Study Title

Assessment of Total DMO Protein Levels in Soybean Tissues Collected from MON 87708 Produced in United States Field Trials During 2008

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Sponsor and Performing Laboratory

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Laboratory Project ID

Study REG-09-411
MSL0022510

The text below applies only to use of the data by the United States Environmental Protection Agency (U.S. EPA) in connection with the provisions of the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA).

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No claim of data confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA § 10(d)(1)(A), (B), or (C).

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Company

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Title

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Signature Date

Statement of Compliance

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160 with the following exceptions: Stability of DMO in soybean tissues has not been assessed. One ELISA plate's original data as read by the spectrophotometer was not retained. The data was overwritten, due to the fact that the analyst did not wipe the bottom of the plate to remove smudges prior to the original read in the spectrophotometer.

Submitter	Date
Melinda C. McCann	5 Mar 2010
Sponsor Representative	Date
[signature]	3/5/10
Study Director	Date

Quality Assurance Statement

Study Title: Assessment of DMO Protein Levels in Soybean Tissues Collected from MON 87708 Produced in United States Field Trials During 2008

Study Number: REG-09-411

Reviews conducted by the Quality Assurance Unit confirm that the final report accurately describes the methods and standard operating procedures followed and accurately reflects the raw data of the study.

Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study reported herein.

Dates of Inspection/Audit	Phase	Date Reported to Study Director	Date Reported to Management
01/04/2010	ELISA	01/19/2010	01/19/2010
02/25/2010	Draft Report and Data Audit	03/05/2010	03/05/2010

Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

Date

Study Certification

This report is an accurate and complete representation of the study/project activities.

Signatures of Approval:

3/5/2010
Seth Tauchman, M.S.
Study Director
Date

3/05/2010
Katherine E. Niemeyer, B.S.
GLP Study Execution Team Lead
Date

Study Information

Study Number:	REG-09-411
Report Number:	MSL0022510
Title:	Assessment of Total DMO Protein Levels in Soybean Tissues Collected from MON 87708 Produced in United States Field Trials During 2008
Facility:	Monsanto Company Regulatory Product Characterization Center 800 North Lindbergh Boulevard Saint Louis, Missouri 63167
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Contributors:	Andrew Deffenbaugh, B.A.; Matt Lippold, M.S.; and Kelly Clark, B.S.
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Records Retention:	All study specific raw data (including rejected data), protocols, final reports, and facility records will be retained at Monsanto Company, St. Louis.

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Abbreviations[1] and Definitions

ANOVA	analysis of variance
CTP	chloroplast transit peptide
CV	coefficient of variation
DMO	dicamba mono-oxygenase
DWCF	dry weight conversion factor
dwt	dry weight of tissue
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
fwt	fresh weight of tissue
HRP	horseradish peroxidase
IgG	immunoglobulin G
LOD	limit of detection
LOQ	limit of quantitation
OSL	over-season leaf
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PCR	polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	standard operating procedure
TBS	tris buffered saline
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
TSSP	tissue-specific site pool

[1] Standard abbreviations, e.g., units of measure, were used in this report according to format described in 'Instructions to Authors' in the Journal of Biological Chemistry.

1.0 Summary

Monsanto Company has developed herbicide-tolerant soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. MON 87708 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses the dicamba mono-oxygenase (DMO) protein to confer tolerance to the dicamba herbicide.

The DMO protein in MON 87708 is targeted into chloroplasts for co-localization with the endogenous reductase and ferredoxin proteins that can supply electrons for the DMO oxidative reaction. The plant-expressed DMO contains a chloroplast transit peptide (CTP) from pea (*Pisum sativum*) and 27 amino acids from the N-terminal coding region of the pea Rubisco small subunit that were contained between the CTP and the amino terminal end of the coding region of DMO to potentially stabilize expression of this protein *in planta*. It was anticipated that during translocation into chloroplasts the CTP and the additional 27 amino acids would be cleaved resulting in the appropriate amino terminus for mature DMO. However, analysis of leaf and mature seed tissue by western blot shows the presence of two bands. One band corresponds to the DMO protein with the expected molecular weight of ~37 kDa, whereas the second band has a molecular weight of ~41 kDa. N-terminal sequence analysis of these two bands revealed that the ~37 kDa band corresponds to DMO with the expected N-terminus, while the ~41 kDa band contains the additional 27 amino acids originating from the pea Rubisco small subunit. This form of the protein is designated DMO+27.

