FINAL REPORT

Guideline: EPA-OPPTS (870.1100)

Testing Facility Study No. EUF00221

Monsanto Study No. CRO-2007-325

An Acute Toxicity Study of Cry1Ac Protein Administered by the Oral (Gavage) Route to Mice

TESTING FACILITY:

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DATE:

14 January 2009

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).

Jason W. Smedley 14JAN2009

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

Date

Sponsor/Submitter
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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). 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
31-Jan-2008	Protocol Review	02-Apr-2008	02-Apr-2008
07-Mar-2008	Body Weights	02-Apr-2008	02-Apr-2008
28-Mar-2008	Protocol Amendment Review	02-Apr-2008	02-Apr-2008
28-Mar-2008	Protocol Amendment Review	02-Apr-2008	02-Apr-2008
28-Mar-2008	Data Audit	02-Apr-2008	02-Apr-2008
28-Mar-2008	Draft Report Review	02-Apr-2008	02-Apr-2008
28-Jul-2008	Data Audit	28-Jul-2008	28-Jul-2008
28-Jul-2008	Protocol Amendment Review	28-Jul-2008	28-Jul-2008
28-Jul-2008	Draft Report Review	28-Jul-2008	28-Jul-2008
07-Jan-2009	Final Report Review	07-Jan-2009	07-Jan-2009

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 3

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.

Jennifer D. McGue 13 Jan 2009

Jennifer D. McGue Date
Associate Quality Assurance Auditor
Charles River Laboratories
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4. INTELLECTUAL PROPERTY RIGHTS

5. NOTES TO REVIEWER

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6. RESPONSIBLE PERSONNEL

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Kathleen S. Crowley, Ph.D.	Principal Investigator, Dose Preparation and Analysis

TABLE OF CONTENTS

LIST OF TABLES

LIST OF APPENDICES

7. SUMMARY

The purpose of this study was to evaluate the short-term toxicity of *E.* coli-produced Cry1Ac protein following a single day oral gavage administration to mice. The study design was as follows:

Experimental Design

Group No.	No. of Animals		Treatment	Analytically-Determined Dose Level[a] (mg protein/kg body weight)
	Male	Female		
1	10	10	Bovine Serum Albumin (BSA)	1280
2	10	10	Cry1Ac protein	1290
3[b]	10	-	Bovine Serum Albumin (BSA)	1620
4[b]	10	-	Cry1Ac protein	1460

[a]Administered as two 33.3 mL/kg body weight doses separated by approximately 4 hours.
[b]To clarify possible effects in Group 2 males, an additional set of male mice (Groups 3 and 4) were dosed at slightly higher target concentrations of BSA and Cry1Ac protein.

The following variables 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 was no effect on body weight or body weight gain in female animals. No differences in food consumption were detected in Cry1Ac protein-treated animals when compared to BSA-treated control animals. No test article-related gross necropsy findings were present.

A statistically significant decrease in body weight gain was noted in Group 2 males (Cry1Ac, 1290 mg/kg) compared to Group 1 males (BSA, 1280 mg/kg) during the first week of the study; however, interpretation of this finding was complicated by an interruption in water delivery to a Group 1 male animal (BSA, 1280 mg/kg) during this period. Additional groups of male animals were treated (Groups 3 and 4) to clarify the finding. No evidence of a body weight effect was noted in the male animals dosed in this additional phase of the study nor were there any body weight effects in the females dosed during the initial phase of the study. Therefore, the finding of decreased body weight gain in male animals was not considered related to treatment.

Conclusion:

There were no treatment-related adverse effects of Cry1Ac protein when administered to mice by oral gavage at a dose of 1290 mg/kg in male and female mice or at a dose of 1460 mg/kg in male mice.

8. INTRODUCTION

The purpose of this study was to evaluate the short-term toxicity of *E.* coli-produced Cry1Ac protein following a single day oral gavage administration to mice.

The protocol was signed by the Study Director on 05 February 2008 (GLP initiation date). The experimental start date was 14 February 2008 and the experimental completion date was 08 July 2008. The in-life phase of the original study phase was initiated on 22 February 2008 and the in-life completion date was 07 March 2008. Findings in male animals during the initial phase of the study resulted in the addition of a second dose level for male animals. The in-life phase of the additional study phase was initiated on 24 June 2008 and the in-life completion date was 08 July 2008.

9. MATERIALS AND METHODS

9.1. Test Materials

9.1.1. Test Article

The test article is defined as follows:

Identification	Cry1Ac Protein
ID Number	10000804

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) – Concentrated Cry1Ac Protein
ID Number	10000804-D
Assigned Testing Facility ID	S08.001.EUF
Receipt Date	21-Feb-2008
Expiration Date	May-2008
Physical Description	Clear, slightly cloudy liquid
Storage Conditions	Frozen in a -70°C freezer

A second dosing solution was prepared by the Sponsor (used to dose Group 4) from the test article and identified as follows:

Identification	Test Dosing Solution (TDS) – Concentrated Cry1Ac Protein
ID Number	10000805-D3F
Assigned Testing Facility ID	S08.011.EUF
Receipt Date	24-Jun-2008
Expiration Date	Sep-2008
Physical Description	Clear yellow liquid
Storage Conditions	Frozen in a -70°C freezer

9.1.2. Control Article

The control article is defined as follows:

Identification	Bovine Serum Albumin (BSA)
ID Number	D19305
Manufacturer	Calbiochem

A dosing solution was prepared by the Sponsor (used to dose Group 1) from the control article and identified as follows:

Identification	Control Dosing Solution (CDS) – Solubilized Bovine Serum Albumin (BSA)
ID Number	D19305-C
Assigned Testing Facility ID	S08.002.EUF
Receipt Date	21-Feb-2008
Expiration Date	May-2008
Physical Description	Clear, slightly yellow liquid
Storage Conditions	Frozen in a -70°C freezer

A second dosing solution was prepared by the Sponsor (used to dose group 3) from the control article and identified as follows:

Identification	Control Dosing Solution (CDS) – Solubilized Bovine Serum Albumin (BSA)
ID Number	D19305-C3F
Assigned Testing Facility ID	S08.012.EUF
Receipt Date	24-Jun-2008
Expiration Date	Sep-2008
Physical Description	Clear, slightly yellow 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 article.

9.1.5. Inventory and Disposition

An inventory of the test materials supplied 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 administered as received from the Sponsor. The dosing solutions were removed from the freezer, allowed to warm to room temperature, stirred at room temperature for at least 20 minutes prior to dosing, and stirred continuously 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

Time Point[a]	Concentration/Stability	Homogeneity
Day 0 (pre-dose)	Groups 1-4	Groups 2 and 4
Day 0 (post-dose)	Groups 1-4	N/A

Note: Group 2 and 4 samples were also analyzed for functional activity.
N/A = not applicable.
[a]Samples were collected for the original and additional study phases.

9.1.7.1. Concentration, Stability, and Functional Activity

Two 500-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 Group 2 and Group 4 samples were also analyzed for functional activity.

9.1.7.2. Homogeneity

Three 500-µL samples were collected, one each from the top, middle, and bottom of the Group 2 and Group 4 dosing formulations, on Day 0 (pre-dose) for homogeneity analysis.

9.1.7.3. Analytical Sample Storage and Shipment

All samples and unused dosing solutions were stored frozen (in a -70°C freezer) and shipped overnight 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 14 February 2008 from Charles River Laboratories, St. Constant, Quebec and on 19 June 2008 from Charles River Laboratories, Portage, Michigan. Twenty-three animals per sex were assigned to the original phase. Twenty-two male animals were assigned to the additional phase of the study. The animals were examined and weighed on the day following receipt, and all were allowed to acclimate to the laboratory environment for a minimum of five 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 preclinical toxicity testing by regulatory agencies. The oral route of exposure was selected since this is a 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 a minimum of once daily and ranged from 67 to 74°F (21 to 23°C) and 43 to 62%, 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, except during fasting 2 to 3 hours prior to dosing. 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 for the original study phase, 46 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 28.4 to 33.4 grams for the males and approximately 10 weeks of age with body weights ranging from 23.1 to 29.4 grams for the females.

Prior to randomization procedures for the additional study phase, 22 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 criterion of acceptance of the randomization. At the time of randomization, the males selected for study use were approximately 6 weeks of age with body weights ranging from 26.5 to 29.4 grams.

9.3. Experimental Design

The experimental design was as follows:

Experimental Design

Group No.	No. of Animals		Test Material [Control/Test Article]	Target Dose Level[a] (mg protein/kg BW)	Dose Volume (mL/kg BW/ dose)
	Male	Female			
1	10	10	Control Dosing Solution (CDS) [Bovine Serum Albumin (BSA)]	1280 (640/dose)	33.3
2	10	10	Test Dosing Solution (TDS) [Cry1Ac protein]	1290 (647/dose)	33.3
[a]Administered as two doses separated by approximately 4 hours.					

A BSA-treated male animal (Group 1) was found with a faulty water sipper during the first week of the study (Days 0-7). The animal suffered a substantial body weight loss (-36%) during this period. The animal was given a water bottle and the sipper was repaired at this time. The animal regained all of the lost weight during the second week of the study (Days 7-14). Additionally, a statistically significant decrease in body weight gain was noted in the Cry1Ac protein-treated male animals (Group 2) when compared to the BSA-treated control group. This finding was unexpected and given the water sipper issue in the BSA-treated animal's cage, two additional groups of male animals (designated Groups 3 and 4) were added to clarify this finding. The BSA and Cry1Ac protein doses were targeted slightly higher than those attained during the initial dosing. The target of the second dosing could only be increased slightly above the first set because the dosing solution concentrations were very near the maximum concentration of Cry1Ac protein that would remain suspended in the buffer.

Experimental Design (Set 2)

Group No.	No. of Animals		Test Material [Control/Test Article]	Target Dose Level[a] (mg protein/kg BW)	Dose Volume (mL/kg BW/ dose)
	Male	Female			
3	10	-	Control Dosing Solution (CDS) – [Bovine Serum Albumin (BSA)]	1620 (809/dose)	33.3
4	10	-	Test Dosing Solution (TDS) – [Cry1Ac protein]	1460 (730/dose)	33.3
[a]Administered as two doses separated by approximately 4 hours.					

9.4. Administration of Test Materials

On Day 0 (for the original and additional study phases), 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 articles were administered once to the appropriate mice as two 33.3 mL/kg doses separated by approximately 4 hours (± 20 minutes). 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. The day of dosing for each phase 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 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 the 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, 10 (Group 3 and 4 only), 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	10/10	14	x	x
2	10/10	14	x	x
3	10/-	14	x	x
4	10/-	14	x	x
Note: "x" = procedure conducted.				

10.5.1. Gross Necropsy

All animals were euthanized by carbon dioxide inhalation 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.

10.5.2. Tissue Collection and Preservation

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

10.6. Protocol Deviations

The following protocol deviation was noted during the course of the study. This deviation did not affect the integrity or validity of the study from a scientific or GLP perspective.

On Day 9 of the additional study phase (Groups 3 and 4), all animals were fed on a non-scheduled food consumption interval. An additional food consumption interval was added on Day 10 to allow data comparison, since no food consumption data was available for the Day 7 to Day 10 interval based on the feeding on Day 9.

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 1.0.0 or higher. The temperature and humidity were recorded on a Systems 600 Apogee Insight System, Version 3.0 or higher. The following computer study numbers were used to collect data for the various study phases: EUF221, original main phase data; EUF221B, additional phase data; MS0805, acclimation data for the original main phase; and MS0817, acclimation data for the additional 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. Groups 1 and 2 were compared to each other as a data set for all analyses; Groups 3 and 4 were compared to each other as a separate data set.

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[6] 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, wet tissues, slides, and blocks will be archived at the Testing Facility 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 (Analytical Chemistry Report)

The CDS (Group 1) was determined to be a stable solution. The TDS (Group 2) was determined to be a stable, biologically active, homogeneous suspension.

Dosing Solution Concentrations and Protein Dose Levels

Group	CDS (BSA) Concentration (mg/mL)	BSA Dose (mg/kg BW)		Group	TDS (Cry1Ac) Concentration (mg/mL)	Cry1Ac Dose (mg/kg BW)
1	19.2	1280		2	19.4	1290

For the second set of male animals, the CDS (Group 3) was determined to be a stable solution. The TDS (Group 4) was determined to be a stable, biologically active, homogeneous suspension.

Dosing Solution Concentrations and Protein Dose Levels (Set 2)

Group	CDS (BSA) Concentration (mg/mL)	BSA Dose (mg/kg BW)		Group	TDS (Cry1Ac) Concentration (mg/mL)	Cry1Ac Dose (mg/kg BW)
3	24.3	1620		4	21.9	1460

13.2. Survival

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

No mortality occurred during the study.

13.3. Clinical Observations

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

No test article-related clinical signs occurred during the study.

A single Group 1 male treated with BSA had few feces, rough coat, thin appearance, and dehydration. These findings were associated with a faulty water sipper. The animal was provided a new sipper and a water bottle and recovered.

