FINAL REPORT

Guideline: EPA-OPPTS (870.1100)

Testing Facility Study No. EUF00229

Monsanto Study No. CRO-09-419

An Acute Toxicity Study of Dicamba Mono-Oxygenase (DMO) Enzyme from MON 87708 Administered by Oral Gavage to Mice

PERFORMING LABORATORIES:

Charles River Laboratories
Preclinical Services
640 North Elizabeth Street
Spencerville, OH 45887

Monsanto Company
800 North Lindbergh Blvd
St. Louis, MO 63167

AUTHOR:

Jason W. Smedley

SPONSOR:

Monsanto Company
800 N. Lindbergh Blvd.
St. Louis, MO 63167

DATE:

21 May 2010

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

The inclusion of this page in all studies is for quality assurance purposes and does not necessarily indicate that this study has been submitted to the U.S. EPA.

1. STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

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

"We submit this material to the United States Environmental Protection Agency specifically under the requirements set forth in FIFRA as amended, and consent to the use and disclosure of this material by EPA strictly in accordance with FIFRA. By submitting this material to EPA in accordance with the method and format requirements contained in PR Notice 86-5, we reserve and do not waive any rights involving this material that are or can be claimed by the company notwithstanding this submission to EPA."

Company: Monsanto Company

Company Agent: _____

Title: _____

Signature: _____ Date: _____

2. COMPLIANCE STATEMENT

This study was conducted in compliance with the Good Laboratory Practice (GLP) regulations as described by the EPA (40 CFR Part 160), OECD [ENV/MC/CHEM(98)17], and JMAFF (11 Nousan No. 6283) with the following exception:

The dose formulation analysis was performed in compliance with the Good Laboratory Practice (GLP) regulations as described by the EPA (40 CFR Part 160) only.

Jason W. Smedley 21 May 2010

Jason W. Smedley, B.S. Date
Study Director
Charles River Laboratories
Preclinical Services

Sponsor/Submitter Date
Monsanto Company

3. QUALITY ASSURANCE STATEMENT

This study has been inspected by the Quality Assurance Unit to assure conformance with the Good Laboratory Practice (GLP) regulations promulgated by the EPA (40 CFR Part 160), OECD [ENV/MC/CHEM(98)17], and JMAFF (11 Nousan No. 6283). The dose formulation analysis was performed in compliance with the Good Laboratory Practice (GLP) regulations as described by the EPA (40 CFR Part 160).

Reports were submitted in accordance with Standard Operating Procedures as follows:

QA INSPECTION DATES

		Date Findings Submitted to:	
Dates of Inspection	Phase(s) Inspected	Study Director	Study Director Management
05-Nov-2009	Protocol Review	05-Nov-2009	05-Nov-2009
10-Nov-2009	Animal Receipt	10-Nov-2009	10-Nov-2009
17-Nov-2009	Protocol Amendment Review	17-Nov-2009	17-Nov-2009
19-Nov-2009	Randomization Procedure	19-Nov-2009	19-Nov-2009
19-Nov-2009	Dosing	19-Nov-2009	19-Nov-2009
03-Dec-2009	Necropsy	03-Dec-2009	03-Dec-2009
12-Dec-2009	Protocol Amendment Review	14-Dec-2009	14-Dec-2009
12-Dec-2009	Data Audit	14-Dec-2009	14-Dec-2009
12-Dec-2009	Data Audit	29-Dec-2009	29-Dec-2009
28-Dec-2009	Draft Report Review	29-Dec-2009	29-Dec-2009
21-May-2010	Final Report Review	21-May-2010	21-May-2010

QA statement(s) provided by the following test site(s) have been reviewed:

Test Site(s)	Phase	QA Statement Location
Monsanto Company	Analytical Chemistry Report	Appendix 2

The final report has been reviewed to assure that it accurately describes the materials and methods, and that the reported results accurately reflect the raw data.

Candace E. Brewer 21-May-2010

Candace E. Brewer, M.Ed. Date
Quality Assurance Auditor II
Charles River Laboratories
Preclinical Services

4. INTELLECTUAL PROPERTY RIGHTS

5. NOTES TO REVIEWER

(Page intentionally left blank)

6. RESPONSIBLE PERSONNEL

Jason W. Smedley, B.S.	Study Director/Associate Research Scientist
Amy C. Pohlman, B.S.	Study Coordinator II
Rusty E. Rush, M.S., DABT	Site Director
Mark A. Morse, Ph.D., DABT	Director of Research
William H. Baker, M.S., D.V.M., DACVP	Division Director, Pathology
Lynn M. Brown, D.V.M.	Staff Veterinarian
Todd N. Merriman, B.S., M.B.A., LATG	Director of Technical Operations
Beth A. Hoover, M.S., PCT, CPhT	Associate Supervisor, Formulations
Anita M. Bosau, RQAP-GLP	Division Director, Regulatory Compliance
Deanna M. Talerico, RQAP-GLP	Senior Manager, Regulatory Compliance
Janet A. Gales, RLATG	Study Supervisor, In-life
Jeremy D. Johnson, B.A., LAT	Primary Technician
Sarah C. Zahn, B.S.	Supervisor, Necropsy
Cheryl A. Bellamy	Manager, Report Coordination
Jennifer A. Rich, B.S.	Supervisor, Study Coordination
Rebecca R. English, B.S.	Archivist
Adam Evans	Principal Investigator, Dose Solution Analysis

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

7. SUMMARY

The purpose of this study was to evaluate the potential short-term toxicity of dicamba mono-oxygenase (DMO) enzyme from soybean MON 87708 when administered as a single dose to mice by oral gavage, followed by a 14-day observation period. The study design was as follows:

Experimental Design

Group No.	No. of Animals		Dose Material (Test/Control Article)	Targeted Dose Level (mg protein/kg BW)	Administered Dose Level (mg protein/kg BW)	Dose Volume (mL dosing solution/kg BW)
	Males	Females				
1	5	5	CDS (BSA)	250	205	33.3
2	5	5	TDS (DMO enzyme)	250	140	33.3

The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, and gross necropsy findings.

Results:

No mortality occurred during the study and no test article-related clinical findings were observed. There were no DMO enzyme-related differences in body weights, body weight changes, or food consumption. No abnormal gross findings were present at necropsy.

Conclusion:

There were no adverse effects of the DMO enzyme when administered by oral gavage at a dose of 140 mg/kg in male and female mice.

8. INTRODUCTION

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) enzyme to confer tolerance to dicamba herbicide.

The DMO enzyme 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. The DMO enzyme contains DMO and DMO+27.

The purpose of this study was to evaluate the potential toxicity of the DMO enzyme from soybean MON 87708 when administered as a single dose to mice by oral gavage, followed by a 14-day observation period.

The protocol was signed by the Study Director on 05 November 2009 (GLP initiation date). The experimental start date was 10 November 2009 and the experimental completion date was 03 December 2009. The in-life phase of the study was initiated on 19 November 2009 and the in-life completion date was 03 December 2009.

9. MATERIALS AND METHODS

9.1. Test Materials

9.1.1. Test Article

The test article was the DMO enzyme produced in MON 87708 and is defined as follows:

Identification	DMO (dicamba mono-oxygenase) enzyme, DMO proteins
ID Number	11261646

A dosing solution was prepared by the Sponsor (used to dose Group 2) from the above material and identified as follows:

Identification	Test Dosing Solution (TDS)
Lot Number	87708-D
Assigned Testing Facility ID	090073
Receipt Date	18-Nov-2009
Expiration Date	Stable throughout duration of study
Physical Description	Cloudy amber liquid
Storage Conditions	Frozen in a -70°C freezer

9.1.2. Control Article

The control article is defined as follows:

Identification	BSA (bovine serum albumin)
ID Number	D00068037

The Control Dosing Solution (CDS) was prepared by the Sponsor (used to dose Group 1) from the control article. The total protein concentration of the CDS was targeted to the total protein concentration of the TDS and identified as follows:

Identification	Control Dosing Solution (CDS)
Lot Number	68037-C
Assigned Testing Facility ID	090072
Receipt Date	18-Nov-2009
Expiration Date	17-May-2010
Physical Description	Clear colorless liquid
Storage Conditions	Frozen in a -70°C freezer

9.1.3. Test and Control Article Characterization

Certificates of Analysis for the test and control articles are presented in Appendix 1.

9.1.4. Reserve Sample

The Sponsor was responsible for maintaining a retention sample of the test and control articles.

9.1.5. Inventory and Disposition

Inventory of materials provided by the Sponsor was maintained. All unused dosing solutions were returned to the Sponsor following completion of the in-life phase.

9.1.6. Preparation of Dose Formulations

The dosing solutions were prepared by the Sponsor (the method of formulation is included in Appendix 2) and shipped frozen. On the day of dosing the dosing solutions were removed from the freezer, allowed to thaw at room temperature, and mixed thoroughly by stirring. The dosing

solutions were administered as received following thawing. The dose solutions were stored on wet ice prior to and during dosing.

9.1.7. Analysis of Dose Formulations

Dose formulation samples were collected for analysis as indicated in the following table:

Dose Formulation Samples for Analysis – Test Dose Solutions (TDS)

Time Point	Analysis	Sample Volume (µL)	Sample Identification
Pre-dose	Concentration/Stability/Activity	250	Pre-TDS
	Homogeneity – Top	250	T-TDS
	Homogeneity – Middle	250	M-TDS
	Homogeneity – Bottom	250	B-TDS
Post-dose	Concentration/Stability/Activity	250	Post-TDS

Dose Formulation Samples for Analysis – Control Dose Solutions (CDS)

Time Point	Analysis	Sample Volume (µL)	Sample Identification
Pre-dose	Concentration/Stability	250	Pre-CDS
Post-dose	Concentration/Stability	250	Post-CDS

9.1.7.1. Concentration, Stability, and Functional Activity

Two 250-µL samples were collected from each dosing formulation on Day 0 (one sample prior to dosing and one sample following completion of dosing) for concentration and stability analysis. The TDS samples were also analyzed for functional activity.

9.1.7.2. Homogeneity

Three 250-µL samples were collected prior to dosing, one each from the top, middle, and bottom of the TDS dosing formulation.

9.1.7.3. Analytical Sample Storage and Shipment

All samples and unused dosing solutions were immediately frozen (in a -70°C freezer) and transferred on dry ice to the Sponsor for analysis. The Analytical Chemistry Report is presented in Appendix 2.

9.2. Test System

9.2.1. Receipt and Description

Male and female CD-1 mice were received on 10 November 2009 from Charles River Laboratories, Portage, Michigan. The animals were examined and weighed on the day following

receipt, and all were allowed to acclimate to the laboratory environment for 9 days prior to the first day of dosing.

9.2.2. Justification of Test System/Route

The CD-1 mouse was chosen as the animal model for this study as it is a preferred rodent species for toxicity testing by regulatory agencies. The oral route of exposure was selected since this is the potential route of human exposure.