This study determined the expression levels of total DMO protein (DMO and DMO+27) by a validated enzyme-linked immunosorbent assay (ELISA) in tissues collected from MON 87708 produced in United States field trials during 2008. Tissue samples were collected from plants grown in the United States at five field sites in 2008 under production plan REG-08-083. In this study, forage, over-season leaf (OSL-1-4), root, and seed tissues were used for ELISA analysis. All protein levels for each tissue type were calculated on a microgram (µg) per gram (g) fresh weight (fwt) basis. Moisture content was then measured for all tissue types and all protein levels were converted and reported on a dry weight (dwt) basis.

The mean total DMO protein levels in MON 87708 across all sites were 53 µg/g dwt in forage, 17 µg/g dwt in OSL-1, 31 µg/g dwt in OSL-2, 44 µg/g dwt in OSL-3, 69 µg/g dwt in OSL-4, 6.1 µg/g dwt in root, and 47 µg/g dwt in seed.

2.0 Background

Monsanto Company has developed herbicide-tolerant soybean MON 87708 that is tolerant to dicamba (3,6-dichloro-2-methoxybenzoic acid) herbicide. MON 87708 contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses the dicamba mono-oxygenase (DMO) protein to confer tolerance to the dicamba herbicide.

The DMO protein in MON 87708 is targeted into chloroplasts for co-localization with the endogenous reductase and ferredoxin proteins that can supply electrons for the DMO oxidative reaction. The plant-expressed DMO contains a chloroplast transit peptide (CTP) from pea (*Pisum sativum*) and 27 amino acids from the N-terminal coding region of the pea Rubisco small subunit that were contained between the CTP and the amino terminal end of the coding region of DMO to potentially stabilize expression of this protein *in planta*. It was anticipated that during translocation into chloroplasts the CTP and the additional 27 amino acids would be cleaved resulting in the appropriate amino terminus for mature DMO. However, analysis of leaf and mature seed tissue by western blot shows the presence of two bands. One band corresponds to the DMO protein with the expected molecular weight of ~37 kDa, whereas the second band has a molecular weight of ~41 kDa. N-terminal sequence analysis of these two bands revealed that the ~37 kDa band corresponds to DMO with the expected N-terminus, while the ~41 kDa band contains the additional 27 amino acids originating from the pea Rubisco small subunit. This form of the protein is designated DMO+27.

Total DMO protein levels (DMO and DMO+27) were determined in soybean tissues produced at five United States field sites in 2008. Soybean was planted in a randomized complete block design (RCBD), in replicates of three, at each field site.

2.1 Purpose

The purpose of this study was to determine the levels of total DMO protein in soybean tissues collected from of MON 87708 produced in a United States field trial during 2008.

3.0 Materials

3.1 Test, Control, and Reference Substances

3.1.1 Test Substance

The test substance for this study was MON 87708. Tissue samples were collected as outlined in production plan REG-08-083 from plants grown from starting seed lot 10001256.

3.1.2 Control Substance

The negative control substance for this study was a non-transgenic, conventional soybean with similar background genetics to the test substance. Negative control tissue samples were collected as outlined in production plan REG-08-083 from plants grown from starting seed lot 10001257.

3.1.3 Characterization of Test and Control Substances

The identities of the test and control substances were confirmed by verifying the chain-of-custody documentation prior to analysis. To further confirm the identities of the test and control substances, event-specific polymerase chain reaction (PCR) analyses were conducted on the harvested seed from each site.

The PCR analyses were archived in the Monsanto Regulatory Archives under the starting seed lot numbers described in sections 3.1.1 and 3.1.2.

3.1.4 Reference Substance

The *E. coli* produced DMO protein (lot 11247247) was used as the analytical reference standard. DMO+27 is not present in the *E.coli* produced protein standard. The concentration of the protein standard was determined by amino acid composition analysis and the purity was determined by SDS-PAGE and densitometric analysis. The purity of the DMO protein standard was 81% and the purity-corrected concentration was 0.34 mg/ml.

Copies of the certificates of analysis for the DMO reference standard, which contain a record of characterization and stability, were included in the study file.

4.0 Methods

4.1 Generation of Plant Samples

4.1.1 Summary of Field Design

Production plan REG-08-083 was initiated during the 2008 planting season to generate test and control substances at various soybean-growing locations in the United States. The tissue samples from the following field sites were used in this study: Jefferson County, Iowa (IARL); Stark County, Illinois (ILWY) Clinton County, Illinois (ILCY), Parke County, Indiana (INRC), and Berks County, Pennsylvania (PAHM). These field sites were representative of soybean producing regions suitable for commercial production. At each site, three replicated plots of plants containing MON 87708, as well as a conventional control were planted using a randomized complete block field design. Tissues were collected from each replicated plot at each field site.