A single Group 1 female treated with BSA was noted to have an ocular lesion. This finding was isolated in nature, is common in mice of this age and strain, and therefore, was not considered related to the control article. Unkempt appearance in a Group 2, Cry1Ac protein-treated male and rough coat in a Group 4, Cry1Ac protein-treated male were also observed; these findings were not considered meaningful based on the isolated nature of each observation.

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 were noted during the study.

A Group 1, BSA-treated male had a body weight decrease of 10 grams between Day 0 and Day 7; this was associated with the faulty water sipper. Following provision of a water bottle and replacement sipper the animal gained weight during the second week of the study. A Group 3, BSA-treated male had a decrease of approximately 3 grams between Day 7 and Day 14; no specific reason for the decrease was identified.

There were minor losses of body weight in a few BSA-treated females from Day 0 to Day 7; these changes were not considered meaningful. A single Cry1Ac protein-treated female had no net body weight gain between Day 0 and Day 7.

There was a statistically significant difference in body weight change during the Day 0 to Day 7 interval between Group 1 (1280 mg/kg BSA) and Group 2 males (1290 mg/kg Cry1Ac protein). Interpretation of this finding was complicated by the interruption in the water supply in a Group 1 male animal during this period. There was no statistically significant difference between Group 3 and Group 4 male animals treated with slightly higher concentrations of the proteins (1620 mg/kg BSA and 1460 mg/kg Cry1Ac protein) during this period, nor were there any body weight effects in females during the initial phase of the study. Therefore, the initial weight changes are not considered to be test article related.

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 test article-related gross necropsy findings occurred during the study.

One female treated with bovine serum albumin had a periovarian cyst, which is a common finding in this strain and age of mouse in this laboratory and, therefore, not related to treatment.

14. CONCLUSION

There were no treatment-related adverse effects of Cry1Ac protein when administered to mice by oral gavage at a dose of 1290 mg/kg body weight in male and female mice and at a dose of 1460 mg/kg body weight in male mice.

Jason W. Smedley 14JAN2009

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

15. REPORT APPROVAL SIGNATURE

Mark A. Morse 13-JAN-2009

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.

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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.

6. Glantz SA. *Primer of Biostatistics*. 4th ed. The McGraw Hill Companies, Inc.; 1997.

Table 1
Summary of Survival and Clinical Observations

TABLE 1

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

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

GROUP:	1	2
LEVEL MG/KG:	1280	1290
DOSE MATERIAL:	BSA	Cry1Ac
DAY 1 to 14		
NORMAL		
WITHIN NORMAL LIMITS	138/10	133/10
SCHEDULED EUTHANASIA	10/10	10/10
EXCRETA/EMESIS		
FEW FECES	2/ 1	0/ 0
BODY		
DEHYDRATION	1/ 1	0/ 0
THIN APPEARANCE	1/ 1	0/ 0
ROUGH COAT	1/ 1	0/ 0
UNKEMPT APPEARANCE	0/ 0	7/ 1

TABLE 1

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

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

GROUP:	1	2
LEVEL MG/KG:	1280	1290
DOSE MATERIAL:	BSA	Cry1Ac
DAY 1 to 14		
NORMAL		
WITHIN NORMAL LIMITS	133/10	140/10
SCHEDULED EUTHANASIA	10/10	10/10
EYE(S)		
OCULAR LESION(S)	7/ 1	0/ 0

TABLE 1

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

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

	GROUP:	3	4
	LEVEL MG/KG:	1620	1460
	DOSE MATERIAL:	BSA	Cry1Ac
DAY 1 to 14			
NORMAL			
WITHIN NORMAL LIMITS		140/10	137/10
SCHEDULED EUTHANASIA		10/10	10/10
BODY			
ROUGH COAT		0/ 0	3/ 1

Table 2
Summary of Body Weight Data

TABLE 2

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF BODY WEIGHT DATA (GRAMS)

	GROUP:		1	2
	LEVEL MG/KG:		1280	1290
	DOSE MATERIAL:		BSA	Cry1Ac
DAY 0 (NON-FASTED)		MEAN	31.5 d	31.4
		S.D.	1.58	1.59
		N	10	10
	% difference vs. control			-0.3
DAY 0 (FASTED)		MEAN	30.2 d	30.2
		S.D.	1.40	1.44
		N	10	10
	% difference vs. control			0.0
DAY 7		MEAN	32.4 k	32.3
		S.D.	5.39	1.69
		N	10	10
	% difference vs. control			-0.2
DAY 14		MEAN	35.6 d	34.7
		S.D.	2.26	1.76
		N	10	10
	% difference vs. control			-2.7

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST k=KRUSKAL-WALLIS/DUNN'S

TABLE 2

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

FEMALES SUMMARY OF BODY WEIGHT DATA (GRAMS)

	GROUP:		1	2
	LEVEL MG/KG:		1280	1290
	DOSE MATERIAL:		BSA	Cry1Ac
DAY 0 (NON-FASTED)		MEAN	26.8 k	26.8
		S.D.	1.85	1.88
		N	10	10
	% difference vs. control			0.0
DAY 0 (FASTED)		MEAN	25.6 d	25.6
		S.D.	1.68	1.85
		N	10	10
	% difference vs. control			0.2
DAY 7		MEAN	26.8 d	27.2
		S.D.	1.73	2.00
		N	10	10
	% difference vs. control			1.7
DAY 14		MEAN	29.4 d	29.4
		S.D.	1.55	1.34
		N	10	10
	% difference vs. control			0.2

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST k=KRUSKAL-WALLIS/DUNN'S

TABLE 2

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF BODY WEIGHT DATA (GRAMS)

	GROUP:		3	4
	LEVEL MG/KG:		1620	1460
	DOSE MATERIAL:		BSA	Cry1Ac
DAY 0 (NON-FASTED)		MEAN	27.7 d	27.8
		S.D.	0.95	0.96
		N	10	10
	% difference vs. control			0.1
DAY 0 (FASTED)		MEAN	26.5 d	26.4
		S.D.	1.20	0.79
		N	10	10
	% difference vs. control			-0.5
DAY 7		MEAN	30.6 d	30.0
		S.D.	1.24	0.97
		N	10	10
	% difference vs. control			-1.6
DAY 14		MEAN	32.2 d	33.0
		S.D.	2.86	1.20
		N	10	10
	% difference vs. control			2.5

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 3
Summary of Body Weight Changes

TABLE 3

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

	GROUP:		1	2
	LEVEL MG/KG:		1280	1290
	DOSE MATERIAL:		BSA	Cry1Ac
DAY	0 (FASTED) TO 7	MEAN	2.2 k	2.1*
		S.D.	4.29	1.22
		N	10	10
DAY	7 TO 14	MEAN	3.3 k	2.4
		S.D.	3.31	0.91
		N	10	10

STATISTICAL KEY: k=KRUSKAL-WALLIS/DUNN'S * = P<0.05

TABLE 3

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

FEMALES SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

	GROUP:		1	2
	LEVEL MG/KG:		1280	1290
	DOSE MATERIAL:		BSA	Cry1Ac
DAY	0 (FASTED) TO 7	MEAN	1.2 d	1.6
		S.D.	1.13	1.20
		N	10	10
DAY	7 TO 14	MEAN	2.6 d	2.2
		S.D.	1.25	1.48
		N	10	10

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

TABLE 3

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

MALES SUMMARY OF BODY WEIGHT CHANGES (GRAMS)

	GROUP:		3	4
	LEVEL MG/KG:		1620	1460
	DOSE MATERIAL:		BSA	Cry1Ac
DAY	0 (FASTED) TO 7	MEAN	4.1 d	3.7
		S.D.	1.28	1.11
		N	10	10
DAY	7 TO 14	MEAN	1.6 d	2.9
		S.D.	1.97	1.04
		N	10	10

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 4
Summary of Food Consumption Data

TABLE 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

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

	GROUP:		1	2
	LEVEL MG/KG:		1280	1290
	DOSE MATERIAL:		BSA	Cry1Ac
DAY	0 to 7	MEAN	6.8 d	6.0
		S.D.	1.22	0.85
		N	10	10
	% difference vs. control			-12.2
DAY	7 to 14	MEAN	7.3 d	6.7
		S.D.	0.96	1.01
		N	10	10
	% difference vs. control			-8.3

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

TABLE 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

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

	GROUP:		1	2
	LEVEL MG/KG:		1280	1290
	DOSE MATERIAL:		BSA	Cry1Ac
DAY	0 to 7	MEAN	5.3 d	5.3
		S.D.	0.62	0.66
		N	10	10
	% difference vs. control			-0.1
DAY	7 to 14	MEAN	5.9 d	5.8
		S.D.	0.55	0.44
		N	10	10
	% difference vs. control			-1.4

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

TABLE 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

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

	GROUP:		3	4
	LEVEL MG/KG:		1620	1460
	DOSE MATERIAL:		BSA	Cry1Ac
DAY 0 to 7		MEAN	7.1 d	7.1
		S.D.	0.68	0.55
		N	10	10
	% difference vs. control			-0.4
DAY 10 to 14		MEAN	6.0 d	6.5
		S.D.	0.81	0.43
		N	10	10
	% difference vs. control			7.9

STATISTICAL KEY: d=ANOVA/DUNNETT-TEST

Table 5
Summary of Gross Necropsy Observations

TABLE 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS
SCHEDULED EUTHANASIA - DAY 14

	GROUP:	1	2
	LEVEL MG/KG:	1280	1290
	DOSE MATERIAL:	BSA	Cry1Ac
MALES:	TOTAL NUMBER EXAMINED	10	10
	WITHIN NORMAL LIMITS	10	10

TABLE 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS
SCHEDULED EUTHANASIA - DAY 14

GROUP:		1	2
LEVEL MG/KG:		1280	1290
DOSE MATERIAL:		BSA	Cry1Ac
FEMALES: TOTAL NUMBER EXAMINED		10	10
WITHIN NORMAL LIMITS		8	10
GENERAL COMMENT	N	1	0
FINAL CLINICAL OBSERVATION NOT APPARENT POSTMORTEM	N	1	0
OVARY	N	1	0
PERIOVARIAN CYST(S)	N	1	0

TABLE 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

SUMMARY OF GROSS NECROPSY OBSERVATIONS
SCHEDULED EUTHANASIA - DAY 14

	GROUP:	3	4
	LEVEL MG/KG:	1620	1460
	DOSE MATERIAL:	BSA	Cry1Ac
MALES:	TOTAL NUMBER EXAMINED	10	10
	WITHIN NORMAL LIMITS	10	10

Appendix 1
Certificates of Analysis

Analytical Protein Standard Certificate of Analysis

MONSANTO ANALYTICAL PROTEIN STANDARDS

Re-characterization No. 1

Sample Information:

Name of APS	Orion Lot Number	Expiration Date
E. coli-produced Cry1Ac [MON 87701]	10000804	May 31, 2008
Common or Alias Name(s)	**Historical APS Lot Number**	**Storage Requirements (until use)**
—	20-100133	–80 °C
Source Fermentation of *Escherichia coli* paste (APS lots 10-100116 through 10-100123, 10-100131 and 10-100132) containing the expression plasmid pMON107800.		**Comment(s)** None
Additional Background Information Historic Lot No. G-818529_26L		

Re-characterization Information		
Characteristic	**Method**	**Assay Date**
Total Protein Concentration	Amino Acid Analysis	4 October 2007
Purity/Molecular weight	SDS-PAGE/Densitometry	9 October 2007
Activity	Insect Bioassay	11 October 2007

Characteristic	Method	Assay Date	Result
Concentration	Amino Acid Composition	15 May 2007	1.4 mg/mL (total protein)
Purity	SDS-PAGE/Densitometry	17 May 2007	*80%
Molecular weight	SDS-PAGE/Densitometry	17 May 2007	131.7 KDa
Identity	Immunoblot	27 July 2007	Confirmed – immuno reactive band observed
Identity	N-terminal sequence	26 May 2007	Confirmed – XMQAMDNN(P)(N)(I)
Identity	MALDI-TOF MS (Trypsinized)	24 May 2007	Confirmed sequence 43.4 % coverage of expected sequence
Identity	MALDI-TOF MS (Native)	24 May 2007	Results inconclusive
Activity	Insect Bioassay	8 June 2007	EC_{50} = 2.1 ng of Cry1Ac [MON 87701] protein/mL diet

Buffer composition: 50 mM CAPS, pH 10.25, 1 mM EDTA, 2.5 mM DTT, and 1 mM benzamidine-HCl
Physical description: Clear solution
Short-term storage stability (41 days) was evaluated during the certification process. Based upon the criteria provided in Characterization Plan 20-100133, no significant degradation was observed for samples stored at 4°C, -20°C and –80°C.

* Purity is determined by summing all bands detected between the full-length protein (~130 KDa) and the tryptic core (~55 KDa) as well as a band immediately above the full-length POI.

Purity corrected concentration is 1.1 mg/mL (1.4 mg/mL × 0.80 ≈ 1.1 mg/mL)

Quality Assurance Specialist

Exact Copy of Original as of 11/30/07 Date
Certified By _____ Initials or Signature

11/28/07
Date

Analytical Protein Standards Officer

11/28/07
Date

Calbiochem®

Certificate of Analysis

Albumin, Bovine Serum, Fraction V, Fatty Acid-Free, Nuclease- and Protease-Free	
Batch Number:	D00019305
Material Number:	126609-5GM
Molecular Weight:	66,000
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 %	>98.0 %
Endonuclease:		None detected
Proteases:		None detected
Loss on drying:	≤5.0 %	2.8 %
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

29-Oct-2007
Date

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Appendix 2
Analytical Chemistry Report

Analytical Sub-Report Title

Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Cry1Ac Protein

Authors

Kathleen S. Crowley, Ph.D., Erin Bell, Ph.D., Joshua Uffman, and Elena Rice, Ph.D.