9.2.3. Housing

The animals were housed individually in suspended stainless steel cages during acclimation and while on study. Housing and care were as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and as described in the *Guide for the Care and Use of Laboratory Animals*[1]. Targeted environmental conditions were as follows:

Temperature	64-79ºF (18-26ºC)
Humidity	50 ± 20%
Light Cycle	12-hour light/12-hour dark cycle
Air Changes	Ten or more air changes per hour with 100% fresh air

Actual room temperature and relative humidity were recorded continuously at 15-minute intervals and the daily averages ranged from 69 to 72°F (21 to 22°C) and 38 to 52%, respectively.

9.2.4. Animal Identification

The animals were individually identified using metal ear tags and cage cards.

9.2.5. Food

PMI Nutrition International Certified Rodent Chow® #5002 was provided *ad libitum* throughout the study. The lot number and expiration date of each batch of diet used during the study were recorded. The feed was analyzed by the supplier for nutritional components and environmental contaminants. Results of the dietary analyses were provided by the manufacturer and are maintained on file at the Testing Facility. Based on these results, there were no contaminants that would interfere with the conduct or interpretation of the study.

9.2.6. Water

Municipal tap water following treatment by reverse osmosis and ultraviolet irradiation was available *ad libitum* throughout the study. The water is periodically analyzed for total dissolved solids, hardness, microbiological content, and various potential environmental contaminants. Results of these analyses are maintained on file at the Testing Facility. Based on these results, there were no contaminants that would interfere with the conduct or interpretation of the study.

9.2.7. Veterinary Care

Veterinary care was available throughout the study and animals were examined by the Attending Veterinarian as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments were documented in the study records. No veterinary medicinal treatments were administered during the study.

9.2.8. Assignment to Study Groups

Prior to randomization procedures, 26 animals were weighed and examined in detail. Animals determined to be suitable as test subjects were then randomly assigned to groups by a stratified randomization scheme designed to achieve similar group mean body weights. Homogeneity of groups by weight was the criteria of acceptance of the randomization. At the time of randomization, the animals selected for study use were approximately 8 weeks of age with body weights ranging from 29.9 to 32.3 grams for the males and 24.4 to 29.2 grams for the females.

9.3. Experimental Design

The experimental design was as follows:

Experimental Design

Group No.	No. of Animals		Dose Material (Test/Control Article)	Targeted Dose Level (mg protein/kg BW)	Administered Dose Level (mg protein/kg BW)	Dose Volume (mL dosing solution/kg BW)
	Males	Females				
1	5	5	CDS (BSA)	250	205	33.3
2	5	5	TDS (DMO proteins)	250	140	33.3

9.4. Administration of Test Materials

On Day 0, the animals chosen for use on study were weighed and fasted for approximately 2 to 3 hours prior to dose administration. The test and control dosing solutions were administered as a single dose to the appropriate group of 5 male and 5 female mice. The dose volume for each animal was based on the Day 0 non-fasted body weight measurement. The doses were given using a syringe with attached gavage cannula and the animals were returned to *ad libitum* feeding. The day of dosing was designated as Study Day 0.

10. EXPERIMENTAL PROCEDURES

10.1. Mortality/Moribundity Checks

General health/mortality and moribundity checks were performed twice daily, in the morning and afternoon.

10.2. Clinical Observations

Detailed clinical observations were performed once prior to randomization, two times on Day 0 (post dose), and once daily thereafter (Days 1-14). Each animal was removed from the cage and observed in detail as described in Appendix 3.

10.3. Body Weights

Individual body weights were recorded on Day 0 prior to fasting, Day 0 prior to dosing, and on Days 7 and 14.

10.4. Food Consumption

Food consumption measurements were recorded on Days 0, 7, and 14.

10.5. Terminal Procedures

Terminal procedures are summarized in the following table:

Terminal Procedures

Group No.	No. of Male/Female Mice	Scheduled Euthanasia Day	Terminal Procedures	
			Gross Necropsy	Tissue Collection
1	5/5	14	x	x
2	5/5	14	x	x
Note: "x" = procedure conducted.				

10.5.1. Gross Necropsy

All animals were euthanized by carbon dioxide inhalation followed by exsanguination and subjected to a complete gross necropsy examination. The necropsy examination included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. A board-certified veterinary pathologist monitored scheduled gross necropsy examinations.

10.5.2. Tissue Collection and Preservation

The lungs and GI tract of each animal were perfused and the entire animal was retained in 10% neutral buffered formalin for possible future analysis.

10.6. Protocol Deviations

The dose formulation analysis was not performed in compliance with the Good Laboratory Practice (GLP) regulations as described by the OECD [ENV/MC/CHEM(98)17] and JMAFF (11 Nousan No. 6283), however by the EPA (40 CFR Part 160) only. This deviation had no impact on the study because GLP assessment of the dose formulations did occur allowing assessment of the formulations provided to the test system to be shown as acceptable. It was an

oversight by the laboratory performing the analysis of dose formulation samples that the OECD and JMAFF regulations were not followed as outlined in the study protocol.

11. DATA ACQUISITION AND ANALYSIS

11.1. Electronic Data Acquisition/Systems

The in-life and gross pathology data were recorded on the Compaq Alpha DS10 Computer using the Toxicology Analysis System Customized, General Toxicology Module, Version 1.48.1. The temperature and humidity were recorded on a Systems 600 Apogee Insight System, Version 3.9.1. The test material receipt and accountability were recorded on Instem Life Science Systems, DISPENSE, Version 7.03. The following computer study numbers were used to collect data for the various study phases: EUF229, main phase data; and MS0916, acclimation data for main phase. The tables and appendices within this report display the applicable computer study number.

11.2. Statistical Analysis

Inferential statistical analyses were performed for the animals using the Compaq Alpha DS10 Computer. The following parameters and end points were analyzed: body weights, body weight changes, and food consumption.

Each data set was subjected to a statistical decision tree. Data sets for each interval were initially analyzed for homogeneity of variance using Levene's test[2] followed by the Shapiro-Wilk test[3] for normality. A $p < 0.001$ level of significance was required for each test to reject the null hypothesis.

If neither Levene's test nor the Shapiro-Wilk test were significant, a single-factor parametric ANOVA[4] was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the parametric ANOVA was significant at $p < 0.05$, Dunnett's test was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

If either Levene's test and/or the Shapiro-Wilk test were significant, then the Kruskal-Wallis non-parametric ANOVA[5] was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the non-parametric Kruskal-Wallis ANOVA was significant at $p < 0.05$, Dunn's test was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

12. MAINTENANCE OF RAW DATA, RECORDS, AND SPECIMENS

Following issuance of the Final Report, materials including but not limited to the protocol and protocol amendment(s), in-life records, pathology records, formulation records, correspondence related to the study, Final Report, micro-slides, specimens, and wet tissues will be archived at Charles River Laboratories, Preclinical Services, Pennsylvania, Horsham, Pennsylvania, for a period of 3 years, after which the Sponsor will be contacted concerning continued storage or return of the materials.

Analytical and dosing preparation data will be archived by the Sponsor.

13. RESULTS

13.1. Dose Formulation Analysis

Appendix 2 (Dose Formulation Analysis Report)

The TDS was stable and homogeneous and contained biologically active DMO enzyme. The total DMO protein concentration in the TDS was 4.20 mg/ml. The administered dose was 140 mg DMO enzyme/kg body weight.

The CDS was stable. The BSA concentration in the CDS was 6.16 mg/ml. The administered dose was 205 mg BSA/kg body weight.

Analytical values for the dose levels were lower than the target value of 250 mg/kg BW. Further discussion of the analytically-confirmed dose levels is provided in Appendix 2.

13.2. Survival

Table 1 (Summary Data)
Appendix 4 (Individual Data)

No mortality occurred during the study; all animals survived until study termination.

13.3. Clinical Observations

Table 1 (Summary Data)
Appendix 4 (Individual Data)

No test article-related clinical findings were observed.

Clinical signs noted during the study were limited to two DMO enzyme-treated males. Animal No. 9971 did not extend its right hind limb fully during open-field observations on Days 4 through 6. This finding may have been a result of an injury to the limb; however, no indications of external injury or internal injury were noted during the in-life phase or at the time of gross necropsy. Animal No. 9972 had unkempt appearance on Days 13 and 14; this is a common background finding of mice of this age and strain, and was therefore considered incidental. While no abnormal findings were present in the control animals, these two findings were not considered to be treatment related, based on the isolated and transient nature of the findings and the lack of similar findings in the female mice treated with the DMO enzyme.

13.4. Body Weights

Table 2 and Table 3 (Summary Data)
Appendix 5 and Appendix 6 (Individual Data)

No test article-related effects on body weight or body weight gain were noted during the study.

There was a statistically significant increase in mean body weight gain in DMO enzyme-treated male mice compared to the BSA-treated control animals during the Day 7 to Day 14. However, there was a decrease in body weight gain, though not statistically significant, of similar magnitude during the Day 0 to Day 7 interval and there was no corresponding difference in female animals during this period; therefore, it is not considered related to treatment. Overall body weight gain was similar for the male and female BSA and DMO enzyme-treated animals.

Individually, a minor decrease in body weight was noted in one DMO enzyme-treated female between Day 0 and Day 7, while all other animals maintained or gained weight. A minor body weight decrease was noted between Day 7 and Day 14 in one BSA-treated male and one BSA-treated female. These body weight decreases were not considered treatment-related, based on the sporadic occurrence and the small magnitude of the differences. All DMO enzyme-treated animals gained body weight during the second week of the study, and all animals exceeded their starting weight by study termination.

13.5. Food Consumption

Table 4 (Summary Data)
Appendix 7 (Individual Data)

There were no test article-related effects on food consumption during the study.

13.6. Gross Necropsy

Table 5 (Summary Data)
Appendix 8 (Individual Data)

No abnormal gross findings were observed at necropsy.

14. CONCLUSION

There were no adverse effects of the DMO enzyme when administered by oral gavage at a dose of 140 mg/kg in male and female mice.

Jason W. Smedley 21 May 2010

Jason W. Smedley, B.S. Date
Study Director
Charles River Laboratories
Preclinical Services

15. REPORT APPROVAL SIGNATURE

Mark A. Morse 21 MAY 2010

Mark A. Morse, Ph.D., DABT Date
Director of Research
Charles River Laboratories
Preclinical Services

16. REFERENCES

1. Guide for the care and use of laboratory animals. Washington, D.C.: National Academy Press. NRC (National Research Council); 1996.

