-230°C). At the end of the half hour period, the oil sample is allowed to cool to 276-302°F (135-150°C). At this time a citric acid solution (0.005%) is added to the oil sample at a rate of one milliliter per 100 grams of oil. The sample is allowed to cool to 220-239°F (105-115°C) before breaking vacuum. Resulting fractions from the oil deodorization step are deodorized oil and distillates. **Dewaxing (Winterization) -** A weighed oil sample is mixed with a small amount of filter aid to serve as crystallization sites (Celite 545 for example). Incubate in cold room, or water bath, at a temperature ranging from 4-10°C (39-50°F), for 12 hours minimum. At end of incubation period, mix sample with filter aid (1% w/w) and filter promptly. Note: the amount of filter aid can be adjusted by operator to facilitate filtration, as can the size of the Buchner funnel.

The processing procedures are outlined on the Material Balance forms.

Comparison to Industrial Practice:

The corn was wet and dry milled in a way that closely simulates commercial practices. Slight variations in industrial milling practices are designed to suit the buyer's needs.

The majority of commercial plants will remove the oil from the germ by expelling (hardpressing). A small percentage will utilize direct solvent extraction to remove the crude oil. Due to equipment available to the GLP Program, hardpressing is not possible.

In comparison, the program's goal is to produce the same component parts for each sample within a study to be used in residue determination. Because of compliance monitoring requirements and sample size, the samples were processed by batch rather than continuous, as in commercial operation.

Due to equipment limitations and batch processing the material balance values for wet milling products will be estimated using percentages from the CRC Handbook of Processing and Utilization in Agriculture. Fraction yields obtained by the industry are not made public. Yields from commercial wet milling plants will vary between plants depending on quality of the corn and differences in milling practice and fiber and germ washing operations. The following table is for approximate yields.
The following percentages are used for material balance forms.

Solubles from steeping	-	7.5%
Starch	-	67.5%
Gluten	-	5.8%
Germ	-	7.5%
Hull	-	11.5%

Processing Results:

An unprocessed sample was taken before processing.

Whole corn from both the control and test substance was processed through dry and wet milling including oil refining into the following key fractions:

Dry Milling Products
Flour
Germ

Wet Milling Products
Gluten
Starch
Dried Germ

Oil Products
Refined, bleached, dewaxed (winterized) oil from dry-milled germ
Refined, bleached, dewaxed (winterized) oil from wet-milled germ

All other processing fractions collected during this study are listed in the original raw data and disposed of (SOP 5.13) if not requested by the Protocol.

Other Circumstances Pertaining to Study:

The following protocol deviations were noted during processing:

During Dry & Wet Mill Processing the following protocol deviation was reported to the Study Director.

1. Pressing of corn germ not done. Small sample amounts were not sufficient to operate our press for processing. Processing of germ was completed following SOP 8.6 Revision # 12, Small Scale Dry Milling of Corn, Section # 6 which included heating of germ and flaking before extraction of oil with hexane.

2. Additional water was inadvertently added for steeping step. SOP 8.6 Rev. 13 requires steeping at 21% +/- 1% moisture. Our moisture level was 29.02%, Sample was dried to 21.8%.

3. Processor inadvertently added water to germ fraction before sample was taken. SOP 8.5 Rev 16 Section 4 requires taking fraction before moisture is added before solvent extraction processing. A 100 gram sample was put in a metal tray and left in the lab @ 72°F to air dry for the afternoon. A sample was taken at the end of the day and the moisture was 9.16%.

Fraction Shipment:

Processed corn fractions were shipped packed with ice blocks, priority overnight delivery to Syngenta Biotechnology, Inc. Research Triangle Park, North Carolina by Federal Express on April 21, May 19, and June 09, 2009. A Chain of Custody accompanied fraction shipment.

CONCLUSIONS:

Corn grain samples were processed into commercially representative fractions.