Analytical Sub-Report Completed On

January 6, 2009

Performing Laboratory

Monsanto Company
800 North Lindbergh Boulevard
Saint Louis, Missouri 63167

Laboratory Project ID

MSL #: 0021276
Charles River Study #: EUF00221
Monsanto Study #: CRO-2007-325

Statement of Compliance

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

Sponsor/Submitter: Terry A. Kaempfe Date: 1/6/09

Principal Investigator: Kathleen S. Crowley, Ph.D. Date: 1/6/09

Quality Assurance Statement

Analytical Sub-Report Title: Formulation, and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Cry1Ac protein

Charles River Study No. EUF00221
Monsanto Study No. CRO-2007-325

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 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
March 10 & 13, 2008	Amino Acid Analysis	September 30, 2008	September. 30, 2008
May 23; May 27-28, 2008; September 15-16, 2008; September 22, 2008	Raw Data Audit	May, 2008; September 23, 2008	September. 23, 2008
October 16-17, 2008; October 20, 2008	Draft Report Review	October 21, 2008	October 21, 2008

Quality Assurance Unit
Monsanto Regulatory, Monsanto Company

01/06/09
Date

Analytical Sub-Report Certification

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

Principal Investigator: 

Date: 1/16/07

Study Information

Charles River/Monsanto Study Number:	EUF00221/CRO-2007-325
MSL Number	0021276
Analytical Sub-Report Title:	Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study in Mice with Cry1Ac Protein
Facilities:	Monsanto Company 800 North Lindbergh Boulevard Saint Louis, Missouri 63167, USA
Study Director:	Jason W. Smedley, B.S.
Principal Investigator:	Kathleen S. Crowley, Ph.D.
Contributors:	Chris Dalton, Joshua Uffman, Steve Levine Ph.D.
Study Specific Procedure Initiation Date:	February 06, 2008
Analytical Sub-Report Completion Date:	January 6, 2009
Records Retention:	All Study Specific Procedure raw data, Study Specific Procedure plan and amendments, final sub-report and facility records were retained at Monsanto, St. Louis.
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]

AAA	Amino acid analysis
APS	Analytical Protein Standard
BSA	Bovine serum albumin
BW	Body weight
CDS	Control Dosing Solution
CEW	Corn earworm
CFR	Code of Federal Regulations
COA	Certificate of analysis
CV	Coefficient of variance
EC_{50}	The level of the test article that produces a 50% growth inhibition
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FIFRA	Federal Insecticide, Fungicide, and Rodenticide Act
ID #	Identification number
MALDI-TOF	Matrix assisted laser desorption ionization – time of flight
MW	Molecular weight
MWCO	Molecular weight cutoff
NIST	National Institute of Standards and Technology
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	Standard operating procedure
SSP	Study Specific Procedure
TML	Trimethyllysine
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 *Escherichia coli (E. coli)*-produced full-length Cry1Ac protein, 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). Four groups of mice were used in the study which required that two test dosing solutions (for Group 2 and Group 4 mice) and two control dosing solutions (for Group 1 and Group 3 mice) be prepared and analyzed.

For each group of mice dosed, the TDS and CDS were administered in a split dose within a single day. Samples were collected for both sets of the TDS and CDS immediately prior to the administration of the doses (pre-dose) and immediately following administration of the doses (post-dose) to determine the concentration and stability of the dosing solutions over the dosing period. Stability was assessed by determining the purity of the Cry1Ac 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 each 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 to inhibit corn earworm (CEW) growth, of the Cry1Ac protein in each TDS was evaluated for the pre- and post-dose TDS aliquots using a diet-incorporation insect bioassay.

Analysis of the TDS (Group 2) showed that the purity of the Cry1Ac protein was 82% and 78% in the pre- and post-dose TDS samples, respectively. Since the difference in purity of the Cry1Ac protein between the pre- and post-dose samples was less than 10%, this TDS was considered stable over the duration of the dosing period. Analysis of the CDS (Group 1) showed that the purity of the BSA protein was 77% and 75% in pre- and post-dose CDS samples, respectively. Since the difference in the purity of the BSA protein between the pre- and post-dose samples was less than 10%, this CDS was considered stable over the duration of the dosing period. The concentrations of the Cry1Ac protein in pre- and post-dose TDS (Group 2) samples were determined to be 23.8 mg/ml and 24.7 mg/ml, respectively, and the TDS (Group 2) was determined to be homogeneous. The concentrations of the BSA protein in the pre- and post-dose CDS (Group 1) samples were determined to be 26.0 mg/ml and 24.6 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 dose level of the Cry1Ac protein in the TDS (Group 2) was 1290 mg/kg body weight (BW), and the dose level of the BSA protein in the CDS (Group 1) was 1280 mg/kg BW. The Cry1Ac protein was biologically active in pre- and post-dose TDS (Group 2) samples with an EC_{50} of 0.0056 µg/ml diet for both.

Analysis of the TDS (Group 4) showed that the purity of the Cry1Ac protein was 80% in both the pre- and post-dose samples. Since the difference in the purity of the Cry1Ac protein between the pre- and post-dose samples was less than 10%, this TDS was considered stable over the duration of the dosing period. Analysis of the CDS (Group 3) showed that the purity of the BSA protein was 87% and 88% in the pre- and post-dose CDS samples, respectively. Since the difference in the purity of the BSA protein between the pre- and post-dose samples was less than 10%, this CDS was considered stable over the duration of the dosing period. The concentration of the Cry1Ac protein in pre- and post-dose TDS (Group 4) samples was determined to be 28.3 mg/ml and 26.4 mg/ml, respectively, and the TDS (Group 4) was determined to be homogeneous. The concentration of the BSA protein in the pre- and post-dose CDS (Group 3) samples was determined to be 27.0 mg/ml and 28.2 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 dose level of the Cry1Ac protein in the TDS (Group 4) was 1460 mg/kg body weight (BW), and the dose level of the BSA protein in the CDS (Group 3) was 1620 mg/kg BW. The Cry1Ac protein was biologically active in pre- and post-dose TDS (Group 4) samples with an EC_{50} of 0.0052 µg/ml of diet for the pre-TDS and 0.0089 µg/ml of diet for the post-TDS.

These data established the dose levels and stability of the test and control dosing solutions used in the mouse oral acute toxicity study.

2.0 Introduction

An acute oral toxicity study was performed on mice as part of the safety assessment of the full-length Cry1Ac protein. This sub-report describes the formulation and analyses of test and control dose solutions used in the mouse acute oral toxicity study. Originally, two groups of mice, each containing 10 females and 10 males and designated Group 1 and Group 2, were dosed. The group of mice receiving the CDS was designated as Group 1, and the group of mice receiving the TDS was designated as Group 2. During evaluation of these animals, a statistically significant decrease in body weight gain was noted in Group 2 males compared to Group 1 males during the first week of the study however, interpretation of this finding was complicated by an interruption in water delivery to at least one Group 1 male animal.

Additional groups of male animals were treated (Groups 3 and 4) to clarify the finding. Ten male mice, identified as Group 3, were administered a newly prepared CDS and 10 male mice, identified as Group 4, were administered a newly prepared TDS.

To clarify the dosing solution nomenclature used in this study, the CDS administered to Group 1 mice was identified as CDS (Group 1) and the CDS administered to Group 3 mice was identified as CDS (Group 3). The TDS administered to Group 2 mice was identified as TDS (Group 2) and the TDS administered to Group 4 mice was identified as TDS (Group 4).

Analyses of each CDS and each TDS included the determination of total protein concentration and purity. Functional activity of Cry1Ac protein in each TDS, both before and after dosing of mice, was also assessed. These procedures were performed to determine the administered dose concentrations and to assess if any changes in the test or control article occurred over the course of the dosing period 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 test and control dose solutions used in the mouse acute oral toxicity study for *E. coli*-produced Cry1Ac protein, performed at Charles River Laboratories Inc. (Charles River study number EUF00221; Spencerville, OH).

4.0 Materials

4.1 Test Article

The test article was *E. coli*-produced full-length Cry1Ac. This Cry1Ac protein (Orion lot 10000804-10000805; historical Analytical Protein Standard lot 20-100133) was isolated from a fermentation batch of *E. coli* containing an expression plasmid (pMON107800) that encodes the full-length Cry1Ac protein. This *E. coli*-produced full-length Cry1Ac protein has been shown to be equivalent to the Cry1Ac protein present in MON87701 (MSL #: 0021146). The identity, concentration, purity, stability, and functional activity of the Cry1Ac protein were previously determined under characterization plan 20-1000133 and re-characterization plan 10000804 No. 1 and 2 and are described in the Certificate of Analysis (COA) for this protein. Copies of the COAs are archived with this study file. It should be noted that due to limitations in the number of aliquots that can be described in Orion, the Cry1Ac protein has been assigned both lot 10000804 and 10000805, although these lots are equivalent and information regarding lot 10000804 applies to lot 10000805 as well. Lot 10000804-10000805 has a total protein concentration of 1.4 mg/ml and a purity of 80%. Activity was confirmed using a diet-incorporation insect bioassay where the EC_{50} for CEW was determined to be 0.0021 µg of Cry1Ac protein/ml of diet. These characteristics did not change over the course of this study as documented in the COAs from re-characterization plan 10000804 No. 1 and 2. Prior to use, the test article was stored in a –80 ºC freezer in a buffer containing 50 mM CAPS, pH 10.25, 1mM EDTA, 2.5 mM DTT, 1 mM benzamidine-HCl.

4.2 Control Article

The control article was bovine serum albumin (BSA) protein (lot D00019305) purchased from Calbiochem (material # 126609). The vendor's COA is archived with this study file. According to the vendor's characterization, the protein has a purity of 98%. The relative amount of the protein content in the solid BSA powder was determined by amino acid analysis to be 85%.

4.3 Assay Controls

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

5.0 Methods for Dose Preparation

The original target dose level of the BSA and Cry1Ac protein in CDS (Group 1) and TDS (Group 2) was set to be at least 1000 mg protein/kg body weight (BW). A new target dose level was set for the CDS and TDS to be administered to Groups 3 and 4 mice to ensure that the dose level was at least as high as that experimentally determined for Cry1Ac protein in the TDS (Group 2) administered to the Group 2 mice. Since the dose level for the TDS (Group 2) was measured to be ~1300 mg/kg BW (see Section 10.7), this was the minimum target dose level set for the new CDS and TDS, i.e., CDS (Group 3) and TDS (Group 4). The calculations of the test and control article dosing solutions are described below.

5.1 Calculation of the Control and Test Article Dosing Solution Concentrations for Group 1 and 2 Mice.

The theoretical protein dose solution concentrations for the formulation of the CDS (Group1) and TDS (Group 2) used in this study were calculated based on the assumptions described below.

a) The target dose level is at least 1000 mg protein/kg body weight (BW)

b) The average mouse BW is 0.030 kg.

c) Dosing solutions are administered as two 33 ml/kg BW doses.

d) The minimum total volume of sample required is 40 ml.

Therefore, the test and control article concentration in the CDS (Group 1) and TDS (Group 2) should be at least:

$$\frac{(1000\ mg/kg\,BW)\times 0.030kg}{(33.33\ ml/kg\,BW\times 2\ \ doses)\times 0.030kg}\cong 15mg/ml$$

Because the estimated purity of the Cry1Ac protein is 80%, the TDS (Group 2) should contain at least 18.8 mg/ml of total protein:

$$\frac{15\ mg/ml}{0.80}\cong 18.8\ mg/ml$$

The estimated purity of the BSA protein is 98% and protein content is 85%, therefore, the CDS (Group 1) should contain at least 18 mg/ml of BSA powder:

$$\frac{15\ mg/ml}{0.85 \times 0.98} \cong 18.0\ mg/ml$$

The experimentally obtained dose levels were determined after administration to mice by assessing the concentration and purity for the BSA and Cry1Ac proteins in CDS and TDS, respectively.

5.2 Formulation of the Test Article in the Test Dosing Solution (TDS) for Group 2 mice.

Because the target Cry1Ac protein concentration for the TDS was significantly higher than the Cry1Ac protein concentration of the test article, it was necessary to concentrate the test article in order to formulate the TDS. To concentrate the test article, approximately 1420 ml of Cry1Ac protein preparation (lot 10000804-10000805) was first thawed and split between four large dialysis bags (Spectra Por, Gardena, CA, Cat. No. 132682, 12-14 kDa MWCO). Each bag was dialyzed four times against 4 L of 1 mM carbonate-bicarbonate buffer, pH 10-11, 0.125 mM reduced glutathione at ~4 ºC. The total dialysis time was approximately 2.5 days. Following dialysis, the contents of all four dialysis bags were pooled. The final recovered volume was 1610 ml. The dialyzed protein solution was quick-frozen in a dry ice/ethanol bath along the sides of lyophilization flasks. The flasks were connected to a VirTis 12ES freeze dryer and lyophilized for approximately five days. A total of 4.72 g of lyophilized powder, estimated to contain approximately 1600 mg of Cry1Ac protein, was recovered. All 4.72 g of the lyophilized powder was placed in a wide mouth container and was dissolved in 1 mM carbonate-bicarbonate buffer, pH 10-11 (lot # G826361C), added to final volume of 60 ml. The solution was mixed with a stir bar for ~1 hr at room temperature to completely solubilize the protein.