2. Levene H. *Contributions to Probability and Statistics*. Stanford University Press; 1960.

3. Royston P, A remark on algorithm AS 181: the W-test for normality. *Applied Statistics*. 1995 44(4):547-551.

4. Gad SC and Weil CS. *Principles and Methods of Toxicology*. 3rd ed. New York, NY: Raven Press, Ltd.; 1994.

5. Siegel S. Nonparametric Statistics for the Behavioral Sciences; 1956.

Table 1
Summary of Survival and Clinical Observations

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

TABLE 1

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

MALES SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (TOTAL OBSERVATIONS/ANIMALS AFFECTED)

GROUP:	1	2
TREATMENT:	BSA	DMO
DAY 0 to 14		
NORMAL		
WITHIN NORMAL LIMITS	85/ 5	80/ 5
SUBMITTED FOR SCHEDULED EUTHANASIA	5/ 5	5/ 5
BODY		
UNKEMPT APPEARANCE	0/ 0	2/ 1
OPEN-FIELD OBS		
GAIT EVALUATION	0/ 0	3/ 1

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

TABLE 1

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

FEMALES SUMMARY OF SURVIVAL AND CLINICAL OBSERVATIONS (TOTAL OBSERVATIONS/ANIMALS AFFECTED)

GROUP:	1	2
TREATMENT:	BSA	DMO
DAY 0 to 14		
NORMAL		
WITHIN NORMAL LIMITS	85/ 5	85/ 5
SUBMITTED FOR SCHEDULED EUTHANASIA	5/ 5	5/ 5

Table 2
Summary of Body Weight Data

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

TABLE 2

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

MALES SUMMARY OF BODY WEIGHT DATA (GRAMS)

	GROUP:		1	2
	TREATMENT:		BSA	DMO
DAY	0 (NON-FASTED)	MEAN	31.1 d	31.3
		S.D.	0.92	0.84
		N	5	5
	% difference vs. control			0.8
DAY	0 (FASTED)	MEAN	30.4 d	30.6
		S.D.	0.92	0.99
		N	5	5
	% difference vs. control			0.5
DAY	7	MEAN	32.0 d	31.6
		S.D.	0.29	0.84
		N	5	5
	% difference vs. control			-1.3
DAY	14	MEAN	32.5 d	33.2
		S.D.	0.59	0.91
		N	5	5
	% difference vs. control			2.0

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

TABLE 2

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

FEMALES SUMMARY OF BODY WEIGHT DATA (GRAMS)

			GROUP:	1	2
			TREATMENT:	BSA	DMO
DAY	0 (NON-FASTED)	MEAN		26.6 d	26.9
		S.D.		1.85	2.24
		N		5	5
	% difference vs. control				1.1
DAY	0 (FASTED)	MEAN		25.2 d	25.7
		S.D.		1.58	1.97
		N		5	5
	% difference vs. control				1.8
DAY	7	MEAN		26.6 d	26.6
		S.D.		1.17	1.67
		N		5	5
	% difference vs. control				-0.2
DAY	14	MEAN		28.3 d	28.1
		S.D.		1.70	2.45
		N		5	5
	% difference vs. control				-0.6

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 3
Summary of Body Weight Changes

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

TABLE 3

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

MALES SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

GROUP:			1	2
TREATMENT:			BSA	DMO
DAY	0 (FASTED) TO 7	MEAN	1.6 d	1.0
		S.D.	0.89	0.81
		N	5	5
DAY	7 TO 14	MEAN	0.5 d	1.6*
		S.D.	0.66	0.55
		N	5	5

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST * = P<0.05

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

TABLE 3

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

FEMALES SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

GROUP:			1	2
TREATMENT:			BSA	DMO
DAY	0 (FASTED) TO 7	MEAN	1.4 d	0.9
		S.D.	1.10	1.13
		N	5	5
DAY	7 TO 14	MEAN	1.7 d	1.5
		S.D.	1.33	1.00
		N	5	5

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 4
Summary of Food Consumption Data

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

TABLE 4

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

MALES SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

			GROUP:	1	2
			TREATMENT:	BSA	DMO
DAY	0 to 7	MEAN		6.6 d	6.4
		S.D.		0.67	0.68
		N		5	5
	% difference vs. control				-2.6
DAY	7 to 14	MEAN		7.0 d	6.8
		S.D.		0.88	0.65
		N		5	5
	% difference vs. control				-2.0

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

TABLE 4

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

FEMALES SUMMARY OF FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

	GROUP:		1	2
	TREATMENT:		BSA	DMO
DAY	0 to 7	MEAN	6.1 d	6.5
		S.D.	0.49	0.36
		N	5	5
	% difference vs. control			6.3
DAY	7 to 14	MEAN	6.5 d	7.0
		S.D.	0.42	0.57
		N	5	5
	% difference vs. control			7.4

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 5
Summary of Gross Necropsy Observations

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

TABLE 5

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS
SCHEDULED EUTHANASIA - DAY 14

	GROUP:	1	2
	TREATMENT:	BSA	DMO
MALES:	TOTAL NUMBER EXAMINED	5	5
	WITHIN NORMAL LIMITS	5	5

TABLE 5

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS
SCHEDULED EUTHANASIA - DAY 14

	GROUP:	1	2
	TREATMENT:	BSA	DMO
FEMALES:	TOTAL NUMBER EXAMINED	5	5
	WITHIN NORMAL LIMITS	5	5

Appendix 1
Certificates of Analysis

Analytical Protein Standard Certificate of Analysis

MONSANTO ANALYTICAL PROTEIN STANDARDS

Sample Information:

Name of APS	**APS Lot Number**	**Expiration Date**
Dicamba Mono-Oxygenase (DMO) Enzyme Isolated from the Seed of MON 87708	11261646	July 31, 2010
Common or Alias Name(s)	**Historical APS Lot Number**	**Storage Requirements (until use)**
DMO enzyme, DMO soy	G-854854	–80 °C
Source		**Comment(s)**
Purified from the seed of MON 87708, which contains a demethylase gene from *Stenotrophomonas maltophilia* that expresses the dicamba mono-oxygenase (DMO) protein.		None
Additional Background Information		
None		

Characteristic	Method	Assay Date	Result
Total Protein Concentration	Amino Acid Composition	2 December 2009	0.18 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	3 December 2009	81 %
Apparent Molecular Weight	SDS-PAGE/Densitometry	3 December 2009	DMO+27: 42.0 kDa DMO: 39.8 kDa
Identity	Immunoblot	8 December 2009	Confirmed – immunoreactive bands at 42.0 and 39.8 kDa were observed.
Identity	N-terminal sequence	4 December 2009	DMO+27: M*QVWPPIGKKKFETL DMO: ATFVRNAWYVAALPE
Identity	MALDI-TOF MS (Trypsinized)	7 December 2009	DMO+27: 82.0% coverage of expected sequence DMO: 77.4 % coverage of expected sequence
Glycosylation	GE ECL Glycosylation Kit	8 Decmeber 2009	Not Glycosylated
Activity	DMO Activity Assay	14 December 2009	62 nmoles/minute/mg DMO enzyme

Buffer composition: 50 mM Potassium Phosphate pH 8.0, 100 mM NaCl, 5% Glycerol, 1 mM DTT
Short-term storage stability (12 days) was evaluated during the characterization process. Based upon the criteria provided in REG-09-576, no significant degradation was observed for samples stored at -20°C and –80°C. However, significant degradation was observed for samples stored at 4°C.

*Methionine (position 1) for the DMO+27 protein was methylated.

Purity-corrected concentration is 0.15 mg/mL (0.18 mg/mL × 0.81 ≈ 0.15 mg/mL)

Quality Assurance Specialist — 03/01/2010 Date

Susan Elliott
Analytical Protein Standards Officer — 3/1/2010 Date

Calbiochem®

Certificate of Analysis

Albumin, Bovine Serum, Fraction V, Fatty Acid-Free, Nuclease- and Protease-Free

Batch Number:	D00068037
Material Number:	126609-10GM
Molecular Weight:	66000
CAS Number:	9048-46-8

Analytical Data

Test	Tolerance	Result
Solubility:		H_2O (10 mg/ml) or 150 mM NaCl (10 mg/ml)
Color:		Off-white
Appearance:		Flakes
Form:		Powder
Solubility:		In water
Purity by SDS-PAGE:	⩾98.0 %	100.0 %
Endonuclease:		None detected
Proteases:		None detected
Loss on drying:	⩽5.0 %	2.7 %
Heavy Metals:	⩽10.0 ppm	<10.0 ppm
pH:	6.8 - 7.2	7.0
Sulfated ash:	⩽2.0 %	1.8 %
Free Fatty Acid:	⩽0.02 %	<0.02 %

Storage and Handling:	+2C to +8C

Ellen Kroninger

Ellen Kroninger, Quality Assurance Manager

11-Jun-2009

Date

USA and Canada
Tel (800) 628-8470
technical@calbiochem.com

Europe
France Freephone 0800 126 461
Germany Freecall 0800 100 3496
Ireland Toll Free 1800 409 445
United Kingdom Freephone 0800 622 935
All Other European Countries +44 115 943 0840
techservice@merckbio.eu

All Other Countries
Contact Your Local Distributor
www.calbiochem.com
technical@calbiochem.com

Appendix 2
Dose Formulation Analysis Report

Analytical Sub-Report Title

Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study of MON 87708-Produced Dicamba Mono-Oxygenase (DMO) Administered by the Oral (Gavage) Route to Mice

Authors

Adam J. Evans, MPH, Luis A. Burzio, Ph.D., and John J. Finnessy, M.S.

Analytical Sub-Report Completed On

April 23, 2010

Performing Laboratory

Monsanto Company
800 North Lindbergh Boulevard
Saint Louis, Missouri 63167

Laboratory Project ID

MSL #: 0022527
Charles River Study #: EUF00229
Monsanto Study #: CRO-09-419

Statement of Compliance

This portion of the study meets the U.S. EPA Good Laboratory Practices specified in 40 CFR Part 160.

Submitter: _____ Date: _____

Sponsor: _____ Date: 4/22/2010
Terry A. Kaempfe

Principal Investigator: _____ Date: 4/23/2010
Adam J. Evans, MPH

Quality Assurance Statement

Analytical Sub-Report Title: Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity of MON 87708-Produced Dicamba Mono-Oxygenase (DMO) Administered by the Oral (gavage) Route to Mice.

Charles River Study No. EUF00229
Monsanto Study No. CRO-09-419

Reviews conducted by the Quality Assurance Unit confirm that the final analytical sub-report accurately describes the methods and standard operating procedures followed and accurately reflect 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
December 10, 2009	SDS-PAGE	December 11, 2009	December 11, 2009
February 18, 2010	Draft Report Review	February 18, 2010	February 18, 2010
February 12, 2010	Raw Data Audit	February 17, 2010	February 17, 2010

Date: 04/22/2010

Todd Butzlaff
Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

Analytical Sub-Report Certification

This sub-report is an accurate and complete representation of the study activities conducted under the study-specific procedure for the formulation and confirmation of dose solutions.