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 Mr. Mark Bednarcik at Syngenta Biotechnology, Inc., Regulatory Science P.O. Box 12257 3054 East Cornwallis Road Research Triangle Park, North Carolina 27709 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 refrigerator temperature records
-original equipment logs (includes scales, temperature recording devices, and processing equipment records)
-CVs of personnel and training records

SOPs Used:

SOP 5.13 Revision 01, "Disposal of unprocessed RACs and processing waste"
SOP 8.6 Revision 13 "Small-Scale Dry Milling of Corn"
SOP 8.5 Revision 16 "Small-Scale Wet Milling of Corn"
SOP 8.11 Revision 8 "Laboratory Bleaching of Vegetable Oil"
SOP 8.13 Revision 8 "Laboratory Deodorization of Vegetable Oil"
SOP 8.51 Revision 00 "Laboratory Dewaxing of Vegetable Oil"

The GLP Program at Texas A&M University followed relevant Standard Operating Procedures (SOPs) during the course of the processing portion of the study. The program retains copies of all SOPs including each SOP revision.

MATERIAL BALANCE of DRY CORN

Sample Number: **Sample A - Nontransgenic**

WHOLE CORN 25.0 lbs.

Drying (130-160°F) 24.4 lbs. (after drying)

Aspiration N/A lbs. **LIGHT IMPURITIES**

Screening N/A lbs. **SMALL SCREENINGS**
N/A lbs. **LARGE SCREENINGS**

24.4 lbs. Dry Milled

Steeping 2.2 lbs. water added (room temp)

Degermination, Drying, (130-160°F) Screening, Aspiration, and Separation

14.8 lbs. **LARGE GRITS** 2.1 lbs. **COARSE MEAL**
1.5 lbs. **MEDIUM GRITS** 1.4 lbs. **MEAL**
1.5 lbs. **SMALL GRITS** 1.8 lbs. **FLOUR**
0.7 lbs. **HULL MATERIAL**

1.4 lbs. **GERM** (Dried to N/A lbs.)

Conditioning, (160-175°F) & Flaking 1.4 lbs. Germ Conditioned

85.2 g **CRUDE OIL** 1.0 lbs. **SOLVENT EXTRACTED GERM** (120-140°F)

59.4 g Refined 2.5 g NaOH added

51.8 g **REFINED OIL** Step #1(145-153°F) **SOAPSTOCK** 7.3 g
Step #2(140-149°F)
51.8 g Bleached

44.3 g **BLEACHED OIL** (185-212°F)

44.3 g Deodorized

40.5 g **DEODORIZED OIL**(428-446°F) 110.5 g **DEODORIZER DISTILLATES**

31.0 g **Dewaxed Oil**

40.5 g **Dewaxed** (39-50°F)

REVISION# 06 FORM# 300.6

MATERIAL BALANCE of DRY CORN

Sample Number: **Sample B 5307 Positive**

WHOLE CORN 25.0 lbs.

Drying (130-160°F) 24.3 lbs. (after drying)

Aspiration N/A lbs. **LIGHT IMPURITIES**

Screening N/A lbs. **SMALL SCREENINGS**

N/A lbs. **LARGE SCREENINGS**

24.3 lbs. Dry Milled

Steeping 2.2 lbs. water added (room temp)

Degermination, Drying, (130-160°F) Screening, Aspiration, and Separation

15.5 lbs. **LARGE GRITS** 1.7 lbs. **COARSE MEAL**
1.9 lbs. **MEDIUM GRITS** 1.5 lbs. **MEAL**
1.8 lbs. **SMALL GRITS** 1.2 lbs. **FLOUR**
0.4 lbs. **HULL MATERIAL**

3.4 lbs. **GERM** (Dried to 3.4 lbs.)