Three 100 µL aliquots were removed to evaluate the solution for suitability for the acute oral toxicity study. Suitability of the solution was assessed based on the following criteria: 1) the solution passes through an 18 or 20-gauge needle, 2) the total protein concentration is $\geq$18.8 mg/ml, and 3) the protein staining pattern of dosing solution observed on SDS-PAGE is similar to that observed for the test article.

The formulated dosing solution was assigned ID #10000804-D (Testing Facility Identification number S08.001.EUF) and is identified as TDS (Group 2). The TDS was stored in a -80 °C freezer in the wide mouth container with a stir bar until shipped on dry ice to Charles River Laboratories, Inc. (Spencerville, OH, ATTN: Jason W. Smedley).

5.3 Formulation of the Protein Control in the Control Dosing Solution for Group 1 mice.

To prepare the CDS for dosing Group 1 mice, the concentrations of carbonate-bicarbonate and reduced glutathione in TDS (Group 2) were first calculated. This was done by calculating the concentration factor for Cry1Ac protein in going from the dialyzed solution to the TDS during the preparation of TDS (Group 2). The concentration factor was then applied to the carbonate-bicarbonate and reduced glutathione concentrations in the dialyzed solution to calculate the concentrations of each in the TDS. These calculated concentrations were then used for preparing a vehicle buffer containing the calculated concentrations of 20 mM carbonate-bicarbonate, pH 10-11, and 2.4 mM reduced glutathione (lot G-826364A).

The vehicle buffer was added to 1703 mg BSA powder (Calbiochem catalogue #126609, lot D00019305) to obtain a final volume of 62.5 ml. The solution was mixed in a wide mouth container with a stir bar for ~1 hr at room temperature to completely solubilize the protein. The formulated CDS was assigned ID# 19305-C (Testing Facility Identification number S08.002.EUF) and identified as CDS (Group 1) in the study. This CDS was frozen, and stored at -80°C until shipped on dry ice to Charles River Laboratories, Inc. (Spencerville, OH, ATTN: Jason W. Smedley).

5.4 Calculation of the Control and Test Article Dosing Solution Concentrations for Group 3 and 4 Mice.

The theoretical protein dose solution concentrations for the formulation of the CDS (Group 3) and TDS (Group 4) were calculated based on the same assumptions as those for formulation of the CDS (Group 1) and TDS (Group 2) except that the target dose level was at least 1300 mg protein/kg BW. Therefore, the test and control article concentration in the CDS (Group 3) and TDS (Group 4) should be at least:

$$\frac{(1300\ mg/kg\,BW)\times 0.030kg}{(33.33\ ml/kg\,BW\times 2\ \ dose)\times 0.030kg}\cong 19.5\,mg/ml$$

Because the estimated purity of the Cry1Ac protein is 80%, the TDS (Group 4) should contain at least 24.4 mg/ml of total protein:

$$\frac{19.5\ mg/ml}{0.80}\cong 24.4\ mg/ml$$

The estimated purity of the BSA protein is 98% and protein content is 85%, therefore, the CDS (Group 3) should contain at least 23.4 mg/ml of BSA powder:

$$\frac{19.5\ mg/ml}{0.85\times 0.98}\cong 23.4\ mg/ml$$

The experimentally obtained dose levels were determined after administration to mice by assessing the concentration and purity for the BSA and Cry1Ac proteins in CDS and TDS, respectively.

5.5 Formulation of the Test Article in the Test Dosing Solution (TDS) for Group 4 mice.

Because the storage stability of the dosing solutions containing high concentration of Cry1Ac protein was not previously evaluated at 4 °C and -80 °C and to avoid loss of the entire dosing solution, two test dosing solutions containing similar concentrations of Cry1Ac protein were prepared. To prepare the two dosing solutions, approximately 1340 ml of Cry1Ac protein (lot 10000805) was thawed and split between three large dialysis bags (Spectra Por, Gardena, CA, Cat. No.132682, 12-14 kDa MWCO). Each bag was dialyzed four times against 4 L of 1 mM carbonate-bicarbonate buffer, pH 10-11 with 0.125 mM reduced glutathione at approximately 4 °C. The total dialysis time was approximately three days. The contents of each bag were pooled. The final recovered volume was 1460 ml. The dialyzed solution was quick-frozen in a dry ice/ethanol bath along the sides of lyophilization flasks and flasks were connected to a VirTis 12ES freeze dryer and lyophilized for approximately four days. A total of 4.46 g of lyophilized powder was recovered. Approximately half of the powder was sealed in a plastic container and temporarily stored at ~4°C. The remainder of the lyophilized powder was used to prepare the first test dosing solution.

To prepare the first test dosing solution, approximately 2.072 g of the powder was dissolved in 1 mM carbonate-bicarbonate buffer, pH 10-11 (lot G836509B), added to a final volume of 27.4 ml. The solution was mixed with a stir bar for ~1 hr at room temperature to completely solubilize the protein and was assigned lot #10000805-D3. An aliquot (100 µl) was removed for amino acid analysis and the remainder of the solution was stored at approximately 4 °C. Once the protein concentration was determined and confirmed to be $\geq$24.4 mg/ml, the stored solution was assigned TDS ID #10000805-D3L. A second 100 µL aliquot was removed for SDS-PAGE to further evaluate the solution for suitability for the acute oral toxicity study. Suitability of the solution was assessed based the following criteria: 1) the solution passes through an 18 or 20-gauge needle, 2) the total protein concentration is $\geq$24.4 mg/ml, and 3) the protein staining pattern of dosing solution observed on SDS-PAGE is similar to that observed for the test article.

The TDS ID #1000085-D3L was stored in at approximately 4 °C in a wide mouth container with a stir bar until shipped on wet ice to Charles River Laboratories, Inc. (Spencerville, OH, ATTN: Jason W. Smedley).

To prepare the second dosing solution, the content of the Cry1Ac protein in the lyophilized powder was calculated based on the concentration values obtained from the first preparation. This was done by calculating the total amount (mg) of Cry1Ac protein in the first dosing solution (ID #10000805-D3L), and dividing that by the

amount (mg) of the powder used to prepare that solution. The Cry1Ac protein content in the lyophilized powder was shown to be 30% (w/w). Based on this determination, a second solution was prepared by solubilizing the remaining lyophilized powder (2.089 g) in 29.2 ml of a 1 mM carbonate-bicarbonate buffer, pH 10-11 (lot # G836509B) to a Cry1Ac protein concentration of 21.5 mg/ml. The solution was mixed with a stir bar for ~1 hr at room temperature to completely solubilize the protein. The solution was assigned TDS ID #10000805-D3F (Testing Facility Identification number S08.011.EUF). Two 100 µl aliquots were removed to evaluate the solution for suitability for the acute oral toxicity study. Suitability of the dosing solution was assessed based the same criteria used to assess TDS ID#10000805-D3L (see above).

TDS ID#10000805-D3F was stored in a -80 °C freezer in a wide mouth container with a stir bar until shipped on dry ice to Charles River Laboratories, Inc. (Spencerville, OH, ATTN: Jason W. Smedley).

5.6 Formulation of the Protein Control in the Control Dosing Solution for Group 3 mice.

Two CDSs (liquid and frozen) were prepared for dosing Group 3 mice; one was paired with TDS ID#10000805-D3L and one was paired with TDS ID#10000805-D3F. As before, the concentration factor for Cry1Ac protein in going from the dialyzed solution to the TDS was calculated (see Section 5.3) and then used to calculate the concentrations of carbonate-bicarbonate and reduced glutathione in TDS ID#10000805-D3L and TDS ID#10000805-D3F. To prepare the liquid CDS, a vehicle buffer containing the same concentrations of carbonate-bicarbonate and reduced glutathione as #10000805-D3L at 23.1 mM and 2.8 mM, respectively, was prepared (lot G-836510C). The vehicle buffer was added to 832 mg BSA powder (Calbiochem catalogue #126609, lot #D00019305) to a final volume of 24.5 ml. The solution was mixed in a wide mouth container with a stir bar for ~1 hr at room temperature to completely solubilize the protein. The formulated CDS was assigned ID# D19305-C3L. This liquid CDS (ID# D19305-C3L) was stored at approximately 4 °C until shipped on wet ice to Charles River Laboratories, Inc. (Spencerville, OH, ATTN: Jason W. Smedley).

To prepare the frozen CDS, a vehicle buffer containing the same concentrations of carbonate-bicarbonate and reduced glutathione as TDS #10000805-D3F at 21.9 mM and 2.6 mM, respectively, was prepared (lot G-836510A). The vehicle buffer was added to 771 mg BSA powder (Calbiochem catalogue #126609, lot D00019305) to a final volume of 24 ml. The solution was mixed in a wide mouth container with a stir bar for ~1 hr at room temperature to completely solubilize the protein. The formulated CDS was assigned ID# D19305-C3F (Testing Facility Identification number S08.012.EUF). This CDS (ID# D19305-C3F) was frozen and then stored at approximately -80°C until shipped on dry ice to Charles River Laboratories, Inc. (Spencerville, OH, ATTN: Jason W. Smedley).

6.0 Analytical Methods

6.1 Amino Acid Analysis

Total protein concentration was estimated by amino acid analysis performed following the current versions of SOPs BR-ME-0990 and BR-EQ-0376. Test samples, NIST BSA (used as a system suitability standard) and a NIST amino acid calibration control standard (Gaithersburg, MD), were spiked with an internal reference (Trimethyllysine, Sigma Chemical Co.) and dried in a Savant SpeedVac centrifuge (Holbrook, NY). Vapor phase acid hydrolysis (6 N HCl containing 1% (v/v) phenol) was performed at 150 °C for 90-120 min. Cooled samples were again evaporated, reconstituted in protein hydrolysate buffer (PH-1, Hitachi Instruments) and loaded onto the Hitachi L-8800 Amino acid Analysis System. Amino acids were detected using ninhydrin and the amino acid concentrations were summed to calculate total protein concentration.

6.2 SDS-PAGE and Purity Analysis

Aliquots of the TDSs and CDSs were subjected to electrophoresis on pre-cast Tris-glycine 4 - 20% polyacrylamide gradient gels (Invitrogen, Carlsbad, CA) as described in SOP BR-ME-0388. For TDS suitability evaluation, as well as TDS and CDS purity evaluation, the protein samples were analyzed at ~ 1, 2, and 3 µg total protein per lane. SDS-PAGE Molecular Weight Standands, Broad Range (Biorad, Hercules, CA) were used to estimate molecular weights. All samples were mixed with 5× Loading Buffer (lot G826318C or G824592A), heated at ~95 °C for 5 min and then applied to a 10-well gel. Electrophoresis was performed at constant voltage (125 V for 1.5 hr followed by 125 V for 10 to15 min) until the dye front reached the bottom of the gel. Proteins were fixed with 40% (v/v) methanol, 7% (v/v) acetic acid, stained by gentle shaking with Brilliant Blue G colloidal stain (Sigma Chemical Co., St. Louis, MO), 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 18 ± 2 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). To measure Cry1Ac purity, the optical density of all protein bands detected from the full length Cry1Ac (~130) down to the tryptic core (~55 kDa) were summed in each lane (accordance with (Characterization Plan 20-1000133); for BSA, the optical density of the main band at ~66 kDa was measured in each lane. Purity was estimated as the percent optical density of the band(s) of interest relative to all bands detected in the lane. Purity was reported as an average of values obtained from all lanes containing the protein under evaluation. The proteins in the dosing solutions were considered stable for the duration of the dosing period if a ≤ 10% change in protein purity between the pre- and post-dose samples was observed.

6.3 Functional Activity Analysis

Pre- and post-dose aliquots of the TDSs were analyzed for the functional activity of the Cry1Ac protein. As described in Appendix 1, protein activity was assessed using an insect bioassay which measures growth inhibition of a target insect, corn earworm (CEW), reported as an EC_{50} value (the effective dose to inhibit the growth of the target insect by 50%). The TDS was considered to be stable for the duration of the dosing period if functional activity was demonstrated in both the pre- and post-dose samples.

7.0 Control of Bias and Quality Measures

Appropriate concentrations of dosing solution samples were analyzed concurrently by SDS-PAGE to establish purity. Multiple dilutions were utilized for the functional assays to ensure that data were collected within operational ranges of the assay.

8.0 Amendments and Deviations to the Study Specific Procedure (SSP)

Three amendments were made to the SSP and two deviations from the SSP were recorded. None of the deviations had an impact on the study. The amendments and deviations are detailed in Appendix 2.

9.0 Data Rejected

During purity analysis of the TDS (Group 4), two “All lane reports” were rejected because the Relative Qty was reported as the “percent of lane” rather than the preferred “percent of bands”. The associated raw data was not changed.