Analytical Principal Investigator: _____ Date: 4/23/2010

Adam J. Evans, MPH
Protein Sciences and Safety Team

Study Information

Charles River/Monsanto Study Number:	EUF00229/CRO-09-419
MSL Number	0022527
Analytical Sub-Report Title:	Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity of MON 87708-Produced Dicamba Mono-Oxygenase (DMO) Administered by the Oral (gavage) Route to Mice
Facilities:	Monsanto Company 800 North Lindbergh Boulevard Saint Louis, Missouri 63167, USA
Study Director:	Jason W. Smedley, B.S.
Principal Investigator:	Adam J. Evans, MPH
Contributors:	Chris Dalton and Robert Heeren
Study Specific Procedure Initiation Date:	November 13, 2009
Analytical Sub-Report Completion Date:	April 23, 2010
Records Retention:	All Study Specific Procedure raw data, Study Specific Procedure plan and amendments, final sub-report and facility records were retained at Monsanto Company, St. Louis, MO.
Disposition of Remaining Dosing Solutions:	Dosing samples, including the end of study samples were returned to Monsanto and disposed of at the close of the study.

Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations[1]

AAbA	Alpha aminobutyric acid
BSA	Bovine serum albumin
BW	Body weight
CDS	Control Dosing Solution
CFR	Code of Federal Regulations
COA	Certificate of analysis
CV	Coefficient of variance
DCSA	3,6-dichlorosalicylic acid
DMO	Dicamba mono-oxygenase protein
DMO+27	DMO protein plus 27 amino acids originating from the pea Rubisco small subunit on the N-terminus
DMO enzyme	Trimer containing DMO and DMO+27
DMO proteins	Both forms of the proteins: DMO and DMO+27
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
ID #	Identification number
MW	Molecular weight
MWCO	Molecular weight cutoff
NIST	National Institute of Standards and Technology
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
SSP	Study Specific Procedure
TDS	Test dosing solution
U.S. EPA	United States Environmental Protection Agency

[1] Standard abbreviations, e.g. units of measure, concentration, mass, time etc., are used without definition according to the format described in “Instructions to Authors” in The Journal of Biological Chemistry.

1.0 Summary

This analytical sub-report describes the formulation and subsequent analyses of the test dosing solutions (TDS), containing the dicamba mono-oxygenase (DMO) enzyme from MON 87708, and control dosing solutions (CDS), containing bovine serum albumin (BSA), used in a mouse acute oral toxicity study performed at Charles River Laboratories, Inc. (Spencerville, OH).

For each group of mice dosed, the TDS and CDS were administered in a single dose within a single day. Samples of both the TDS and CDS were collected immediately prior to the administration of the doses (pre-dose) and immediately following administration of the doses (post-dose) to determine the stability, concentration, homogeneity, and activity of the dosing solutions over the dosing period. Stability was assessed by determining the purity of the DMO and BSA proteins in the pre- and post-dose samples. Purity was measured using densitometric analysis of protein bands in the samples separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with colloidal Brilliant Blue G stain. Total protein concentration of the pre- and post-dose TDS and CDS samples was determined by measuring the total amino acid concentration. In addition, the homogeneity of the TDS was assessed by comparing the protein concentration of aliquots taken from the top, middle, and bottom portions of the TDS container prior to dosing. The functional activity, i.e., the ability of the DMO enzyme in the TDS to convert dicamba to 3,6-dichlorosalicylic acid (DCSA) was evaluated for the pre- and post-dose TDS aliquots using an enzymatic assay.

Analysis of the TDS showed that the purity of the DMO proteins was 82% and 83% in the pre- and post-dose TDS samples, respectively. Since the difference in purity of the DMO proteins between the pre- and post-dose samples was less than 10%, the TDS was considered stable over the duration of the dosing period. Analysis of the CDS showed that the purity of the BSA protein was 88% and 89% in pre- and post-dose CDS samples, respectively. Since the difference in purity of the BSA protein between the pre- and post-dose samples was less than 10%, the CDS was considered stable over the duration of the dosing period. The total protein concentration of the pre- and post-dose TDS samples were determined to be 5.26 mg/ml and 4.86 mg/ml, respectively, and the TDS was determined to be homogeneous. The concentrations of the BSA protein in the pre- and post-dose CDS samples were determined to be 6.97 mg/ml and 6.87 mg/ml, respectively. Dose levels were calculated for the TDS and the CDS using the average purity-corrected concentrations of the pre- and post-dose samples. The average purity-corrected concentration of the DMO proteins in the TDS was 4.20 mg/ml which, based on a dose volume of 33.3 ml/kg body weight (BW), resulted in a dose level of 140 mg/kg BW. Likewise, using a 33.3 mg/kg BW dose volume the average purity-corrected concentration of BSA protein in the CDS was 6.16 mg/ml resulted in a dose level of 205 mg/kg BW The DMO enzyme was biologically active in pre- and post-dose TDS samples with an average specific activity of 43.49 nmoles/min/mg of DMO enzyme.

These data establish the protein concentrations and stability of the test and control dosing solutions used and the dose levels administered in a mouse oral acute toxicity study.

2.0 Introduction

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 dicamba herbicide.

The DMO protein produced in MON 87708 is targeted to chloroplasts for co-localization with the endogenous reductase and ferredoxin proteins that can supply electrons for the DMO oxidative reaction. The plant-produced 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 are located between the CTP and the amino terminal end of the coding region of DMO to potentially stabilize expression of this protein *in planta* (Feng and Malven 2008; Song et al., 2009). 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 (Feng and Malven, 2008; Morey and Niemeyer, 2009 A and B). 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 was designated DMO+27. The DMO enzyme functions as a trimer (D'Ordine et al., 2009; Dumitru et al., 2009) and in the case of MON 87708 the DMO enzyme would be a trimer comprised of DMO and DMO+27 (Feng and Malven 2008).

An acute oral toxicity study was performed on mice as part of the safety assessment of the DMO proteins. This sub-report describes the formulation and analyses of the TDS and CDS used in the mouse acute oral toxicity study. Analyses included protein concentration, purity (for TDS and CDS), homogeneity and functional activity (for TDS only) of the dose solutions both before and after dosing of the mice. These procedures were performed to evaluate the administered dose concentrations and to assess if any changes in the test or control article occurred during the performance of the acute oral toxicity study.

3.0 Purpose

The purpose of this analytical sub-report is to describe the formulation and analysis of the TDS and CDS used in the mouse acute oral toxicity study for MON 87708-produced DMO proteins, performed at Charles River Laboratories Inc. (Charles River study number EUF00229; Spencerville, OH).

4.0 Materials

4.1 Test Article

The test article was comprised of the DMO enzyme produced in MON 87708. The DMO enzyme (Orion lot 11261646) was purified at Monsanto Company and includes the DMO and DMO+27 proteins. The identity, concentration, purity, stability, and functional activity of the DMO proteins were determined concurrently under a separate characterization study. The final total protein concentration determined during the characterization study was 0.18 mg/ml. Activity was confirmed using an *in vitro* assay that measures the conversion of dicamba to DCSA catalyzed by the DMO enzyme. A copy of the COA was archived with this study file.

The preliminary total protein concentration of DMO enzyme (Orion lot 11261646) was reported as 0.39 mg/ml prior to formulation of the TDS. This miscalculated value was used to determine the amount of the DMO enzyme preparation necessary to formulate the TDS at the target protein concentration, and resulted in a lower final concentration of DMO in the TDS. The test article was formulated as a test dosing solution (TDS) at Monsanto Company in an aqueous buffer containing 20 mM potassium phosphate pH 8.0. The TDS was assigned lot number 87708-D.

4.2 Control Article

The control article was bovine serum albumin (BSA) protein (catalog # 126609, lot D00068037) purchased from Calbiochem (Gibbstown, NJ). The vendor's COA is archived with this study file. According to the vendor's characterization, the protein has a purity of 100%. The relative amount of the protein content in the solid BSA powder was determined during development by amino acid analysis to be 77%.

4.3 Assay Controls

Protein molecular weight markers (Broad Range, Bio-Rad, Hercules, CA) were used to calibrate SDS-polyacrylamide gels. National Institutes of Standards and Technology (NIST, Gaithersburg, MD) amino acid calibration control standard was used to calibrate the amino acid analyzer and NIST BSA was used as a hydrolysis control. Alpha aminobutyric acid (AAbA, (NIST, Gaithersburg, MD) Sigma, St. Louis) was used as an internal standard for the amino acid analysis.

5.0 Methods for Dose Preparation

The target dose level of the DMO proteins and BSA in the acute oral toxicity study was 250 mg protein/kg body weight (BW). The calculations to create the appropriate protein concentrations in the test and control article dosing solutions to achieve the target dose level are described below.

5.1 Calculation of the Control and Test Article Dosing Solution Concentrations

The theoretical protein dose concentrations for formulation of the TDS and CDS were calculated using the following assumptions.

a) The target dose level for each mouse was 250 mg protein/kg BW

b) The average mouse BW is 0.030 kg.

c) Doses are administered at 33.33 ml/kg BW, ~1 ml/dose, once:

$$0.030\ kg\ BW \times 33.33\ ml/kg \cong 1\ ml$$

d) the target protein dose concentration is at least 7.5 mg/ml:

$$\frac{250\ mg/kg\,BW}{33.33\ ml/kg\,BW \times 1\ dose} \cong 7.5 mg/ml\ dose$$

e) The minimum total volume of sample required is 10 ml:

$$10\ mice \times 1\ doses \times 1\ ml = 10\ ml$$

A total of approximately 13 ml of the TDS and CDS was produced.

The estimated purity of the BSA protein is 100% and protein content is 77%, therefore, the CDS solution should contain approximately 9.7 mg/ml of BSA powder:

$$\frac{7.5 mg/ml}{0.77 \times 1.00} \cong 9.7\ mg/ml$$

The experimentally obtained dose levels were determined after administration to mice by assessing the concentration and purity for the DMO proteins and the BSA protein in the TDS and CDS, respectively.

5.2 Formulation of the Test Article in the Test Dosing Solution (TDS)

The DMO enzyme preparation was concentrated to target the desired concentration of approximately 7.5 mg/ml. A 330 ml aliquot of DMO enzyme preparation (Orion lot 11261646) was thawed on wet ice and concentrated using a 3 kDa molecular weight cut-off polyethersulfone concentrator (Sartorius-Stedim Biotech, France). The final volume after concentration was 17.5 ml.

The concentrated sample was then diafiltered with 10-fold the concentrated volume (175 ml) to exchange the sample into the TDS buffer (20 mM potassium phosphate, pH 8.0, lot G853668). The concentrated and diafiltered sample was removed from the concentration apparatus, and a final 13.25 ml of sample was recovered. Thirteen milliliters were transferred to a wide mouth container with a Teflon stir bar and frozen on dry ice. This solution was identified as the TDS, assigned lot 87708-D, and shipped to Charles River Laboratories for dosing.