4.2 Tissue Processing and Protein Extraction Methods

4.2.1 Processing Method

All tissue samples produced at the field sites were shipped to Monsanto, St. Louis and were prepared by the Monsanto Sample Management Team. The prepared tissue samples were stored in a -80°C freezer until transferred on dry ice to the analytical facility.

4.2.2 Extraction Methods

The total DMO protein (DMO and DMO+27) was extracted from soybean tissues as described in Monsanto Standard Operating Procedure (SOP) BR-ME-1306-01, draft 10/16/2009. Extraction parameters for the total DMO protein and each tissue type are described in Appendix 2. The extracts were aliquoted and stored in a -80°C freezer until analysis.

4.3 ELISA Reagents and Methods

4.3.1 DMO Antibodies

Goat polyclonal antibodies (lot G-844411) specific for DMO (and DMO+27) protein were purified using Protein-G Agarose affinity chromatography. The concentration of the purified IgG was determined to be 8.1 mg/ml by spectrophotometric methods. The purified antibody was stored in 1X phosphate-buffered saline (PBS), pH 7.4. The purified DMO antibodies were coupled with biotin (Pierce, Rockford, IL)according to the manufacturer's instructions and assigned lot number G-844413. The detection reagent was NeutrAvidin (Pierce, Rockford, IL) conjugated tohorseradish peroxidase (HRP).

4.3.1 DMO ELISA Method

The DMO ELISA was performed according to SOP BR-ME-1306, draft dated 10/16/09.

Goat anti-DMO capture antibodies were diluted in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, and 150 mM NaCl, pH 9.6) to a final concentration of 5.0 µg/ml and immobilized onto 96-well microtiter plates followed by incubation in a 4°C refrigerator for ≥8 h. Plates were washed with 1X PBS containing 0.05% (v/v) Tween-20 (1X PBST). The plates were blocked using 10 % Casein in tris buffered saline(TBS) blocking buffer (Pierce, Rockford, IL) at 200 µl per well for 1 hour a room temperature. The blocking buffer was aspirated and DMO protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37 °C. Prior to the addition of biotinylated antibody, NuetrAvidin-HRP and 3,3',5,5'-tetramethyl-benzidine TMB reagents, plates were washed with 1X PBS containing 0.05% (v/v) Tween-20 (1X PBST). The captured DMO protein was detected by the addition of 100 µl per well of biotinylated goat anti-DMO antibodies and NeutrAvidin-HRP (Pierce). Plates were developed by adding 100 µl per well of 3,3',5,5'-tetramethyl-benzidine (TMB; Kirkegaard & Perry, Gaithersburg, MD). The enzymatic reaction was terminated by the addition of 100 µl per well of 3 M H_3PO_4. Quantification of total DMO protein (DMO and DMO+27) was accomplished by interpolation on a DMO protein standard curve that ranged from 0.313 - 20 ng/ml.

4.3.2 Total DMO ELISA Validation

Appendix 2 summarize the results of validation of the ELISA used to assess the total DMO protein levels in soybean tissues.

4.4 Control of Bias

The test and control substances were planted using a randomized complete block design as described in production plan REG-08-083. The substances were randomly assigned to the plots within a block to prevent any experimental bias. Representative

tissues from each plot were collected as described in the production plan. All tissues were processed by thoroughly grinding to produce a homogeneous sample before extraction to minimize sampling bias. The ELISA method used was optimized to minimize method bias. Protein extracts from the test and control substances were analyzed by ELISA with the appropriate protein standards and inter-assay negative and positive controls.

4.5 Moisture Analysis

All tissues, except seed, were analyzed for moisture content using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO) according to SOP BR-ME-1238-01. Seed tissue was analyzed as described in protocol REG-09-411, Appendix 4. A homogeneous tissue-specific site pool (TSSP) was prepared consisting of samples of a given tissue type grown at a given site. These pools were prepared for each tissue in this study. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

$$DWCF = 1 - [Mean\ \%\ TSSP\ Moisture / 100]$$

The DWCF was used to convert protein levels assessed on a µg/g fresh weight (fwt) basis into levels reported on a µg/g dry weight (dwt) basis using the following calculation:

$$Protein\ Level\ in\ Dry\ Weight = \frac{(Protein\ Level\ Fresh\ Weight)}{(DWCF)}$$

The protein levels (ng/ml) that were reported to be less than or equal to the limit of detection (LOD) or less than the limit of quantitation (LOQ) on a fresh weight basis were not reported on a dry weight basis.