10.0 Results and Discussion

Control and test dosing solutions containing BSA and Cry1Ac, respectively, were prepared for the mouse acute oral toxicity study. In total, four groups of mice were dosed, two with CDS and two with TDS. Group 1 and Group 2 mice were administered CDS (Group 1) (ID#19305-C) and TDS (Group 2) (ID#10000804-D), respectively, each prepared using the calculations and assumption described in Section 5.1. The CDS and TDS administered to Group 3 and Group 4 mice were prepared using the calculations and assumption described in Section 5.4. The TDS ID#10000805-D3F was fully soluble upon thawing at the testing facility, designated TDS (Group 4) and administered to Group 4 mice. The corresponding CDS ID#19305-D3F was designated CDS (Group 3) and administered to Group 3 mice.

10.1 Control and Test Dosing Solution Suitability

Both TDS (Group 2) and TDS (Group 4) met the suitability criteria for dosing solutions. Both solutions passed through a 20-gauge needle and were shown to have a protein banding pattern similar to that of the test article on stained SDS-PAGE (data not shown). In addition, TDS (Group 2) was shown to have a total protein concentration of 23.1 mg/ml which was greater than the minimum acceptable concentration of 18.8 mg/ml, while TDS (Group 4) was formulated to have a total

protein concentration of 26.8 mg/ml which was greater than the minimum acceptable concentration of 24.4 mg/ml.

10.2 Vehicle Formulation

The millimolar concentrations of the carbonate-bicarbonate salts and reduced glutathione in the vehicle were calculated following formulation of the test article. The test article was exhaustively dialyzed in 1 mM carbonate-bicarbonate buffer, pH 10-11, and 0.125 mM reduced glutathione. During preparation of TDS (Group 2) the volume of the sample increased from ~1422 ml to ~1610 ml. The initial total protein concentration of the test article (lot 10000804-10000805) was 1.4 mg/ml. Following dialysis, the total protein concentration of the solution was:

$$\frac{1422\ ml}{1610\ ml} \times 1.4\ mg/ml \cong 1.2\ mg/ml$$

During preparation of TDS (Group 4) the volume of the sample increased from ~1340 ml to ~1460 ml. Following dialysis, the total protein concentration of the solution was:

$$\frac{1340\ ml}{1460\ ml} \times 1.4\ mg/ml \cong 1.3\ mg/ml$$

The dialyzed material was subsequently lyophilized (no loss of buffer salts occurs) and re-suspended in 1 mM carbonate-bicarbonate buffer, pH 10-11. The total protein concentration for the TDS (Group 2) and TDS (Group 4) was 23.1 mg/ml and 26.8 mg/ml, respectively. The concentration factor for the buffer salts could therefore be estimated by the protein concentration factor:

$$\frac{23.1\ mg/ml}{1.2\ mg/ml} \cong 19\ fold \text{ for TDS (Group 2)}$$

and

$$\frac{26.8\ mg/ml}{1.3\ mg/ml} \cong 21\ fold \text{ for TDS (Group 4)}$$

The final buffer salt composition calculated for the vehicle was therefore:

$$1\ mM\ carb-bicarb \times 19 \cong 19\ mM$$
$$1\ mM\ carb-bicarb\ in\ the\ resuspension\ buffer$$
$$0.125\ mM\ reduced\ glutathione \times 19 \cong 2.4\ mM$$
$$\sum = 20\ mM\ carb-bicarb,\ 2.4\ mM\ reduced\ glutathione$$

for preparation of CDS (Group1), and

$$1\,mM\ \ carb-bicarb \times 21 \cong 21\,mM$$
$$1\,mM\ \ carb-bicarb\ in\ the\ resuspension\ buffer$$
$$0.125\,mM\ \ reduced\ \ glutathione \times 21 \cong 2.6\,mM$$
$$\sum = 22\,mM\ \ carb-bicarb,\ 2.6\,mM\ \ reduced\ \ glutathione$$

for preparation of CDS (Group 3).

10.3 Evaluation of TDS Homogeneity

To evaluate the TDSs homogeneity, the protein concentration of the samples taken from the top, middle, and bottom sections of each TDS container was determined. The percent coefficient of variance (%CV) of the average concentration of the three samples of each dosing solution was calculated (Table 1), resulting in a %CV of 2.86% for TDS (Group 2) and 0.99% for TDS (Group 4). These values are below the pre-set acceptance criterion for homogeneity of a %CV ≤ 15% and, therefore, both TDSs were considered to be homogeneous solutions.

10.4 Protein Concentration of the TDS and CDS

Total protein concentration of the TDSs and CDSs were determined before and after dose administration using amino acid analysis (Tables 2 and 3, respectively). Amino acid analyses were performed in triplicate for all the samples. The total protein concentration for the pre- and post-dose TDS (Group 2) samples was determined to be 23.8 mg/ml and 24.7 mg/ml, respectively (Table 2). The average concentration of the TDS (Group 2) was 24.3 mg/ml (Table 2). The total protein concentration for the pre- and post-dose TDS (Group 4) samples was determined to be 28.3 mg/ml and 26.4 mg/ml, respectively (Table 2). The average concentration of the TDS (Group 4) was 27.4 mg/ml (Table 2).

The total protein concentration for the pre- and post-dose CDS (Group 1) samples was 26.0 mg/ml and 24.6 mg/ml, respectively. The average concentration was calculated to be 25.3 mg/ml (Table 3). The total protein concentration for the pre- and post-dose CDS (Group 3) samples was 27.0 mg/ml and 28.2 mg/ml, respectively. The average concentration was calculated to be 27.6 mg/ml (Table 3).

10.5 Purity of the Cry1Ac and BSA Proteins in each TDS and each CDS

To assess the stability of the Cry1Ac and BSA proteins in each TDS and each CDS, respectively, purity of the pre- and post-dose samples was analyzed using densitometric analysis of the Brilliant Blue G - colloidal stained SDS-polyacrylamide gels (Figures 1 to 4). Purity data for the Cry1Ac and BSA proteins in both TDS samples and both CDS samples are summarized in Table 4.

The average percent purity of the Cry1Ac protein in the pre- and post-dose TDS (Group 2) samples was 82% and 78%, respectively. The percent change in purity was 4.8%, which met the stability acceptance criterion of ≤ 10% change in purity (Table 4). Thus, the TDS (Group 2) was considered to be stable for the duration of the dosing period. The average of the pre- and post-dose TDS (Group 2) Cry1Ac protein purity was 80% and was used to calculate the final Cry1Ac dose level for Group 2 mice.

The average percent purity of the Cry1Ac protein in the pre- and post-dose TDS (Group 4) samples was 80% for each. Since the change in the percent purity was ≤ 10%, the stability acceptance criterion was met (Table 4) and, thus, the TDS (Group 4) was considered to be stable for the duration of the dosing period. The average of the pre- and post-dose TDS (Group 4) Cry1Ac protein purity, 80%, was used to calculate the final Cry1Ac dose level for Group 4 mice.

The average purity of the BSA protein was 77% and 75% in pre- and post- dose CDS (Group 1) samples, respectively. The percent change in purity was 1.3% which met the stability acceptance criterion of ≤ 10% change in purity (Table 4). Thus, the CDS (Group 1) was considered to be stable for the duration of the dosing period.

The average purity of the BSA protein was 87% and 88% in pre- and post- dose CDS (Group 3) samples, respectively. The percent change in purity was 1.1% which meets the stability acceptance criterion of ≤ 10% change in purity (Table 4). Thus, the CDS (Group 3) was considered to be stable for the duration of the dosing period.

10.6 Functional Activity of the Cry1Ac Protein in the TDS

Functional activity of each TDS was evaluated using an insect bioassay. The method and dose response modeling are reported in Appendix 1. The dose response curves are shown in Figures 5 and 6. The pre-dose and post-dose samples of TDS (Group 2) were determined to have similar biological activity with identical EC_{50} values of 0.0056 µg Cry1Ac protein/ml of diet (Table 5, Figure 5). The pre-dose and post-dose samples of TDS (Group 4) were determined to have similar biological activity with EC_{50} values of 0.0052 µg Cry1Ac protein/ml of diet and 0.0089 µg Cry1Ac protein/ml of diet, respectively (Table 5, Figure 6). Thus, both TDSs were considered to be biologically active throughout the period of dosing.

10.7 Calculation of the TDS and CDS Dose Levels

The final dose levels were calculated using concentration and purity values determined for each TDS and CDS (see Sections10.4 and 10.5). The data for purity corrected protein concentration and final dose level are shown in Table 6. In each case, the doses met or exceeded the target dosing level.

The dose level[2] for the TDS (Group 2) was determined as 1290 $\frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $24.3\frac{mg}{ml}\times 0.80 = 19.4\frac{mg}{ml}$

b) Protein level per dose: $19.4\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 647\frac{mg}{kgBW}$

c) Final dose level: $647\frac{mg}{kgBW}\times 2\ doses = 1294\frac{mg}{kgBW}$

The dose level[2] for the CDS (Group 1) was determined as 1280 $\frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $25.3\frac{mg}{ml}\times 0.76 = 19.2\frac{mg}{ml}$

b) Protein level per dose: $19.2\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 640\frac{mg}{kgBW}$

c) Final dose level: $640\frac{mg}{kgBW}\times 2\ \ doses = 1280\frac{mg}{kgBW}$

The dose level[2] for the TDS (Group 4) was determined as 1460 $\frac{mg}{kg\ BW}$:

a) Concentration corrected for purity: $27.4\frac{mg}{ml}\times 0.80 = 21.9\frac{mg}{ml}$

b) Protein level per dose: $21.9\frac{mg}{ml}\times 33.3\frac{ml}{kgBW} = 730\frac{mg}{kgBW}$

c) Final dose level: $730\frac{mg}{kgBW}\times 2\ doses = 1460\frac{mg}{kgBW}$

The dose level[3] for the CDS (Group 3) was determined as 1620 $\frac{mg}{kg\ BW}$:

[2] Rounding of numbers in calculations shown was for reporting purposes only. For the final dose level, rounding was deferred until after all calculations have been made.

a) Concentration corrected for purity: $27.6\ \frac{mg}{ml} \times 0.88 = 24.3 \frac{mg}{ml}$

b) Protein level per dose: $24.3\ \frac{mg}{ml} \times 33.3\ \frac{ml}{kgBW} = 809\ \frac{mg}{kgBW}$

c) Final dose level: $809\ \frac{mg}{kgBW} \times 2\ \ doses = 1618\ \frac{mg}{kgBW}$

11.0 Conclusions

The analytical tests performed on TDS samples have established that stable, homogeneous formulations of the Cry1Ac protein were achieved in both TDS preparations described in this report. The %CV of the concentrations of samples taken for the top, middle, and bottom of each TDS container was less than 15% indicating that each TDS was homogeneous. The difference in the Cry1Ac purity in each TDS was less than 10% prior to and after the administration of each TDS to Group 2 and Group 4 mice indicating that both TDS preparations were stable throughout the dosing periods. The Cry1Ac protein in each TDS was biologically active through the course of dosing both the Group 2 and Group 4 mice. The difference in the BSA protein purity was less than 10% prior to and after the administration of each CDS to Group 1 and Group 3 mice, indicating that each CDS was also stable throughout the dosing periods.

The concentration of the Cry1Ac and BSA proteins in the TDS and CDS, respectively, were calculated based upon total protein concentration and percent purity. The experimentally confirmed dosing level of the Cry1Ac protein in the TDS (Group 2) was 1290 mg/kg mouse BW. The experimentally confirmed dosing level for the BSA protein in the CDS (Group 1) was 1280 mg/kg mouse BW. The experimentally confirmed dosing level of the Cry1Ac protein in the TDS (Group 4) was 1460 mg/kg mouse BW. The experimentally confirmed dosing level for the BSA protein in the CDS (Group 3) was 1620 mg/kg mouse BW.

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

[3] Rounding of numbers in calculations shown was for reporting purposes only. For the final dose level reported, rounding was deferred until after all calculations have been made.

Table 1. Homogeneity of the TDS Pre-Dose Samples

Dose ID	Dose ID#	Sample Description	Concentration[1] (mg/ml)	%CV
TDS (Group 2)	10000804-D	Bottom	23.8	2.86
		Middle	23.8	
		Top	25.0	
TDS (Group 4)	10000805-3DF	Bottom	26.5	0.99
		Middle	26.6	
		Top	27.0	

[1] Value represents the mean of three replicates rounded to three significant figures.