The remainder of the solution (~0.25 ml) was used to evaluate the suitability of the TDS for the acute oral toxicity study using the following criteria: (1) the dose solution

passes through an 18 or 20-gauge needle; (2) a total protein concentration of at least 7.5 mg/ml was achieved; (3) the total protein concentration of the CDS should be within 10% of that of the TDS; and (4) a similar pattern of stained protein bands in the TDS relative to the test article should be observed on SDS-PAGE analysis.

5.3 Formulation of the Protein Control in the Control Dosing Solution (CDS)

BSA powder (157 mg, Calbiochem catalog #126609, lot D00068037) was weighed and brought to 18 ml with 20 mM potassium phosphate buffer pH 8.0 (lot G853668). The solution was transferred to a container with a Teflon stir bar and frozen on dry ice. This solution was identified as the CDS, assigned lot 68037-C, and shipped to Charles River Laboratories for dosing.

6.0 Methods

6.1 Total Protein Concentration Determination

The total protein concentration of the samples was determined using amino acid analysis. Each sample was diluted with water and analyzed in quintuplicate. The diluted samples were subjected to vapor phase acid hydrolysis followed by amino acid analysis using AccQ-Tag™ derivatization with fluorescence detection performed following the SOPs BR-ME-1139 and BR-EQ-1138. Final dose solution concentrations were calculated based on the average of the purity-corrected total protein concentration values for the DMO and BSA proteins in both the pre- and post-dose TDS and CDS samples, respectively.

The homogeneity of the TDS solution was assessed using amino acid analysis as described above. Each TDS homogeneity sample (i.e., top, middle, and bottom) was analyzed in quintuplicate, and the total protein concentrations averaged for the bottom, middle, and top samples. The dosing solution was considered homogeneous if the CV of the three averages of the homogeneity samples for total protein concentration was ≤15%.

6.2 Bio-Rad Protein Assay

The Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) was performed using 96-well microtiter plates as described in SOP BR-ME-0525. Total protein concentration of the TDS was determined as the mean of three dilutions, each prepared and read in triplicate. A standard curve ranging from 0.05 to 0.39 mg/ml of DMO (Orion lot 11261646) was prepared in triplicate. All standards and samples were diluted with a 20 mM potassium phosphate buffer pH 8.0 (lot G853668). Plates were read at 595 nm using a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA) interfaced with Softmax Pro GxP software (version 5.0.1).

6.3 SDS-PAGE and Purity Analysis

For TDS suitability, aliquots of the TDS and the test article (Orion Lot 11261646) protein samples were subjected to electrophoresis at ~ 0.5, 1.0, and 1.5 µg total

protein per lane on a pre-cast Tris-glycine 4 - 20% polyacrylamide gradient gel (Invitrogen, Carlsbad, CA) as described in SOP BR-ME-0388. Precision Plus molecular weight markers (Bio-Rad Laboratories, Hercules, CA) were used to estimate molecular weight of the protein bands. All samples were mixed with 5× LB, heated at 100 °C for 3 min and then applied to a 12-well gel. Electrophoresis was performed at a constant voltage of 125 V for 90 min. Proteins were fixed with 40% (v/v) methanol, 7% (v/v) acetic acid for 25 min, stained by gentle shaking with Brilliant Blue G colloidal stain (Sigma, Chemical Co., St. Louis, MO) for 9 h, and destained for 30 s with a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid followed by a solution of 25% methanol for 23 h.

For purity analysis, aliquots of the pre- and post- TDS and CDS were subjected to electrophoresis on pre-cast Tris-glycine 4 - 20% polyacrylamide gradient gels (Invitrogen) as described in SOP BR-ME-0388 and BR-ME-0956. The protein samples were analyzed at ~ 0.5, 1.0, and 1.5 µg total protein per lane. Broad Range molecular weight markers (Bio-Rad Laboratories) were used to estimate molecular weight of the protein bands. All samples were mixed with 5× LB, heated at 100 °C for 3 - 5 min and then applied to a 10-well gel. Electrophoresis was performed at a constant voltage of 125 V for 90 min. Proteins were fixed with 40% (v/v) methanol, 7% (v/v) acetic acid for 25 min, stained by gentle shaking with Brilliant Blue G colloidal stain (Sigma) for 17.5 h, and destained for 30 - 45 s with a solution containing 25% (v/v) methanol and 10% (v/v) acetic acid followed by a solution of 25% methanol for 6.5 h

Purity was determined using densitometric analysis of the stained SDS-PAGE gels. Analysis was performed using a Bio-Rad GS-800 densitometer with the supplied Quantity One™ software (version 4.4.0, Hercules, CA). The optical density of protein bands corresponding to DMO (~39 kDa) and DMO+27 (~ 42 kDa), the two forms of the protein that are present in the DMO enzyme, and BSA (~66 kDa) protein was measured within each lane. Purity was estimated as the percent optical density of the bands of interest relative to all bands detected in the lane. Purity was reported as an average of the six values obtained from all three duplicate lanes containing the DMO proteins or the BSA protein. Molecular weight was evaluated for positional reference only. Dose solutions were considered stable throughout dosing if a ≤10% decrease in protein purity of the post-dose samples compared to the pre-dose samples was observed.

6.4 Activity Analysis

Samples of the TDS taken both pre- and post-dose were used to verify the functional activity of the DMO enzyme in the TDS. An end-point assay based on the DMO-dependent conversion of dicamba to 3,6-dichlorosalicylic acid (DCSA) was used to determine the functional activity of the TDS samples, according to the November 16th, 2009 draft SOP BR-ME-1244. Demonstration of functional activity in both the

pre- and post-TDS samples would confirm that the TDS was biologically active throughout dosing.

A calibration curve using DCSA was constructed to final concentrations of 0.1, 0.3, 0.6, 0.9, 1.2, 2.4, and 4.8 nmoles of DCSA/250 µl. The assay samples were prepared by adding 165 µl of an assay master mix containing 25 mM KPi, pH 7.2, 10 mM $MgCl_2$, 15.4 µg reductase, 2.5 µg ferredoxin, and ~0.085 U of formaldehyde dehydrogenase. After pre-incubating at 30 °C for 15 min, NADH and $FeSO_4$ were added to each tube, followed by the addition of 10 µl of 6 mM dicamba. The assay samples were incubated at 30 °C for 15 min, after which the reaction was stopped with the addition of 50 µl of 5% H_2SO_4. The samples were filtered and loaded onto an HPLC, where the DCSA and dicamba were separated by reverse phase chromatography using a Phenomenex C-18 column (Torrance, CA). The formation of DCSA was monitored by fluorescence at 424 nm (excitation at 306 nm).

The DMO enzyme was considered to have retained its activity throughout the dosing regimen if activity was observed both in the pre- and post-dosing TDS samples.

7.0 Control of Bias and Quality Measures

Appropriate concentrations of dosing solution samples were analyzed concurrently on SDS-PAGE gels to establish purity. Multiple dilutions were utilized for the Bio-Rad protein assay to ensure that data was collected within operational range of the assay. The functional assay samples were analyzed in quintuplicate to ensure an accurate determination of the DMO enzyme activity.

8.0 Amendments and Deviations to the Study Specific Work Procedure

There were 3 amendments to the Study Specific procedure. There were 4 deviations in the study:

Amendments

1. The study-specific procedure was amended to add the DMO enzyme preparations test article (Orion Lot #: 11242646) as an additional standard protein used in the Bio-Rad protein concentration assay. This amendment was made to more accurately determine the amount of protein in the TDS. The concentration estimate using the DMO enzyme preparation as a standard was chosen, since the characteristics of that standard were more similar to the TDS solution than was the BSA standard.

2. The study-specific procedure was amended to correct the draft date and version of the Dicamba Mono-Oxygenase Activity Assay SOP. The original protocol referenced an erroneous date of draft of November 23rd, 2009 for SOP BR-ME-1244. The amendment corrected that draft date to November 16th, 2009. The change in the protocol had no effect on the study because the date was a typographical error.

3. The study-specific procedure was amended to correct a typographical error. The intended samples for the stability analysis, as indicated in the table in sections 4.2 and 5.0, are the pre and post- TDS and CDS samples, not the test article (Orion lot 11261646) as was originally written. This amendment had no impact on the study because the TDS and CDS were always the intended samples for stability analysis.

Deviations

1. On the day the purity gels for the TDS and CDS pre- and post-dose samples for gavage were scanned, the initial Image Reports were not printed as required in the last operation of Step 9 in the SOP, but were printed at a later date. There was no impact on the study specific procedure due to printing the initial Image Reports at a later date because the history log indicates no changes were made during that period.

2. The stained gel generated for the test dose solution (TDS) suitability evaluation was scanned and saved as "20091118 aaevan DMOsoy TDS87708DGel v2.1sc", but the data file was not transferred to the Regulatory Fileshare within 10 days of scanning, as specified by the SOP. It was transferred to the Regulatory Fileshare on a later date. There was no adverse impact on the study specific procedure, because the files were ultimately transferred to the Regulatory Fileshare.

3. When determining the total protein concentration of the pre-dose TDS sample by amino acid analysis according to BR-ME-1139 it was observed that the pmol yield of the internal calibrant (AAbA) was lower for test sample replicate C2 than that of the other replicates despite the same gravimetric loading amount of AAbA for other test sample replicates (C1, C3, C4, and C5) in the analysis. This led to test sample replicate C2 having a significantly higher concentration than the other replicates and producing an absolute error relative to the average total protein concentration of all five replicates of more than 20%. The entire analysis was not repeated as required by the SOP, instead the Pre-TDS sample replicate C2 was removed from the analysis due to this issue. With test sample replicate C2 removed the absolute error for each replicate relative to the average total protein concentration of the remaining four replicates (C1, C3, C4, and C5) fell below the 20% as specified by the SOP. There was no adverse impact on the study by removing test sample replicate C2 and it was estimated that the remaining replicates provided a reliable total protein concentration.

4. While each AAA data package that is intended to support a regulatory submission to an outside agency must include the Pro_And_Events Atlas report, the report was not included with this data package. While attempting to print the Pro_And_Events report an error was displayed on the screen that prevented printing. Monsanto IT is currently in the process of identifying and resolving the

print error issue. The Pro_And_Events Atlas report is provided as supporting data within the data package and does not contain any information that is critical to the generation of the total protein concentration value therefore, its absence from the data package has no effect on the study.

9.0 Data Rejected or Not Reported

The first Bio-Rad Protein Assay (BR-ME-0525) for estimating the TDS concentration on 16-November-2009 was rejected because the estimated total protein concentration of the test article (Orion Lot 11242646), using BSA as the standard, was significantly lower than that established previously by amino acid analysis. The BSA standard was subsequently tested in parallel against the test article (Orion Lot 11261646), which confirmed significant differences in TDS concentration estimates between the two standards.