Conditioning, (160-175°F) & Flaking 3.4 lbs. Germ Conditioned (120-140°F)

167.9 g **CRUDE OIL** 2.8 lbs. **SOLVENT EXTRACTED GERM**

131.6 g Refined 5.4 g NaOH added

116.9 g **REFINED OIL** Step #1(145-153°F) **SOAPSTOCK** 17.7 g

Step #2(140-149°F)

116.9. g Bleached

107.5 g **BLEACHED OIL** (185-212°F)

107.5 g Deodorized

101.1 g **DEODORIZED OIL** (428-446°F) 100.2 g **DEODORIZER DISTILLATES**

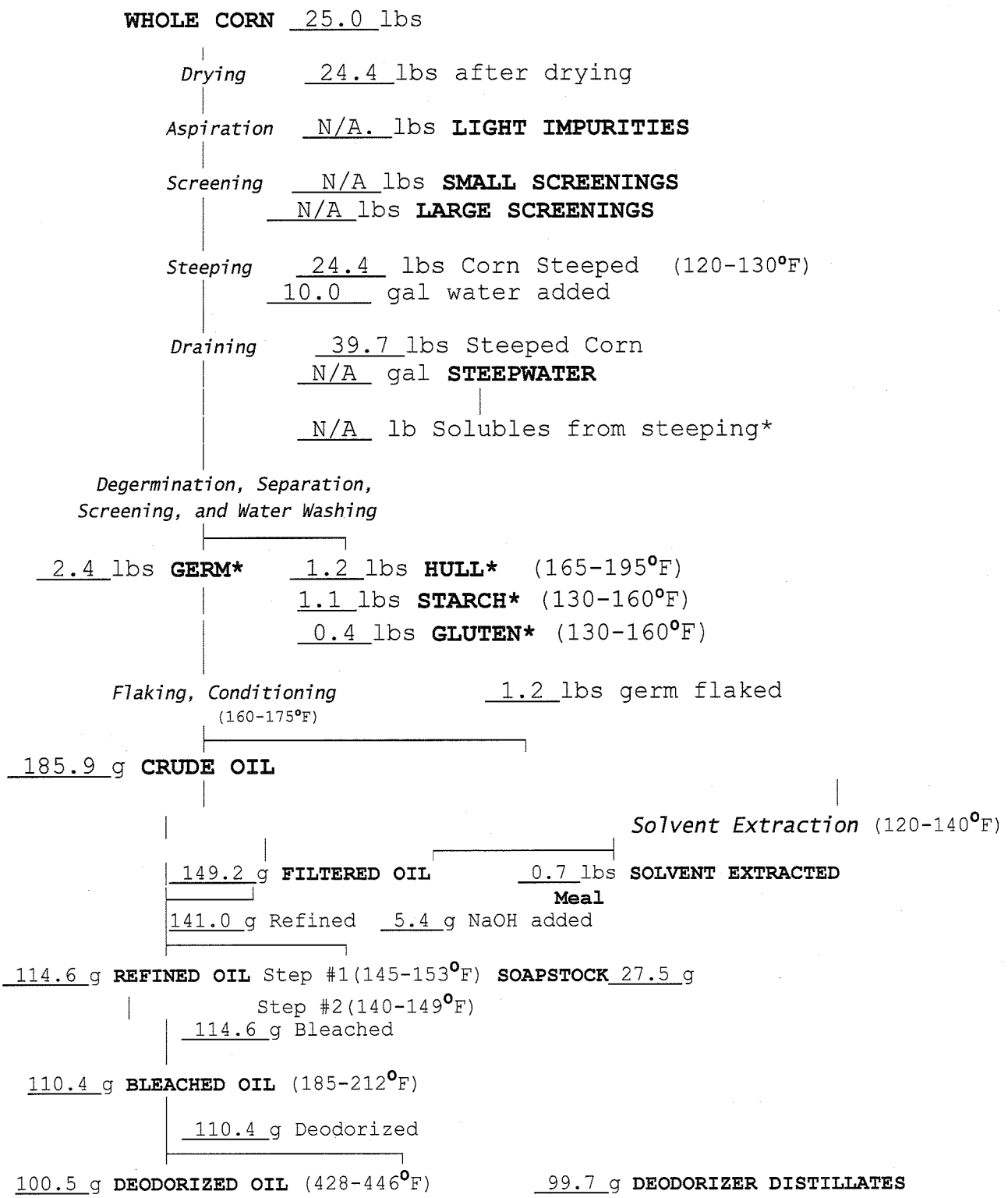
74.0 g **Dewaxed Oil**

101.1 g **Dewaxed** (39-50°F)

REVISION# 06 FORM# 300.5

MATERIAL BALANCE of WET CORN

Sample Number: **Sample A Nontransgenic**



78.9 g **Dewaxed Oil** 100.5 g **Dewaxed** (39-50°F)

* Calculated amounts based on commercial recovery percentages and starting weight of corn used for wet milling.
** Refer to form 300.21.