Table 2. Total Protein Concentrations of the TDS Samples

Dose ID	Dose ID#	Sample Description	Concentration[1] (mg/ml)	TDS Average Protein Concentration (mg/ml)
TDS (Group 2)	10000804-D	Pre-dose	23.8	24.3
		Post-dose	24.7	
TDS (Group 4)	10000805-3DF	Pre-dose	28.3	27.4
		Post-dose	26.4	

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

Table 3. Total Protein Concentrations of the CDS Samples

Dose ID	Dose ID#	Sample Description	Concentration[1] (mg/ml)	CDS Average Protein Concentration (mg/ml)
CDS (Group 1)	19305-C	Pre-dose	26.0	25.3
		Post-dose	24.6	
CDS (Group 3)	19305-C3F	Pre-dose	27.0	27.6
		Post-dose	28.2	

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

Table 4. Purity of the Cry1Ac and BSA in the TDS and CDS Samples

Dose ID	Dose ID#	Sample Description	Purity[1] (%)	Percent Change[2]	Purity (%) Used in Dose Calculation[3]
TDS (Group 2)	10000804-D	Pre-Dose	82	4.8	80
		Post-Dose	78		
CDS (Group 1)	19305-C	Pre-Dose	77	1.3	76
		Post-Dose	75		
TDS (Group 4)	10000805-D3F	Pre-Dose	80	0.0	80
		Post-Dose	80		
CDS (Group 3)	19305-C3F	Pre-Dose	87	1.1	88
		Post-Dose	88		

[1] Each value represents the mean of three purity values estimated from loadings of 1, 2, and 3 µg total protein.

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

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

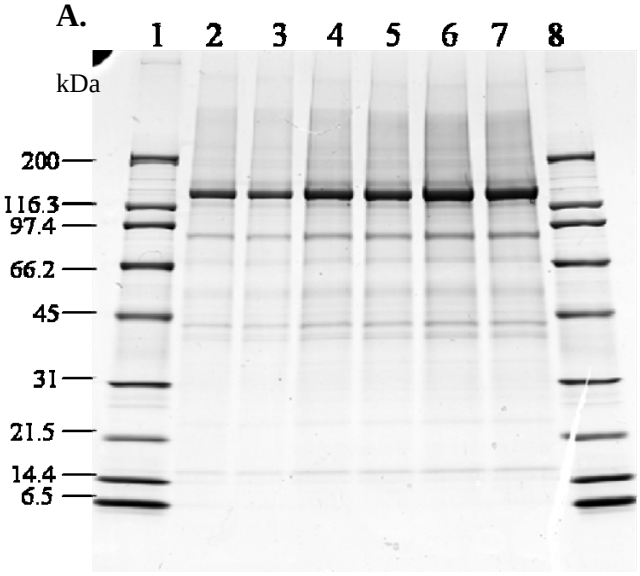
Table 5. Functional Activity of Pre- and Post-Dose TDS Samples

Dose ID	Dose ID#	Sample Description	EC_{50} (µg/mL diet Cry1Ac)	Standard Error (µg/mL diet)
TDS (Group 2)	10000804-D	Pre-Dose	0.0056	0.00074
		Post-Dose	0.0056	0.00073
TDS (Group 4)	10000805-D3F	Pre-Dose	0.0052	0.00053
		Post-Dose	0.0089	0.00097

Table 6. Experimentally Determined Dose Level

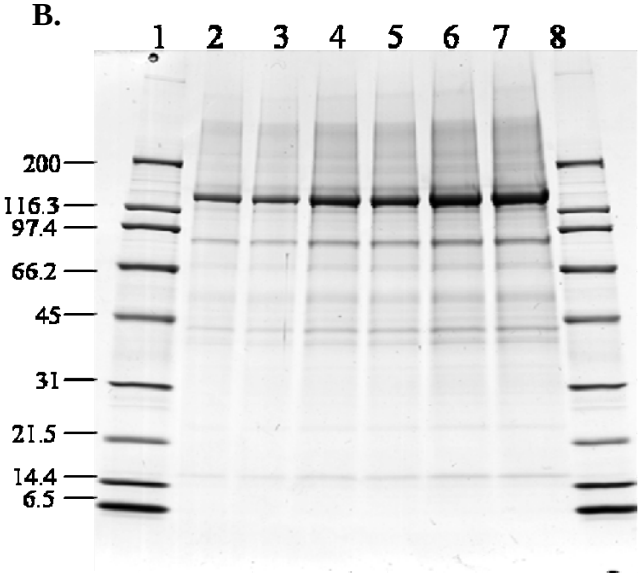
Dose ID	Dose ID#	Purity Corrected Concentration (mg/ml)	Dose Level (mg/kg BW)
TDS (Group 2)	10000804-D	19.4	1290
CDS (Group 1)	19305-C	19.2	1280
TDS (Group 4)	10000805-D3F	21.9	1460
CDS (Group3)	19305-C3F	24.3	1620

A.



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

B.

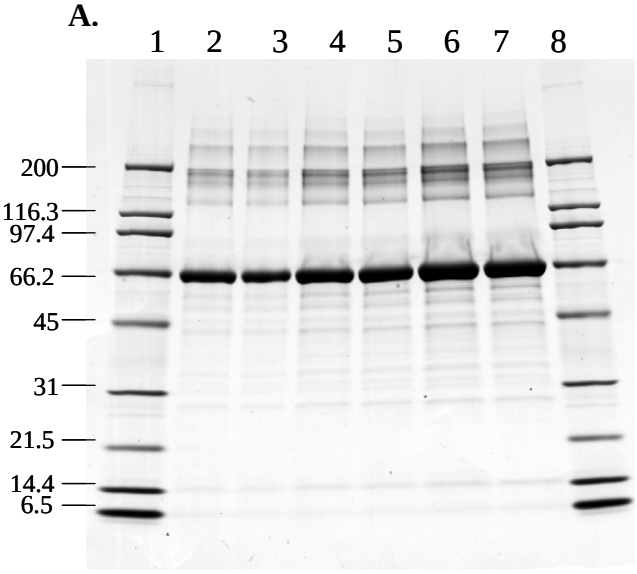


Lane	Sample	Amount (µg)
1	MW markers	-
2	Post-Dose TDS	1
3	Post-Dose TDS	1
4	Post-Dose TDS	2
5	Post-Dose TDS	2
6	Post-Dose TDS	3
7	Post-Dose TDS	3
8	MW markers	-

Figure 1. SDS-PAGE Analysis of Full-length Cry1Ac in the Pre- and Post-Dose TDS (Group 2) ID# 10000804-D Samples.

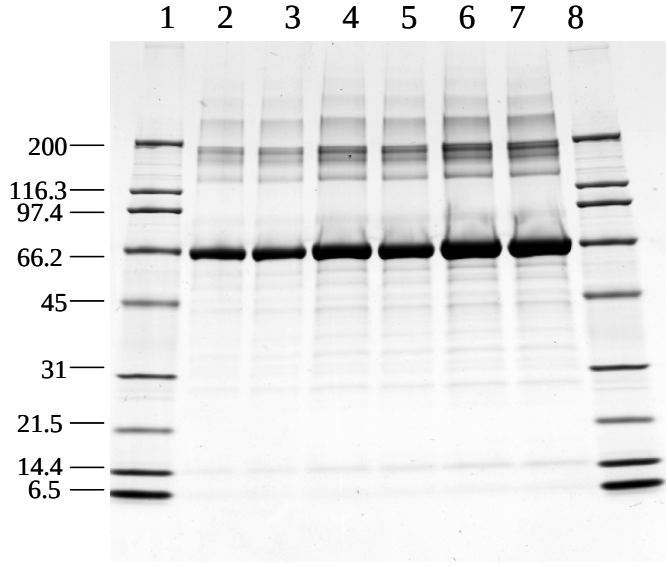
A. Lanes 2-7 correspond to the samples taken prior to dosing of the mice. B. Lanes 2-7 correspond to samples taken after dosing of the mice. Tris-glycine 4-20% polyacrylamide gradient gels were stained with colloidal Brilliant Blue G. Amounts loaded correspond to total protein. Approximate molecular weights (kDa) are shown on the left and correspond to the markers loaded in Lanes 1 and 8. Note: Empty lanes were cropped from original gel image and lanes were re-numbered, as cited above.

A.



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

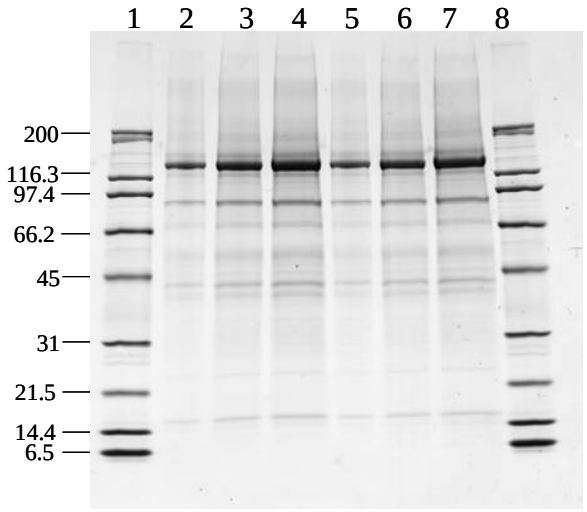
B.



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

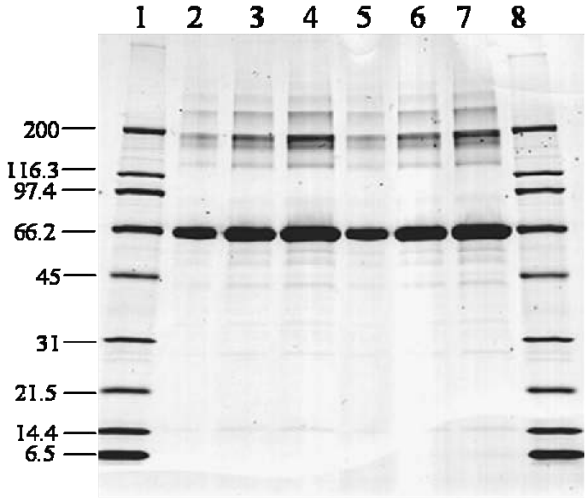
Figure 2. SDS-PAGE Analysis of BSA Protein in the Pre- and Post-Dose CDS (Group 1) ID # 19305-C Samples.

A. Lanes 2-7 correspond to samples taken prior to dosing of the mice. B. 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. Note: Empty lanes were cropped from initial gel image and lanes were re-numbered, as cited above.



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

Figure 3. SDS-PAGE Analysis of Full-length Cry1Ac in the Pre- and Post-Dose TDS (Group 4) ID# 10000805-D3F Samples.
Lanes 2-4 correspond to the samples taken prior to dosing of the mice. Lanes 5-7 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. Note: Empty lanes were cropped from initial gel image and lanes were re-numbered, as cited above.



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

Figure 4. SDS-PAGE Analysis of BSA Protein in the Pre- and Post-Dose CDS (Group 3) ID # 19305-C3F Samples.
Lanes 2-4 correspond to samples taken prior to dosing of the mice. Lanes 5-7 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. Note: Empty lanes were cropped from initial gel image and lanes were re-numbered, as cited above.

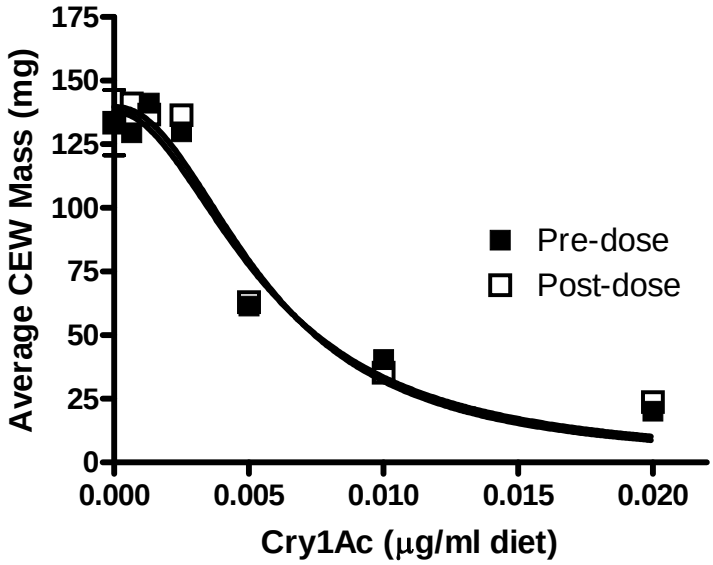


Figure 5. Functional Activity of the Cry1Ac Protein in the TDS (Group 2) ID# 10000804-D.
Corn earworm dose-response relationships for Cry1Ac Pre-dose and Post-dose samples in a diet-incorporation bioassay (prepared with GraphPad Prism software v.4.02).

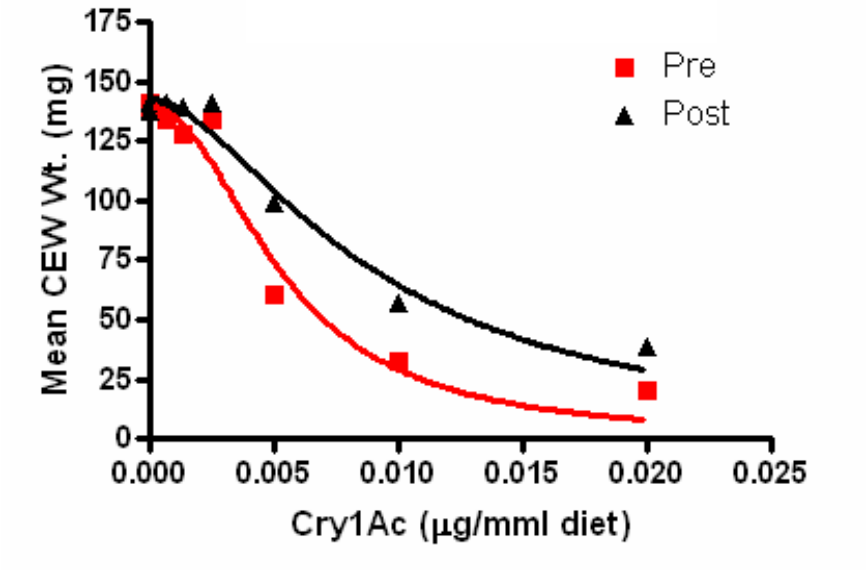


Figure 6. Functional Activity of the Cry1Ac Protein in the TDS (Group 4) ID# 10000805-D3F.
Corn earworm dose-response relationships for Cry1Ac Pre-dose and Post-dose samples in a diet-incorporation bioassay (prepared with GraphPad Prism software v.4).