The initial purity analysis of the CDS Pre-Dose purity gel scanned image was performed on a compressed image rather than the original high resolution raw image. The first analysis was rejected and the high resolution image was analyzed to determine the purity of the CDS pre-dose sample.

10.0 Results and Discussion

10.1 Dosing Solution Preparation and Suitability Assessment

Dosing solutions containing the DMO and BSA proteins were prepared for the mouse acute oral toxicity study using the calculations and assumptions described in Section 5.1. Several tests were performed to assess the suitability of the TDS for use in the study. The TDS passed through a 20-gauge needle. The total protein concentration, determined using a Bio-Rad colorimetric protein assay, was 8.5 mg/ml, (using the preliminary concentration of DMO). A similar protein banding pattern was observed for the TDS and the test article on a stained SDS-polyacrylamide gel.

10.2 Homogeneity of the TDS

TDS homogeneity was determined by evaluating the protein concentration of samples taken from the top, middle, and bottom sections of the TDS solution container. The %CV of the average concentration of the three samples was 6.8 % (Table 1). This value was below the pre-set acceptance criterion for homogeneity of ≤ 15% and, therefore, the TDS was considered to be a homogeneous solution.

10.3 Protein Concentration of the TDS and CDS

Total protein concentration was determined using amino acid analysis on the dosing solution samples taken before and after administration of doses (Tables 2 and 3). Amino acid analyses were performed in quintuplicate for all the samples, except one. The total protein concentration for the pre- and post- dose TDS samples was determined to be 5.26 mg/ml and 4.86 mg/ml, respectively (Table 2). The average

concentration of the TDS sample was 5.06 mg/ml (Table 2). Though this value is below the concentration determined during the suitability assessment (Section 10.1), it reflects the concentration in the TDS at the time of dosing, is accurate, and, therefore, was used to calculate the dose levels (see Section 10.6).

Total protein concentration for the pre- and post- dose CDS samples was 6.97 mg/ml and 6.87 mg/ml, respectively. The average concentration was calculated to be 6.92 mg/ml (Table 3).

10.4 Purity of the DMO and BSA Proteins in the TDS and CDS

Stability of the DMO and BSA proteins in the TDS and CDS, respectively was determined by measuring the protein purity in the pre- and post-dose samples using densitometric analysis of the colloidal Brilliant Blue G stained SDS-polyacrylamide gels (Figures 1 - 4). Purity data for the DMO and BSA proteins are summarized in Table 4.

The average percent purity of the DMO proteins in the pre and post- dose TDS sample was 82 and 83%, respectively. There was a 1% change in the percent purity of the DMO proteins (Table 4), thus the TDS was considered to be stable for the duration of the dosing period.

The average purity of the BSA protein was 88% and 89% in pre- and post- dose CDS samples, respectively. The percent change in purity was 1% (Table 4). This is also within the established acceptance criteria for stability of a decrease of ≤ 10% in purity. Thus, the CDS was considered to be stable for the duration of the dosing period. These values were lower than those used to formulate the CDS as reported by the manufacturer.

10.5 Activity of the DMO Enzyme in the TDS

The activity of the DMO enzyme in the TDS was evaluated. The DMO enzyme was active in pre- and post-dose samples with specific activities of 41.09 and 45.89 nmoles DCSA/min/mg DMO, respectively (Table 5), resulting in an 11.7% difference. This difference is within the intra-assay variability of the enzyme assay, confirming that the biological activity of the DMO enzyme in the TDS was retained throughout the dosing period.

10.6 Calculation of the Dose Levels

The final dose levels were calculated using total protein concentration and purity values determined for the TDS and CDS as described in Sections 10.3 and 10.4. Because the TDS was determined to be a homogeneous suspension, the administered test article dose level was calculated from the average concentration of the pre- and post- dose values. The calculations are shown below and the results are summarized in Table 6.

The dose level for the DMO proteins was determined to be $140\frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $5.06\frac{mg}{ml}\times 0.83 = 4.20\frac{mg}{ml}$

b) Protein level per dose: $4.20\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 140\frac{mg}{kgBW}$

c) Final dose level: $140\frac{mg}{kg\ BW}\times 1\ \ dose = 140\frac{mg}{kg\ BW}$

The dose level for BSA protein was determined to be $205\frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $6.92\frac{mg}{ml}\times 0.89 = 6.16\frac{mg}{ml}$

b) Protein level per dose: $6.16\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 205\frac{mg}{kgBW}$

c) Final dose level: $205\frac{mg}{kg\ BW}\times 1\ \ dose = 205\frac{mg}{kg\ BW}$

11.0 Conclusions

The analytical tests performed for the TDS samples established that a stable, homogenous formulation of the DMO proteins in the TDS was achieved. No change in the purity of the DMO proteins was observed prior to and after the administration of the TDS to mice, indicating that the TDS was stable throughout the dosing. In addition, the DMO enzyme was biologically active throughout the dosing. There was no decrease in the BSA protein purity observed after the administration of the CDS to mice, indicating that the CDS was stable throughout the dosing period.

The concentration of the DMO and BSA proteins in the TDS and CDS, respectively, were calculated based upon total protein concentration and percent purity. The experimentally confirmed protein concentration of the DMO proteins in the TDS was 4.20 mg/ml resulting in a dose level of 140 mg/kg BW. The experimentally confirmed protein concentration of the BSA protein in the CDS was 6.16 mg/ml resulting in a dose level of 205 mg/kg BW.

These data establish the protein concentrations and stability of the test and control dosing solutions used and the doses levels administered in a mouse oral acute toxicity study.

Table 1. Homogeneity of the TDS Pre- Dose Samples

Sample Description	Sample ID	Concentration[1] (mg/ml)	CV(%)
TDS- Bottom	B-TDS	4.86	6.8
TDS-Middle	M-TDS	4.72	
TDS-Top	T-TDS	5.36	

[1] Each value represents the mean of five replicates rounded to three significant figures.

Table 2. Total Protein Concentrations of the TDS Samples

Sample	Concentration (mg/ml)	TDS Average Protein Concentration (mg/ml)
Pre-TDS	5.26[1]	5.06
Post-TDS	4.86[2]	

[1]The value represents the mean of four replicates rounded to three significant figures.
[2]The value represents the mean of five replicates rounded to three significant figures.

Table 3. Total Protein Concentrations of the CDS Samples

Sample	Concentration (mg/ml)[1]	CDS Average Protein Concentration (mg/ml) [1]
Pre-Dose	6.97	6.92
Post-Dose	6.87	

[1]Each value represents the mean of five replicates rounded to three significant figures.

Table 4. Purity of the DMO and BSA proteins in the TDS and CDS Samples

Dose Identification	Sample	Purity (%)[1]	Percent Change[2]	Purity (%) Used in Dose Calculation[3]
TDS	Pre-Dose	82	1	83
	Post-Dose	83		
CDS	Pre-Dose	88	1	89
	Post-Dose	89		

[1] Each value represents the mean of three purity values estimated from loadings of 0.5, 1.0, and 1.5 µg total protein. The purity is determined by adding the purity of the DMO proteins (DMO and DMO+27)

[2] Calculated as follows: $\left|\frac{(\text{pre Dose}) - (\text{post Dose})}{(\text{pre Dose})}\right| \times 100\% \cdot$

[3] Calculated as the mean of the pre- and post dose purity values, and rounded to two significant figures.

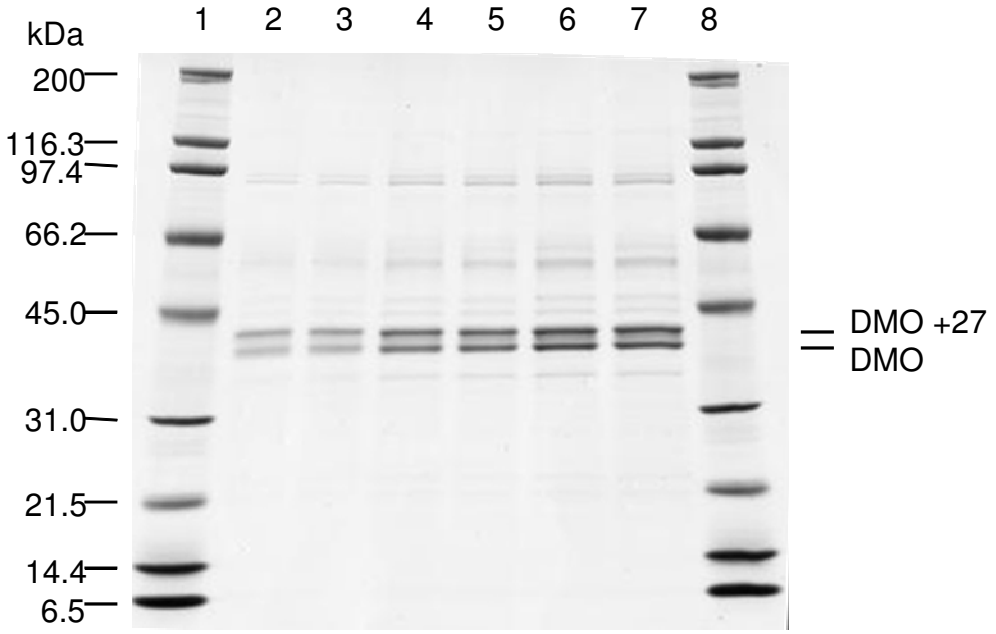
Table 5. Functional Activity of Pre- and Post-Dose TDS Samples

Sample	Dose	Specific Activity (nmoles DCSA/min/mg DMO)	Difference (%)[1]
TDS	Pre-Dose	41.09	11.7
	Post-Dose	45.89	

[1] Calculated as follows: $\left|\frac{(\text{pre Dose Activity}) - (\text{post Dose Activity})}{(\text{pre Dose Activity})}\right| \times 100\% \cdot$