REVISION# 06 FORM# 300.5

MATERIAL BALANCE of WET CORN

Sample Number: **Sample B 5307 Positive Grain**

WHOLE CORN 24.5 lbs

Drying 24.1 lbs after drying (°F)

Aspiration N/A. lbs **LIGHT IMPURITIES**

Screening N/A lbs **SMALL SCREENINGS**
N/A lbs **LARGE SCREENINGS**

Steeping 24.1 lbs Corn Steeped (120-130°F)
10 gal water added

Draining 38.4 lbs Steeped Corn
N/A gal **STEEPWATER**
N/A lb Solubles from steeping*

Degermination, Separation, Screening, and Water Washing

1.0 lbs **GERM*** 0.9 lbs **HULL*** (165-195°F)
2.0 lbs **STARCH*** (130-160°F)
0.4 lbs **GLUTEN*** (130-160°F)

Flaking, Conditioning (160-175°F) 1.4 lbs germ flaked

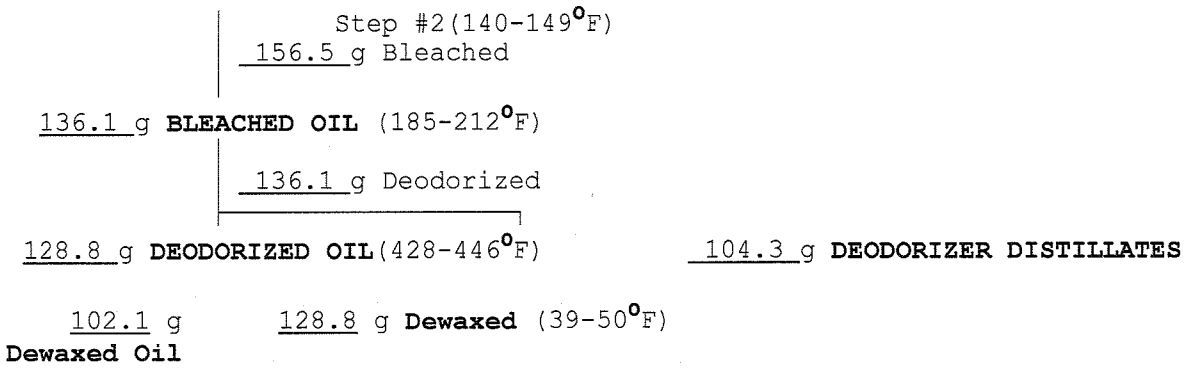
212.6 g **CRUDE OIL**

lbs germ flaked
(120-140°F)
Solvent Extraction

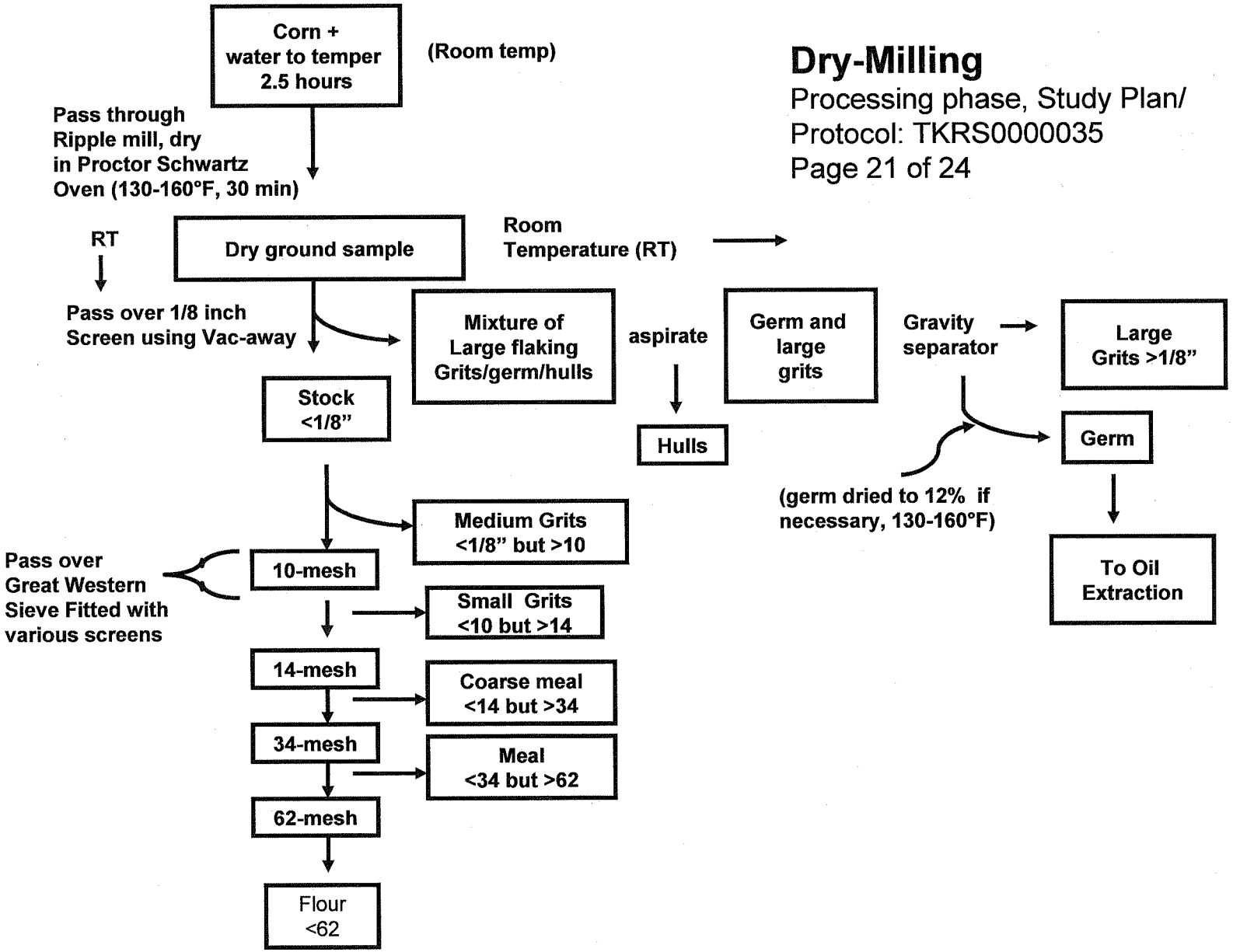
178.5 g **FILTERED OIL** 227.0 g **SOLVENT EXTRACTED Meal**

170.2 g Refined 7.1 g NaOH added

156.5 g **REFINED OIL** Step #1(145-153°F) **SOAPSTOCK** 18.7 g



*Calculated amounts based on commercial recovery percentages and starting weight of corn used for wet milling.
** Refer to form 300.21.
Raw Data in support of this study are available under separate cover.