Appendix 1. Insect Bioassay

Insect Bioassay Summary for:

Study-Specific Work Procedure for the Formulation and Confirmation of Dose Solutions for an Acute Oral Toxicity Study of Cry1Ac Protein Administered by the Oral (gavage) Route to Mice

Purpose:

Corn earworm (CEW), *Helicoverpa zea*, diet-incorporation insect bioassays were performed to verify that Cry1Ac mouse gavage test dosing solutions (TDS) collected pre and post-dosing were biologically active. Biological activity was measured as a 7-day EC_{50} value, the level of Cry1Ac protein that results in 50% CEW growth inhibition at 7days.

Materials & Methods:

TDS samples collected to evaluate the biological activity of Pre-Dosing and Post-Dosing solutions were received from the Regulatory Product Characterization Team (RPCT). TDS samples received on March 5, 2008 were labeled as pre-TDS and post-TDS (used in bioassay ID: 080305.CEW.Cry1Ac.MG), while TDS samples received on July 9, 2008 were labeled as Pre-TDS-1 and Post-TDS-1. Hereafter, TDS1 is used to refer to the first set of TDS samples received, while TDS2 is used to refer to the second set of TDS samples received. Total protein concentrations and purity from the RPCT for each of the samples was indicated as listed in Table 1.

Table 1. Total protein concentration and purity values for the TDS samples.

	TDS1 Pre-dose	**TDS1 Post-dose**	**TDS2 Pre-dose**	**TDS2 Post-dose**
Total Protein Concentration (mg/mL)	23.8	24.7	28.3	26.4
Purity (%)	82	78	80	80

TDS1 samples were received on wet ice in 20 mM carbonate/bicarbonate, pH 10-11, 2.4 mM reduced glutathione buffer. The control substance for TDS1 was identified as 20 mM carbonate/bicarbonate, pH 10-11, 2.4 mM reduced glutathione buffer (lot #: G-826370A). For TDS1, the protein samples and the control substance were used upon receipt from the RPCT.

TDS2 samples were received frozen on dry ice in 21 mM carbonate/bicarbonate, pH 10-11, 2.6 mM reduced glutathione buffer, and stored at approximately -80º C upon receipt.

The control substance for TDS2 was identified as 21.9 mM carbonate/bicarbonate, pH 10-11, 2.6 mM reduced glutathione buffer (lot #: G-836510C). The control substance for the TDS2 samples was stored at 4° C upon receipt from the RPCT.

Insects. CEW were obtained from Benzon Research Inc. Insect eggs were incubated at temperatures ranging from 10° C to 27° C to achieve the desired hatch time.

Bioassays. CEW larvae were used to measure the biological activity of the TDS samples received in accordance with the Monsanto SOP BR-ME-0044-03 entitled "Diet Incorporation Insect Bioassay for Use in Determining Biological Activity". The dosing solutions for the pre-dose and post-dose were prepared by diluting the pre-dose and post-dose Cry1Ac samples with purified water and incorporating the dilution into an agar-based multiple species diet (i.e., Southland diet). The doses for the pre-dose and post-dose samples were tested at the following geometrically spaced levels: 0.00065, 0.0013, 0.0025, 0.0050, 0.010, 0.020 µg Cry1Ac protein/ml diet. These dose levels were chosen in order to elicit a dose response to allow for estimation of EC_{50} values. Additionally, for each insect bioassay two control replicates were included, each containing buffer of the same composition used to suspend the Cry1Ac protein in the TDS samples. The amount of buffer included in these controls was at a level equivalent to the volume of buffer in the highest Cry1Ac dose level. The bioassay for the pre-dose and post-dose TDS1 samples were run in parallel. Similarly, the bioassay for the pre-dose and post-dose TDS2 samples were run in parallel.

Dosing solutions were incorporated into the agar-based multiple species diet and was then dispensed in 1 mL aliquots into 16 wells per treatment in a 128-well tray. Each well was infested with a single CEW larvae that was ≤ 30 hours from the first observation of hatching. Larvae were allowed to feed for 7-days in an environmental chamber programmed at 27° C, ambient relative humidity and a lighting regime of 14 hours light: 10 hours dark. The number of surviving insects and the combined weight of the surviving insects was recorded for each treatment level at the end of the incubation period.

Dose Response Modeling and Results:

The following 3-parameter logistic regression model was run under PROC NLMIXED using the SAS Software Release 9.1 (TS1M3) to model each dose-response curve and for EC_{50} estimation:

$$y_{ij} = \frac{w_0}{1 + (Dose_i / EC_{50})^{B}} + e_{ij}$$

where y_{ij} is the average larvae weight (mg) under dose level i and $Dose_i$ is the Cry1Ac protein diet dose level (µg/mL diet). The parameters included in the model are as follows: W_0 represents the weight in mg at $Dose_i$ = 0.0 µg/mL diet, EC_{50} represents of the effective dose to inhibit the growth of the target insect by 50%, and B reflects the rate of weight loss as $Dose_i$ increases, and e_{ij} denotes the residual effect.

All TDS samples produced similar dose-dependent decreases in average CEW body mass and 7-day EC_{50} estimates were accordingly very similar (Tables 2 and 3).

Conclusion:

The TDS1 Pre-dose and Post-dose samples were shown to be biologically active and have similar biologically activity with identical EC_{50} values, estimated to be 0.0056 µg Cry1Ac protein/mL. The TDS2 Pre-dose and Post-dose samples were also shown to be biologically active and have comparable EC_{50} values. EC_{50} values were estimated to be 0.0052 µg Cry1Ac protein/mL for the Pre-dose sample and 0.0089 µg Cry1Ac protein/mL for the Post-dose sample.

Table 2. EC_{50} estimates and standard errors for the TDS1 samples

Cry1Ac Sample	EC_{50} (µg/mL diet)	Standard Error (µg/mL diet)
Pre-dose	0.0056	0.00074
Post-dose	0.0056	0.00073

Table 3. EC_{50} estimates and standard errors for the TDS2 samples

Cry1Ac Sample	EC_{50} (µg/mL diet)	Standard Error (µg/mL diet)
Pre-dose	0.0052	0.00053
Post-dose	0.0089	0.00097

References:

SAS Software Release 9.1 (TS1M3). Copyright© 2002-2003 by SAS Institute Inc. Cary, NC.

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Appendix 2. Amendments and Deviations to SSP CRO-2007-325.

SSP Amendments.

Amendment 1. The target Cry1Ac dose level was originally set to be at least 1500 mg/kg BW and therefore, the target Cry1Ac concentration for the TDS was set to at least 22.5 mg/ml (28.1 mg/ml total protein). The Cry1Ac protein is susceptible to losing solubility as its concentration is increased. Therefore, the target dose level was changed to 1000 mg/kg BW so that the target Cry1Ac protein concentration in the TDS could be lower to be at least 15 mg/ml (18.8 mg/ml total protein).

Amendment 2. This amendment was made to the SSP to allow for the formulation of a second set of the TDS and CDS for dosing of a group of animals added to the Study Protocol EUF00221 (Protocol Amendment 3). SSP Amendment 2 states that the SSP will be carried out a second time. To ensure that the dose levels administered to the new group of mice was at least as high as the experimentally determined dose level of Cry1Ac protein administered to Group 2 mice, the target Cry1Ac dose level was set to be at least 1350 mg/kg BW. The target Cry1Ac concentration for the TDS, therefore, was set to be at least 20.3 mg/ml (25.4 mg/ml total protein). The TDS and CDS prepared under this amendment were not used in the study.

Amendment 3. This amendment was made to the SSP to allow for the formulation of a third set of the TDS and CDS for dosing male mice added to the Study Protocol EUF00221 (Protocol Amendment 5). This was necessary because the Cry1Ac protein in the TDS formulated under SSP Amendment 2 was not completely soluble upon thawing at Testing Facility and therefore was not suitable for dosing the added male mice. Under this amendment, the minimum target dose level for the new TDS and CDS was set to be at least 1300 mg/kg BW to ensure that the dose levels for the new mice were at least as high as the experimentally determined dose level administered to the Group 2 mice. Amendment 3 also allowed for the preparation of twice as much of the TDS and CDS as needed so that each could be split into 2 aliquots. One aliquot of each the TDS and the CDS was stored and shipped in liquid form at approximately 4 °C and one aliquot of each stored and shipped frozen at approximately -70 °C or lower. This was needed to ensure availability of the TDS if the freezing thawing cycle induced aggregation of protein in the TDS stored and shipped approximately -70 °C or lower.

SSP Deviations

1. The SSP states that Cry1Ac purity of samples containing test articles will be based on a summation of stained bands from just above the full length Cry1Ac protein (~151 kDa) down to and including the trypsin resistant core protein (~53). Instead, Cry1Ac purity was calculated based on a summation of stained bands from the full length Cry1Ac protein (~130 kDa) down to and including the trypsin resistant core protein (~55). Because the purity of Cry1Ac protein in samples analyzed during this study was consistent with the purity reported for E. coli-produced Cry1Ac [MON 87701], (Orion lot # 10000804 and 10000805 COAs, characterization and re-characterization), this deviation had no negative impact on the study.

2. SSP Amendment 3 states that ~60 ml of the TDS and CDS will be produced and each divided into two ~30 ml aliquots. Instead, the TDS and CDS intended for storage at 4 ºC were prepared separately from the TDS and CDS intended for storage at -80 C. As both dosing solutions met the suitability criteria, and only one TDS and CDS were used, this change had no impact on the study.

Appendix 3. List of Applicable SOPs

BR-ME-0388-02	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
BR-ME-0527-01	Brilliant Blue G-Colloidal Staining of Polyacrylamide Gels
BR-EQ-0599-02	Bio-Rad GS-800 Densitometer
BR-ME-0956-03	Protein Percent Purity and Apparent Molecular Weight Determination
BR-EQ-0376-02	Hitachi L-8800 Amino Acid Analysis System
BR-ME-0990-01	Vapor Phase Acid Hydrolysis Using 6 N HCl and Subsequent Amino Acid Analysis

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)

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 1: BSA - 1280 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8453	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8454	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8455	DEAD	14	7-MAR-08	08:57	SCHEDULED EUTHANASIA
8456	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8457	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8458	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8459	EXCRETA/EMESIS	7	29-FEB-08	10:28	FEW FECES
	BODY	7	29-FEB-08	10:28	DEHYDRATION
	BODY	7	29-FEB-08	10:28	THIN APPEARANCE
	BODY	7	29-FEB-08	10:28	ROUGH COAT
	EXCRETA/EMESIS	8	1-MAR-08	08:23	FEW FECES
	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8460	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8461	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8462	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 2: Cry1Ac - 1290 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8463	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8464	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8465	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8466	BODY	8	1-MAR-08	08:50	UNKEMPT APPEARANCE
	BODY	9	2-MAR-08	08:11	UNKEMPT APPEARANCE
	BODY	10	3-MAR-08	13:04	UNKEMPT APPEARANCE
	BODY	11	4-MAR-08	13:16	UNKEMPT APPEARANCE
	BODY	12	5-MAR-08	12:11	UNKEMPT APPEARANCE
	BODY	13	6-MAR-08	07:51	UNKEMPT APPEARANCE
	BODY	14	7-MAR-08	07:51	UNKEMPT APPEARANCE
	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8467	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8468	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8469	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8470	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8471	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8472	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES GROUP 1: BSA - 1280 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8473	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8474	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8475	EYE(S)	8	1-MAR-08	08:38	OCULAR LESION(S)
	EYE(S)	9	2-MAR-08	08:02	OCULAR LESION(S)
	EYE(S)	10	3-MAR-08	13:01	OCULAR LESION(S)
	EYE(S)	11	4-MAR-08	13:13	OCULAR LESION(S)
	EYE(S)	12	5-MAR-08	12:03	OCULAR LESION(S)
	EYE(S)	13	6-MAR-08	07:48	OCULAR LESION(S)
	EYE(S)	14	7-MAR-08	07:39	OCULAR LESION(S)
	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8476	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8477	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8478	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8479	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8480	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8481	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA
8482	DEAD	14	7-MAR-08	08:58	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

FEMALES GROUP 2: Cry1Ac - 1290 MG/KG 22-FEB-08 to 7-MAR-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
8483	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8484	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8485	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8486	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8487	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8488	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8489	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8490	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8491	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA
8492	DEAD	14	7-MAR-08	08:59	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 3: BSA - 1620 MG/KG 24-JUN-08 to 8-JUL-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
7853	DEAD	14	8-JUL-08	08:10	SCHEDULED EUTHANASIA
7854	DEAD	14	8-JUL-08	08:10	SCHEDULED EUTHANASIA
7855	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7856	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7857	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7858	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7859	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7860	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7861	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7862	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA

APPENDIX 4

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL SURVIVAL AND CLINICAL OBSERVATIONS

MALES GROUP 4: Cry1Ac - 1460 MG/KG 24-JUN-08 to 8-JUL-08

ANIMAL#	CATEGORY	DAY	DATE	TIME	OBSERVATIONS
7863	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7864	DEAD	14	8-JUL-08	08:11	SCHEDULED EUTHANASIA
7865	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA
7866	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA
7867	BODY	12	6-JUL-08	13:47	ROUGH COAT
	BODY	13	7-JUL-08	08:59	ROUGH COAT
	BODY	14	8-JUL-08	08:02	ROUGH COAT
	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA
7868	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA
7869	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA
7870	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA
7871	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA
7872	DEAD	14	8-JUL-08	08:12	SCHEDULED EUTHANASIA

Appendix 5
Individual Body Weight Data

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 1: BSA - 1280 MG/KG

ANIMAL#	DAY OF STUDY 0 (NON-FASTED)	0 (FASTED)	7	14
8453	31.3	30.5	34.3	36.2
8454	33.2	31.6	35.4	37.8
8455	32.9	31.1	35.2	37.3
8456	32.0	30.8	34.6	36.4
8457	31.3	30.2	34.1	35.2
8458	32.2	30.5	33.5	35.4
8459	28.4	27.5	17.5	30.0
8460	30.2	28.9	32.0	35.2
8461	33.4	32.1	35.4	37.8
8462	30.1	28.9	31.6	34.9
MEAN	31.5	30.2	32.4	35.6
S.D.	1.58	1.40	5.39	2.26
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF STUDY 0 (NON-FASTED)	0 (FASTED)	7	14
8463	32.7	31.3	31.6	34.4
8464	28.5	27.5	30.3	33.0
8465	30.1	29.2	31.8	32.6
8466	33.3	31.3	32.9	36.7
8467	30.3	28.8	30.9	33.9
8468	31.4	30.2	31.5	33.8
8469	33.4	32.0	34.5	36.7
8470	31.3	30.3	35.0	36.0
8471	30.5	29.8	30.6	32.6
8472	32.6	31.8	34.0	36.9
MEAN	31.4	30.2	32.3	34.7
S.D.	1.59	1.44	1.69	1.76
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES GROUP 1: BSA - 1280 MG/KG

ANIMAL#	DAY OF STUDY 0 (NON-FASTED)	0 (FASTED)	7	14
8473	29.1	27.7	27.5	31.8
8474	26.2	24.5	26.8	28.3
8475	27.0	26.5	26.4	30.5
8476	29.4	27.8	30.4	31.0
8477	27.9	26.3	27.8	30.4
8478	26.0	24.4	24.9	28.9
8479	27.1	26.0	25.7	27.5
8480	23.9	23.0	24.4	27.0
8481	27.6	26.2	27.7	29.3
8482	24.1	23.4	25.9	28.9
MEAN	26.8	25.6	26.8	29.4
S.D.	1.85	1.68	1.73	1.55
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

FEMALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF STUDY 0 (NON-FASTED)	0 (FASTED)	7	14
8483	24.7	23.1	26.3	28.6
8484	26.7	25.2	26.0	30.8
8485	27.8	26.8	27.0	29.3
8486	26.9	26.3	27.2	29.2
8487	23.1	22.6	22.6	27.1
8488	28.0	26.6	28.0	30.2
8489	25.8	24.1	27.8	28.2
8490	29.4	28.4	29.9	31.2
8491	28.6	27.1	28.9	30.9
8492	27.4	26.1	28.3	28.6
MEAN	26.8	25.6	27.2	29.4
S.D.	1.88	1.85	2.00	1.34
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 3: BSA - 1620 MG/KG

ANIMAL#	DAY OF STUDY 0 (NON-FASTED)	0 (FASTED)	7	14
7853	29.4	28.5	31.7	34.2
7854	27.3	26.2	29.6	30.5
7855	26.8	25.1	28.7	25.5
7856	27.6	25.9	30.4	32.7
7857	27.5	26.1	30.2	32.8
7858	28.3	27.2	32.7	33.7
7859	26.6	25.0	31.9	36.3
7860	28.7	27.5	30.5	32.2
7861	28.5	27.8	30.5	33.0
7862	26.7	25.5	29.3	30.9
MEAN	27.7	26.5	30.6	32.2
S.D.	0.95	1.20	1.24	2.86
N	10	10	10	10

APPENDIX 5

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT DATA (GRAMS)

MALES GROUP 4: Cry1Ac - 1460 MG/KG

ANIMAL#	DAY OF STUDY 0 (NON-FASTED)	0 (FASTED)	7	14
7863	28.7	27.4	30.5	33.8
7864	29.1	27.4	30.5	33.4
7865	28.5	27.2	30.9	34.6
7866	27.1	25.6	29.1	33.3
7867	27.5	26.4	30.1	32.0
7868	28.9	26.1	29.1	32.2
7869	27.5	26.4	30.8	32.1
7870	27.2	26.3	28.2	32.1
7871	26.5	25.3	31.3	34.9
7872	26.6	25.4	30.0	31.4
MEAN	27.8	26.4	30.0	33.0
S.D.	0.96	0.79	0.97	1.20
N	10	10	10	10

Appendix 6
Individual Body Weight Changes

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 1: BSA - 1280 MG/KG

ANIMAL#	DAY OF STUDY 0 (FASTED)-7	7-14
8453	3.8	1.9
8454	3.8	2.4
8455	4.1	2.1
8456	3.8	1.8
8457	3.9	1.1
8458	3.0	1.9
8459	-10.0	12.5
8460	3.1	3.2
8461	3.3	2.4
8462	2.7	3.3
MEAN	2.2	3.3
S.D.	4.29	3.31
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF STUDY 0 (FASTED)-7	7-14
8463	0.3	2.8
8464	2.8	2.7
8465	2.6	0.8
8466	1.6	3.8
8467	2.1	3.0
8468	1.3	2.3
8469	2.5	2.2
8470	4.7	1.0
8471	0.8	2.0
8472	2.2	2.9
MEAN	2.1	2.4
S.D.	1.22	0.91
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES GROUP 1: BSA - 1280 MG/KG

ANIMAL#	DAY OF STUDY 0 (FASTED)-7	7-14
8473	-0.2	4.3
8474	2.3	1.5
8475	-0.1	4.1
8476	2.6	0.6
8477	1.5	2.6
8478	0.5	4.0
8479	-0.3	1.8
8480	1.4	2.6
8481	1.5	1.6
8482	2.5	3.0
MEAN	1.2	2.6
S.D.	1.13	1.25
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

FEMALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF STUDY 0 (FASTED)-7	7-14
8483	3.2	2.3
8484	0.8	4.8
8485	0.2	2.3
8486	0.9	2.0
8487	0.0	4.5
8488	1.4	2.2
8489	3.7	0.4
8490	1.5	1.3
8491	1.8	2.0
8492	2.2	0.3
MEAN	1.6	2.2
S.D.	1.20	1.48
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 3: BSA - 1620 MG/KG

ANIMAL#	DAY OF STUDY 0 (FASTED)-7	7-14
7853	3.2	2.5
7854	3.4	0.9
7855	3.6	-3.2
7856	4.5	2.3
7857	4.1	2.6
7858	5.5	1.0
7859	6.9	4.4
7860	3.0	1.7
7861	2.7	2.5
7862	3.8	1.6
MEAN	4.1	1.6
S.D.	1.28	1.97
N	10	10

APPENDIX 6

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL BODY WEIGHT CHANGES (GRAMS)

MALES GROUP 4: Cry1Ac - 1460 MG/KG

ANIMAL#	DAY OF STUDY 0 (FASTED)-7	7-14
7863	3.1	3.3
7864	3.1	2.9
7865	3.7	3.7
7866	3.5	4.2
7867	3.7	1.9
7868	3.0	3.1
7869	4.4	1.3
7870	1.9	3.9
7871	6.0	3.6
7872	4.6	1.4
MEAN	3.7	2.9
S.D.	1.11	1.04
N	10	10

Appendix 7
Individual Food Consumption Data

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 1: BSA - 1280 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8453	8.8	9.4
8454	7.4	7.6
8455	7.2	7.4
8456	6.5	6.6
8457	7.2	6.6
8458	7.1	7.3
8459	3.9	8.1
8460	6.8	7.4
8461	6.2	5.8
8462	6.8	7.1
MEAN	6.8	7.3
S.D.	1.22	0.96
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8463	5.3	6.2
8464	4.4	6.0
8465	5.6	5.8
8466	6.3	8.3
8467	6.4	8.7
8468	5.8	6.2
8469	6.6	6.7
8470	7.5	6.6
8471	5.3	5.8
8472	6.4	7.1
MEAN	6.0	6.7
S.D.	0.85	1.01
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES GROUP 1: BSA - 1280 MG/KG

	DAY OF STUDY	
ANIMAL#	0-7	7-14
8473	5.6	6.4
8474	4.8	5.0
8475	4.8	6.7
8476	5.9	6.1
8477	5.9	6.1
8478	4.4	5.3
8479	5.7	6.2
8480	4.6	5.3
8481	5.5	5.7
8482	6.1	6.2
MEAN	5.3	5.9
S.D.	0.62	0.55
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

FEMALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF STUDY 0-7	7-14
8483	5.6	5.8
8484	3.9	5.1
8485	5.2	5.9
8486	5.7	5.2
8487	4.7	6.0
8488	5.5	6.4
8489	5.5	5.5
8490	5.8	6.1
8491	5.3	5.9
8492	6.1	6.2
MEAN	5.3	5.8
S.D.	0.66	0.44
N	10	10

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 3: BSA - 1620 MG/KG

	DAY OF STUDY		
ANIMAL#	0-7	7-10	10-14
7853	6.8	NVTE	5.8
7854	6.2	NVTE	4.9
7855	6.5	NVTE	4.4
7856	6.6	NVTE	6.1
7857	7.0	NVTE	6.2
7858	8.2	NVTE	6.2
7859	7.6	NVTE	7.0
7860	7.8	NVTE	7.0
7861	7.7	NVTE	6.4
7862	6.7	NVTE	5.8
MEAN	7.1		6.0
S.D.	0.68		0.81
N	10		10

NVTE=NO VALUE DUE TO TECHNICAL ERROR

APPENDIX 7

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL FOOD CONSUMPTION DATA (GRAMS/ANIMAL/DAY)

MALES GROUP 4: Cry1Ac - 1460 MG/KG

	DAY OF STUDY		
ANIMAL#	0-7	7-10	10-14
7863	7.1	NVTE	6.8
7864	7.1	NVTE	6.9
7865	7.0	NVTE	6.6
7866	6.6	NVTE	6.3
7867	6.6	NVTE	6.0
7868	6.3	NVTE	6.2
7869	7.2	NVTE	5.7
7870	8.3	NVTE	6.8
7871	7.3	NVTE	6.3
7872	7.4	NVTE	7.0
MEAN	7.1		6.5
S.D.	0.55		0.43
N	10		10

NVTE=NO VALUE DUE TO TECHNICAL ERROR

Appendix 8
Individual Gross Necropsy Observations

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 1: BSA - 1280 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8453	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8454	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8455	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8456	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8457	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8458	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8459	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8460	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8461	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8462	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8463	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8464	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8465	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8466	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8467	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8468	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8469	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8470	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8471	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8472	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES GROUP 1: BSA - 1280 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8473	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8474	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8475	7-MAR-08	14	GENERAL COMMENT: FINAL CLINICAL OBSERVATION NOT APPARENT POSTMORTEM OCULAR LESION(S)	SCHEDULED EUTHANASIA
8476	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8477	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8478	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8479	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8480	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8481	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8482	7-MAR-08	14	OVARY: PERIOVARIAN CYST(S); PRESENT LEFT, 0.5 X 0.3 X 0.3 CM, RED FLUID FILLED	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

FEMALES GROUP 2: Cry1Ac - 1290 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
8483	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8484	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8485	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8486	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8487	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8488	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8489	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8490	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8491	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
8492	7-MAR-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 3: BSA - 1620 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
7853	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7854	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7855	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7856	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7857	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7858	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7859	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7860	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7861	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7862	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA

APPENDIX 8

AN ACUTE TOXICITY STUDY ADMINISTERED BY THE ORAL (GAVAGE) ROUTE
TO MICE

INDIVIDUAL GROSS NECROPSY OBSERVATIONS

MALES GROUP 4: Cry1Ac - 1460 MG/KG

ANIMAL#	DAY OF DEATH	STUDY DAY	OBSERVATION	FATE
7863	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7864	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7865	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7866	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7867	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7868	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7869	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7870	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7871	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA
7872	8-JUL-08	14	ALL TISSUES WITHIN NORMAL LIMITS	SCHEDULED EUTHANASIA