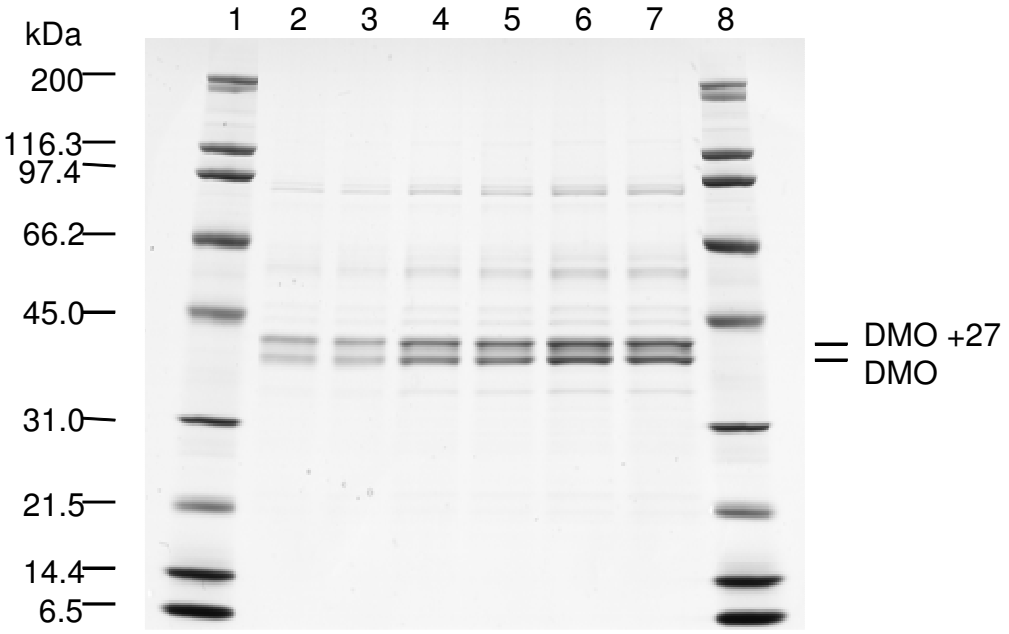
Table 6. Experimentally Determined Dose Levels

Dose Identification	Purity Corrected Concentration (mg/ml)	Dose Level (mg/kg BW)
Avg. Dose TDS	4.20	140
Avg. Dose CDS	6.16	205



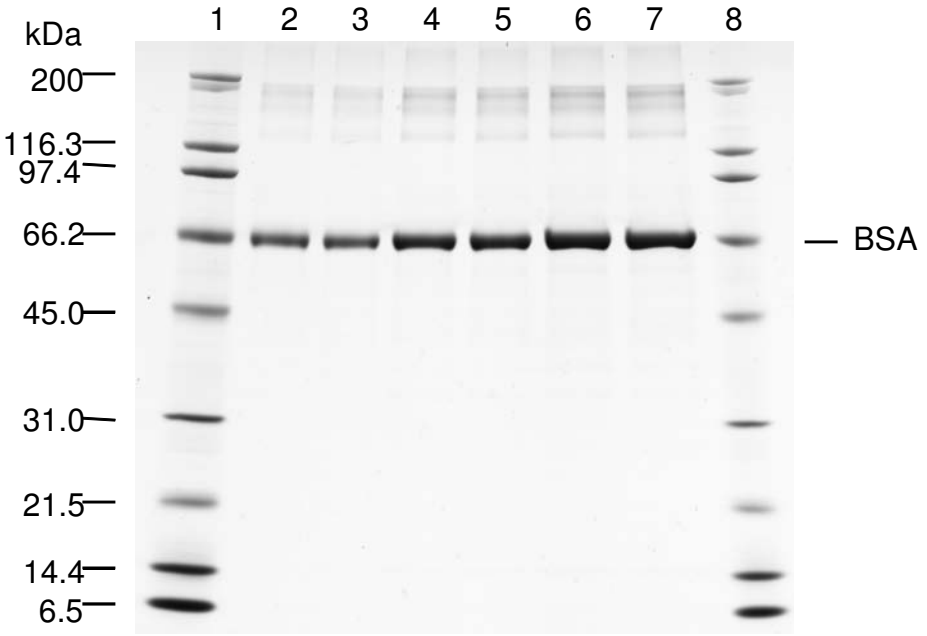
Lane	Sample	Amount (µg)
1	MW markers	-
2	Pre-Dose TDS	0.5
3	Pre-Dose TDS	0.5
4	Pre-Dose TDS	1.0
5	Pre-Dose TDS	1.0
6	Pre-Dose TDS	1.5
7	Pre-Dose TDS	1.5
8	MW markers	-

Figure 1. Purity Analysis of the DMO Proteins in the Pre-Dose TDS Samples
Lanes 2-7 correspond to samples taken prior to dosing of the mice. Tris-Glycine 4-20% polyacrylamide gradient gels were stained with colloidal Brilliant Blue G. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped and lanes re-numbered.



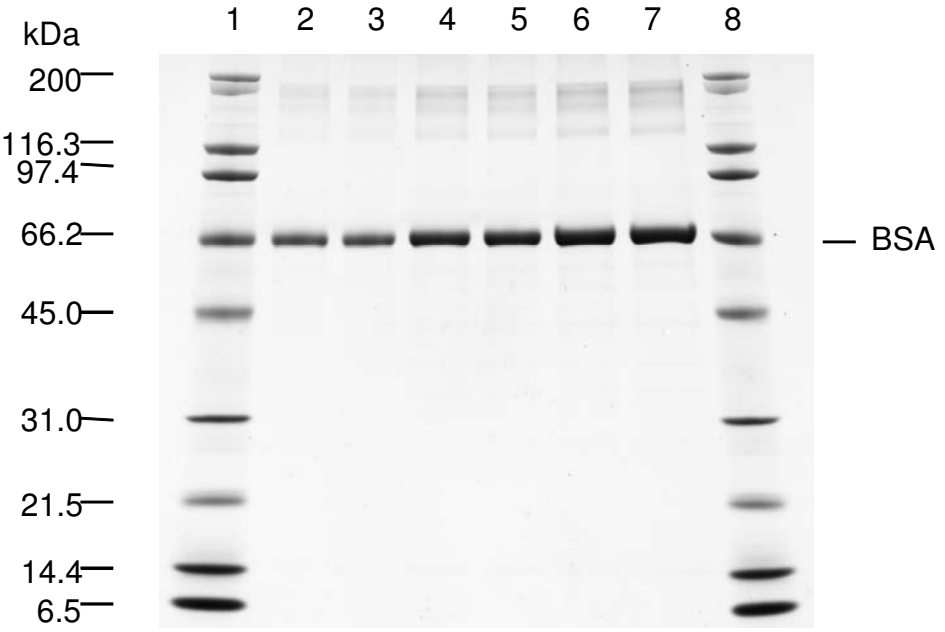
Lane	Sample	Amount (µg)
1	MW markers	-
2	Post-Dose TDS	0.5
3	Post-Dose TDS	0.5
4	Post-Dose TDS	1.0
5	Post-Dose TDS	1.0
6	Post-Dose TDS	1.5
7	Post-Dose TDS	1.5
8	MW markers	-

Figure 2. SDS-PAGE Analysis of the DMO Proteins in Post-Dose TDS Samples
Lanes 2-7 correspond to samples taken after dosing of the mice. Tris-Glycine 4 - 20% polyacrylamide gradient gels were stained with colloidal Brilliant Blue G. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped and lanes re-numbered.



Lane	Sample	Amount (µg)
1	MW markers	-
2	Pre-Dose CDS	0.5
3	Pre-Dose CDS	0.5
4	Pre-Dose CDS	1.0
5	Pre-Dose CDS	1.0
6	Pre-Dose CDS	1.5
7	Pre-Dose CDS	1.5
8	MW markers	-

Figure 3. Purity Analysis of BSA in the Pre-Dose CDS Samples
Lanes 2-7 correspond to samples taken prior to dosing of the mice. Tris-Glycine 4-20% polyacrylamide gradient gels were stained with colloidal Brilliant Blue G. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped and lanes re-numbered.



Lane	Sample	Amount (µg)
1	MW markers	-
2	Post-Dose CDS	0.5
3	Post-Dose CDS	0.5
4	Post-Dose CDS	1.0
5	Post-Dose CDS	1.0
6	Post-Dose CDS	1.5
7	Post-Dose CDS	1.5
8	MW markers	-

Figure 4. SDS-PAGE Analysis of BSA Protein in the Post-Dose CDS Samples
Lanes 2-7 correspond to samples taken after dosing of the mice. Tris-Glycine 4- 20% polyacrylamide gradient gels were stained with colloidal Brilliant Blue G. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Empty lanes were cropped and lanes re-numbered.

Appendix 1. List of Applicable SOPs

AG-ME-0388-03	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0525-02	Bio-Rad Protein Assay
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-EQ-0599-05	Bio-Rad GS-800 Densitometer
BR-ME-0956-03	Protein Percent Purity and Apparent Molecular Weight Determination
BR-EQ-1138-01	Waters 2695 Separations Module for AccQ-Tag Analysis
BR-ME-1139-01	Vapor Phase Acid Hydrolysis Using 6 N HCl and Subsequent Amino Acid Analysis Using AccQ-Tag® Derivatization
BR-ME-1244-draft	Dicamba Mono-Oxygenase Activity Assay

Appendix 3
Detailed Clinical Observation Parameters

Detailed Clinical Observation Parameters

Cage-side Observations	Recorded As
Abnormal movements or behavior	See Categorical
Resistance to removal from cage	Score

Hand-held Observations	Recorded As
Palpebral closure	Score
Lacrimation (non-colored periocular wetness)	Score
Pupil Size	Score
Salivation (non-colored perioral wetness)	Score
Muscle tone	Score
Extensor-thrust response	Score
Reactivity to handling	Score

Open-field observations	Recorded As
Responsiveness to touch	Score
Gait evaluation	Score

Categorical observations (anytime during the DCO)	Recorded As
Abnormal behavior	Description
Abnormalities of the eye	Description
Abnormal urine or feces	Description
Abnormalites of the gastrointestinal (GI) tract	Description
Injury	Description
Missing extremity	Description
Abnormal muscle movements	Description
Palpable mass/swellings	Description
Abnormal posture	Description
Abnormalities of the reproductive system	Description
Abnormal respiration	Description
Abnormal skin or hair-coat/mucous membranes	Description
Excessive soiling	Description
General abnormalities	Description

Explicitly Defined Scales for DCOs

DCO Examination Conduct

The clinical examination is conducted in a careful and systematic format. The examination begins at the head of the animal and gradually works towards the tail as outlined below.

Cage-side observations are made first.

Categorical observations include: Unusual body movements (e.g., tremors, convulsions), abnormal behaviors (e.g., circling, stereotypy) and changes in posture (e.g., arched back, splayed stance).

Resistance to Removal: The degree to which the animal attempts to escape capture is scored. The observer will slowly present a gloved hand into the cage and will grasp the animal over the shoulder area or by the tail.

1 = Decrease – clearly less resistance to capture than typical

2 = Typical – minimally to actively avoids capture and may be mildly aggressive

3 = Increase – clearly more resistance to capture than typical and is very aggressive (attempts to bite).

Eye observations: Eyes are bilaterally examined for these effects; however, if a unilateral observation is made, a concurrent observation is not made for the other eye if it is within normal limits.

Palpebral closure:

1 = Closed (50% to completely closed)

2 = Open

3 = Protruding eyes

Pupil size (aided by penlight): Under typical examination conditions (white light), the typical appearance of the pupils in albino animals is complete constriction. Therefore a decrease in pupil size cannot be observed.