Dry-Milling

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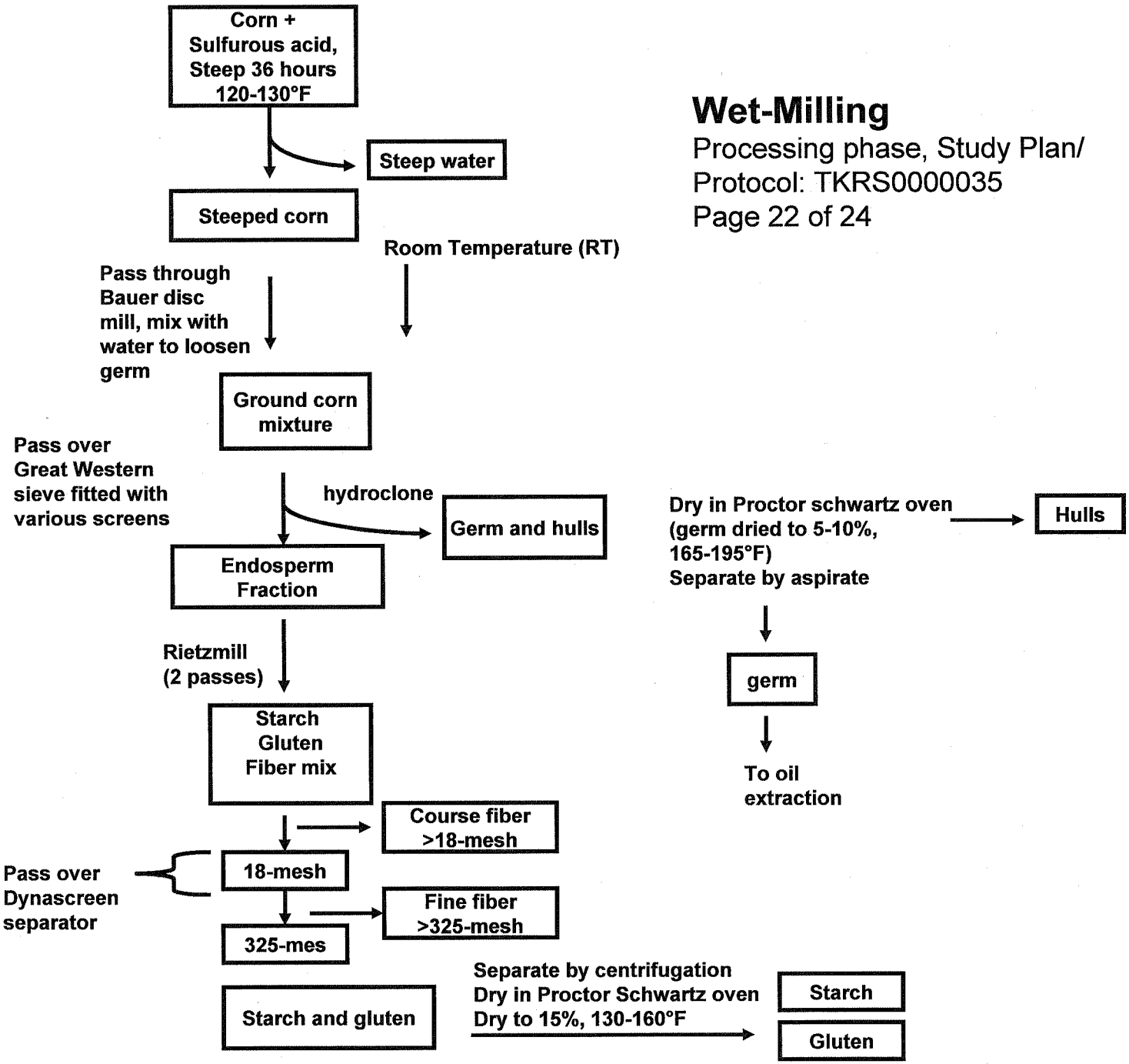
Corn + Sulfurous acid, Steep 36 hours 120-130°F
Steep water
Steeped corn
Room Temperature (RT)
Pass through Bauer disc mill, mix with water to loosen germ
Ground corn mixture
Pass over Great Western sieve fitted with various screens
hydroclone
Germ and hulls
Endosperm Fraction
Rietzmill (2 passes)
Starch Gluten Fiber mix
Course fiber >18-mesh
Pass over Dynascreen separator
18-mesh
Fine fiber >325-mesh
325-mes
Starch and gluten
Separate by centrifugation
Dry in Proctor Schwartz oven
Dry to 15%, 130-160°F
Starch
Gluten
Dry in Proctor schwartz oven (germ dried to 5-10%, 165-195°F)
Separate by aspirate
Hulls
germ
To oil extraction

Extraction of dry or wet mill germ and subsequent refining of resulting crude oil

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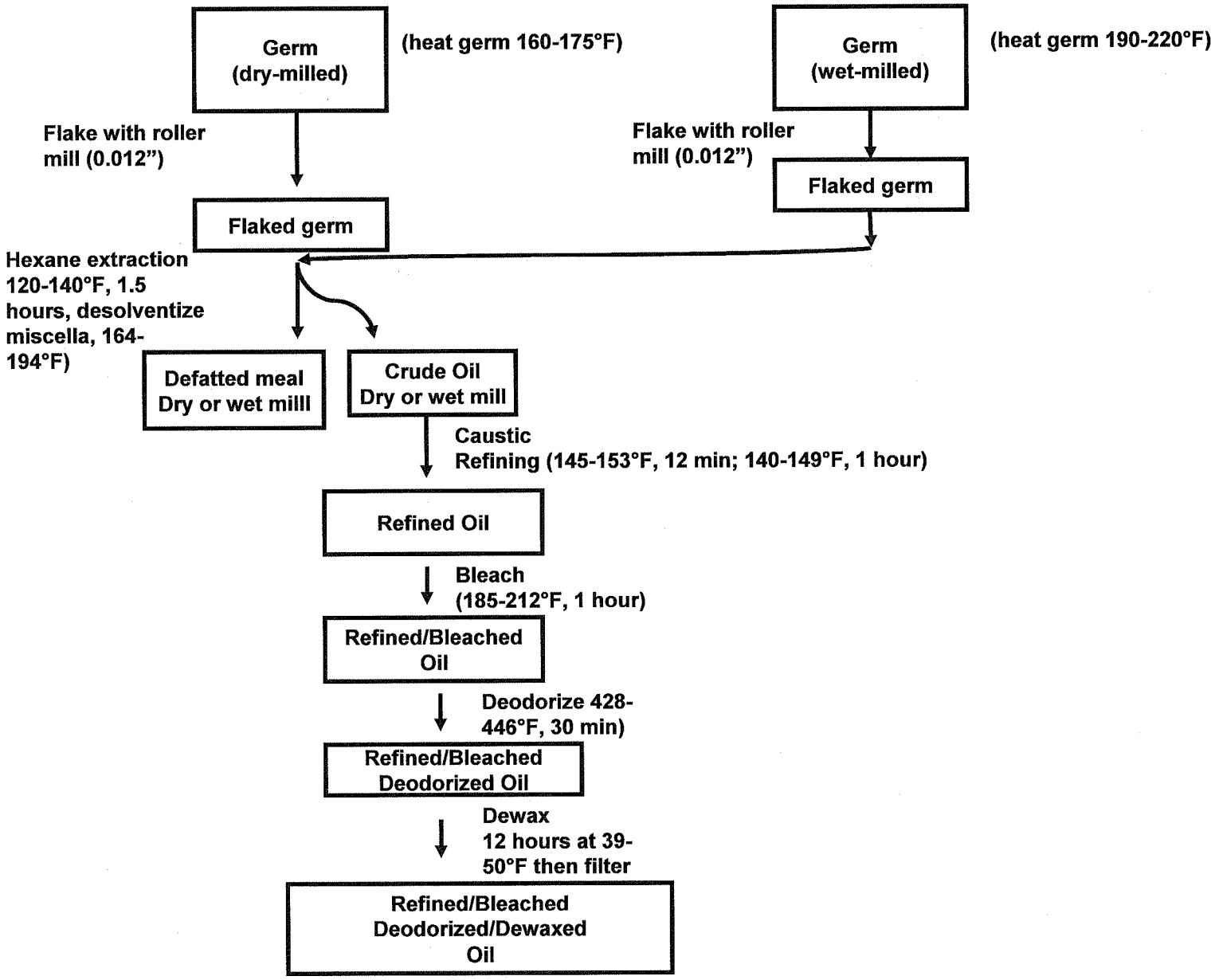

APPENDIX

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