4.6 Data Analyses

All total DMO ELISA plates were analyzed on a SPECTRAmax Plus 384 (Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. All protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620-650 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GxP version 5.0.1 software. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a "µg/g fwt" basis for data that were greater than or equal to the LOQ. For total DMO, this conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values in "µg/g fwt" were also converted to "µg/g dwt" by applying the DWCF. Microsoft Excel 2007 (Version 12.0.6514.5000 SP2 Microsoft, Redmond, WA) was used to calculate the total DMO protein levels in soybean

tissues. The sample mean, standard deviations, and range were also calculated by Microsoft Excel 2007.

4.7 Protocol Amendments/Deviations

There was one protocol deviation in this study. The protocol states there will be five sites with three replicates per site. The conventional control for forage tissue at site PAHM, has only two replicates. The deviation occurred because forage, conventional control, sample number 11209906, from site PAHM, was never received by the sample management team; thus, never analyzed. This had no impact on the study as it was a conventional control sample, thus not used for statistical analysis.

5.0 Results

The across-site mean, standard deviation (SD), and range are reported for total DMO protein levels on a µg/g fwt and µg/g dwt basis in soybean tissues collected from five field sites in 2008 in Table 1.

5.1 Protein Levels in Total DMO

The total DMO protein levels for MON 87708 are presented in Table 1. Results showed that the mean total DMO protein levels in MON 87708 across all sites were 53 µg/g dwt in forage, 17 µg/g dwt in OSL-1, 31 µg/g dwt in OSL-2, 44 µg/g dwt in OSL-3, 69 µg/g dwt in OSL-4, 6.1 µg/g dwt in root, and 47 µg/g dwt in seed.

5.2 Stability of Test Materials

Tissue storage stability of the total DMO proteins in processed soybean tissues has not been determined.

6.0 Conclusions

MON 87708 was grown in United States field trials at five field sites during the 2008 growing season. Tissue samples were collected and analyzed for total DMO protein (DMO + DMO+27) levels using a validated ELISA method. These data provide an estimation of the levels of total DMO protein on a fresh weight and dry weight basis in seven tissue types throughout the growing season.

7.0 Acknowledgments

The authors would like to acknowledge Jack Milligan and the Agronomy and Sample Processing Center for processing the tissue samples and Andre Van Oyen, Jr. and John Lake from the Sample Dispensary for the sample distributions.

Table 1. Summary of Total DMO Protein Levels in Tissues Collected from MON 87708 Produced in United States Field Trials in 2008

Tissue Type[1]	Total DMO	
	Mean (SD)[2] Range[3] (µg/g fwt)[4]	Mean (SD) Range (µg/g dwt)[5]
Forage	12 (2.5) 7.0 – 17	53 (18) 25 – 84
OSL-1	3.1 (1.9) 0.87 – 6.8	17 (7.7) 6.2 – 29
OSL-2	5.2 (2.6) 1.4 – 9.8	31 (13) 12 – 54
OSL-3	6.0 (2.2) 3.5 – 11	44 (14) 25 – 71
OSL-4	16 (12) 4.6 - 43	69 (46) 23 – 180
Root	1.9 (0.73) 1.2 – 3.6	6.1 (2.1) 3.9 – 11
Seed	43 (7.7) 31 - 55	47 (8.7) 34 – 59

1. Tissues were collected at the following growth stages:
   a. OSL - 1: V3 – V4
   b. OSL - 2: V5 – V8
   c. OSL - 3: R2 – V 12
   d. OSL - 4: R5 – V16
   e. Forage: R6
   f. Root: R6
   g.Seed: R8
2. The mean and standard deviation were calculated (n=15). The "n" values for the calculated mean and standard deviations represent the number of samples figured into the calculation.
3. Minimum and maximum values were determined for each tissue type.
4. Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
5. Protein levels are expressed as µg/g on a dry weight (dwt) basis. The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factors obtained from moisture analysis data.

Appendices

Appendix 1. Standard Operating Procedures

BR-ME-1306-01 Draft 10/16/2009	Extraction and Indirect ELISA Analysis of DMO in Soybean Tissues
BR-ME-1238-01	Analysis of Moisture Content Using the Denver Instrument IR-200 Moisture Analyzer

Appendix 2. Summary of the Validation Results for the total DMO Protein ELISA in Soybean Matrices
Accuracy

Description of Method and Scope of Validation:
This validation summary describes the performance of an ELISA developed to detect and quantitate the DMO protein in soybean tissues leaf, root, forage, and grain. The validation results contained within this document demonstrate the accuracy, specificity, and precision of this ELISA method.

1.0 Accuracy

1.1 Extraction Efficiency and Spike and Recovery

Extraction Efficiency acceptance criteria = 70 – 130%.
Spike and Recovery acceptance criteria = 70 – 130%.

Tissue	Tissue-to-Buffer Ratio	Extraction Efficiency[1]	Spike and Recovery[2]
Leaf	1:100	97 %	81%
Root	1:50	79 %	73%-89%
Forage	1:50	100 %	82-89%
Grain	1:100	90 %	90-93 %

1. Extraction efficiency for Leaf, Forage, Grain was determined by comparing an aqueous extract to an extract in harsh buffer (e.g. Laemmli buffer) on a western blot. For root samples successive extractions using an optimized aqueous extraction buffer was analyzed by ELISA.
2. To evaluate the analytical accuracy of the ELISA, extracts prepared from each tissue type of conventional soybean plants were spiked with known quantities of DMO protein at three concentrations spanning the range of the standard curve.

1.2 Matrix Effects

Matrix Effects acceptance criteria = 70 – 130%.
No matrix interferences (non-specific binding) were noted when sample extracts were analyzed at matrix dilutions stated below.

Tissue	Minimal Dilution to Avoid Matrix Effects	Average Percent Recovery
Leaf	1:20	85-99 %
Root	1:2	96-105 %
Forage	1:40	90-98 %
Grain	1:40	97-104%

1.3 Parallelism

Parallelism is defined to mean that the plant-produced DMO protein is immunologically equivalent to the *E. coli*-DMO protein standard. Parallelism acceptance criteria = 70 – 130%.

Tissue	Parallelism
Leaf	100 - 104 %
Root	100 - 114 %
Forage	93 – 100 %
Grain	93 – 100 %

2.0 Precision

Range of Quantitation: 0.313 – 20 ng/ml
Method for Curve Fit 4-parameter

Intra-Assay Precision Acceptance Criteria: ≤ 15%
Inter-Assay Precision Acceptance Criteria: ≤ 25%
Precision Profile Acceptance Criteria: Standards 1-6 ≤ 15%
Standard 7 ≤ 25%

Intra-Assay Precision[3]: 3.4 %
Inter-Assay Precision[3]: 17.3 %

3. The inter- and intra-assay precision were assessed by determining the CV of the concentration of DMO protein measured for the positive control sample from 21 independent ELISAs using one-way analysis of variance (ANOVA).

Precision Profile:

Standard Number	Concentration (ng/ml)	%CV (over 21 runs)
1	20	13.8
2	10	10.5
3	5	8.4
4	2.5	6.0
5	1.25	4.9
6	0.625	7.2
7	0.313	5.5

The total intra-assay precision based on the standard curve was calculated to be 8.0%

3.0 Sensitivity

Limits of Quantitation[4] and Detection[5]:

Tissue Type	Dilution	LOD (ng/ml)	LOD (µg/g fwt)	LOQ (ng/ml)	LOQ (µg/g fwt)
Leaf	1:20	0.10	0.20	0.313	0.63
Root	1:2	0.15	0.015	0.313	0.031
Forage	1:40	0.050	0.10	0.313	0.63
Grain	1:40	0.053	0.21	0.313	1.3

4. The limit of detection (LOD) was calculated as the mean value plus three SD using the data generated with conventional sample extracts for each tissue type. The LOD value in "ng/ml" was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.
5. The limit of quantitation (LOQ) was calculated based on the lowest standard concentration. The "ng/ml" value was converted to "µg/g fwt" using the respective dilution factor and tissue-to-buffer ratio.

4.0 Extraction Parameters[6]

Tissue Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf	1:100	1X PBST/0.5% BSA
Root	1:50	1X PBST/0.5% BSA
Forage	1:50	1X PBST/0.5% BSA
Grain	1:100	1 X Tris Borate Buffer

6. The total DMO protein was extracted from each tissue by adding the appropriate volume of DMO Extraction Buffer, and shaking in a Harbil mixer. The extracted sample was clarified using a serum filter.