0 = Unable to evaluate

1 = Decrease – clearly decreased pupil size compared to typical

2 = Typical – completely constricted pupils

3 = Increase – clearly increased pupil size compared to typical

Lacrimation (clear wetness): Under typical examination conditions, corneal dryness is not observed in rodents, nor are the eyelids excessively wet.

1 = Decrease – extremely dry appearance of cornea

2 = Typical – glistening cornea (moderate dryness or wetness)

3 = Increase – extensive wetness around the eyes

Degree of salivation: Under typical examination conditions, dryness of the oral cavity is not observed in rodents.

1 = Decrease – oral dryness

2 = Typical – limited to moderate perioral wetness, but lips and chin are dry

3 = Increase – extensive wetness around the mouth and lips

Muscle tone: An assessment of muscle tone at the time of the hand-held observations.

1 = Decrease – clearly less muscle tone than typical

2 = Typical – animal is neither very relaxed nor very tense

3 = Increase – clearly more muscle tone than typical

Extensor-thrust response: Extent of reflex response to brisk pushes (by finger) on the plantar surface of the hindfeet.

1 = Decrease – clearly less response than typical

2 = Typical – clearly detectable extensor-thrust response

3 = Increase – clearly more response than typical

Reactivity to handling: The degree to which an animal struggles to get free from hand-held restraint is ranked.

1 = Decrease – very slight or no struggling

2 = Typical – mild to moderate struggling, animal may vocalize

3 = Increase – aggressive escape behavior, may try to bite observer and usually vocalizes

Observations made in the open-field.

Responsiveness to touch: The ventral aspect of the tail is lightly stroked using a finger. Typically, the animal will lift its tail and wrap it around the finger when lightly touched.

1 = Decrease – does not lift tail, but may briefly hold tail in the air when manually lifted; no response to touch

2 = Typical – lifts tail when touched

3 = Increase – lifts tail and acts startled, may turn towards finger in an attack response

Gait evaluation: Open-field observations are used for gait evaluation. If the animal remains motionless in the open-field, it may be forced to walk on its forelegs while the hindlegs are held off the floor of the observation box ("the wheel-barrow test").

1 = Unable to walk

2 = Clear knuckling, stumbling and poor coordination, may include falling and/or dragging of one or more limbs

3 = Typical – smooth and coordinated gait

Categorical Observations: These observations can be made at anytime during the DCOs. For the categories listed below, the observer directly records the positive observation.

1.	Abnormal behavior	Description
2.	Abnormalities of the eye	Description
3.	Abnormal urine or feces	Description
4.	Abnormalities of the gastrointestinal tract	Description
5.	Injury	Description
6.	Missing extremity	Description
7.	Abnormal muscle movements	Description
8.	Palpable mass/swellings	Description
9.	Abnormal posture	Description
10.	Abnormalities of the reproductive system	Description
11.	Abnormal respiration	Description
12.	Abnormal skin or hair coat/mucous membranes	Description
13.	Excessive soiling	Description
14.	General abnormalities	Description

Appendix 4
Individual Survival and Clinical Observations

(Positive Findings)

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

APPENDIX 4

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 1: BSA 19-NOV-09 to 3-DEC-09

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
9963	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA
9964	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA
9965	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA
9966	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA
9967	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 2: DMO 19-NOV-09 to 3-DEC-09

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
9968	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9969	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9970	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9971	OPEN-FIELD OBS	4	23-NOV-09	07:39	GAIT EVALUATION; MODERATE NOT FULLY EXTENDING RIGHT HINDLIMB
	OPEN-FIELD OBS	5	24-NOV-09	07:34	GAIT EVALUATION; MODERATE NOT FULLY EXTENDING RIGHT HINDLIMB
	OPEN-FIELD OBS	6	25-NOV-09	07:51	GAIT EVALUATION; MODERATE NOT FULLY EXTENDING RIGHT HINDLIMB
	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9972	BODY	13	2-DEC-09	07:39	UNKEMPT APPEARANCE
	BODY	14	3-DEC-09	08:16	UNKEMPT APPEARANCE
	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES GROUP 1: BSA 19-NOV-09 to 3-DEC-09

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
9973	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA
9974	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA
9975	DEAD	14	3-DEC-09	08:21	SUBMITTED FOR SCHEDULED EUTHANASIA
9976	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9977	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES GROUP 2: DMO 19-NOV-09 to 3-DEC-09

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
9978	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9979	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9980	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9981	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA
9982	DEAD	14	3-DEC-09	08:22	SUBMITTED FOR SCHEDULED EUTHANASIA

Appendix 5
Individual Body Weight Data

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

APPENDIX 5

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 1: BSA

ANIMAL#	DAY OF STUDY 0 (NON-FASTED)	0 (FASTED)	7	14
9963	30.6	30.1	32.1	33.5
9964	32.0	31.3	32.0	32.5
9965	32.0	31.4	31.9	32.0
9966	29.9	29.2	31.6	32.5
9967	30.8	30.2	32.4	32.1
MEAN	31.1	30.4	32.0	32.5
S.D.	0.92	0.92	0.29	0.59
N	5	5	5	5

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

APPENDIX 5

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 2: DMO

	DAY OF STUDY			
ANIMAL#	0 (NON-FASTED)	0 (FASTED)	7	14
9968	30.3	29.2	31.2	33.3
9969	31.7	31.2	32.8	34.3
9970	32.3	31.7	31.7	33.7
9971	30.6	30.0	30.5	32.1
9972	31.7	30.8	31.7	32.4
MEAN	31.3	30.6	31.6	33.2
S.D.	0.84	0.99	0.84	0.91
N	5	5	5	5

APPENDIX 5

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES GROUP 1: BSA

	DAY OF STUDY			
ANIMAL#	0 (NON-FASTED)	0 (FASTED)	7	14
9973	27.7	26.2	26.8	29.8
9974	26.0	24.8	27.1	28.4
9975	29.2	27.4	28.1	30.0
9976	24.4	23.4	26.3	25.9
9977	25.9	24.3	24.9	27.5
MEAN	26.6	25.2	26.6	28.3
S.D.	1.85	1.58	1.17	1.70
N	5	5	5	5

STUDY NO.: EUF229 PAGE 4
MONSANTO COMPANY REF CRO-09-419

APPENDIX 5

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES GROUP 2: DMO

	DAY OF STUDY			
ANIMAL#	0 (NON-FASTED)	0 (FASTED)	7	14
9978	27.4	26.3	28.1	30.9
9979	24.6	23.8	25.5	26.2
9980	29.2	27.7	26.7	29.1
9981	28.9	27.2	28.3	29.5
9982	24.6	23.4	24.4	25.0
MEAN	26.9	25.7	26.6	28.1
S.D.	2.24	1.97	1.67	2.45
N	5	5	5	5

Appendix 6
Individual Body Weight Changes

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

APPENDIX 6

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 1: BSA

ANIMAL#	DAY OF STUDY 0 (FASTED) -7	7-14
9963	2.0	1.4
9964	0.7	0.5
9965	0.5	0.1
9966	2.4	0.9
9967	2.2	-0.3
MEAN	1.6	0.5
S.D.	0.89	0.66
N	5	5

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

APPENDIX 6

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 2: DMO

ANIMAL#	DAY OF STUDY 0 (FASTED)	-7 7-14
9968	2.0	2.1
9969	1.6	1.5
9970	0.0	2.0
9971	0.5	1.6
9972	0.9	0.7
MEAN	1.0	1.6
S.D.	0.81	0.55
N	5	5

STUDY NO.: EUF229 PAGE 3
MONSANTO COMPANY REF CRO-09-419

APPENDIX 6

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES GROUP 1: BSA

ANIMAL#	DAY OF STUDY 0 (FASTED)	-7 7-14
9973	0.6	3.0
9974	2.3	1.3
9975	0.7	1.9
9976	2.9	-0.4
9977	0.6	2.6
MEAN	1.4	1.7
S.D.	1.10	1.33
N	5	5

STUDY NO.: EUF229 PAGE 4
MONSANTO COMPANY REF CRO-09-419

APPENDIX 6

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES GROUP 2: DMO

ANIMAL#	DAY OF STUDY 0 (FASTED)	-7 7-14
9978	1.8	2.8
9979	1.7	0.7
9980	-1.0	2.4
9981	1.1	1.2
9982	1.0	0.6
MEAN	0.9	1.5
S.D.	1.13	1.00
N	5	5

Appendix 7
Individual Food Consumption Data

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

APPENDIX 7

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 1: BSA

	DAY OF STUDY	
ANIMAL#	0-7	7-14
9963	7.2	8.1
9964	6.0	6.0
9965	6.1	6.5
9966	6.2	6.6
9967	7.4	7.8
MEAN	6.6	7.0
S.D.	0.67	0.88
N	5	5

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

APPENDIX 7

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 2: DMO

	DAY OF STUDY	
ANIMAL#	0-7	7-14
9968	6.4	6.7
9969	7.5	7.9
9970	5.7	6.1
9971	6.4	7.0
9972	6.0	6.6
MEAN	6.4	6.8
S.D.	0.68	0.65
N	5	5

STUDY NO.: EUF229 PAGE 3
MONSANTO COMPANY REF CRO-09-419

APPENDIX 7

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES GROUP 1: BSA

	DAY OF STUDY	
ANIMAL#	0-7	7-14
9973	6.1	6.8
9974	6.3	6.8
9975	5.5	5.9
9976	6.8	6.4
9977	6.0	6.7
MEAN	6.1	6.5
S.D.	0.49	0.42
N	5	5

STUDY NO.: EUF229 PAGE 4
MONSANTO COMPANY REF CRO-09-419

APPENDIX 7

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES GROUP 2: DMO

	DAY OF STUDY	
ANIMAL#	0-7	7-14
9978	6.7	7.0
9979	6.8	6.3
9980	6.4	7.6
9981	6.7	7.6
9982	5.9	6.7
MEAN	6.5	7.0
S.D.	0.36	0.57
N	5	5

Appendix 8
Individual Gross Necropsy Observations

STUDY NO.: EUF229 PAGE 1
MONSANTO COMPANY REF CRO-09-419

APPENDIX 8

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 1: BSA

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
9963	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9964	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9965	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9966	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9967	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

STUDY NO.: EUF229 PAGE 2
MONSANTO COMPANY REF CRO-09-419

APPENDIX 8

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 2: DMO

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
9968	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9969	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9970	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9971	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9972	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

STUDY NO.: EUF229 PAGE 3
MONSANTO COMPANY REF CRO-09-419

APPENDIX 8

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES GROUP 1: BSA

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
9973	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9974	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9975	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9976	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9977	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

STUDY NO.: EUF229 PAGE 4
MONSANTO COMPANY REF CRO-09-419

APPENDIX 8

AN ACUTE ORAL GAVAGE TOXICITY STUDY IN MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES GROUP 2: DMO

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
9978	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9979	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9980	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9981	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
9982	3-DEC-09	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA