Bayer CropScience

2mEPSPS PROTEIN

ACUTE TOXICITY BY ORAL GAVAGE

IN MICE

DATA REQUIREMENTS

Based on the U.S. E.P.A. Health Effects Test Guidelines OPPTS 870.1100 adopted in 2002 and on the O.E.C.D. Test Guideline 425 adopted in 2001

REPORT OF STUDY SA 06175

AUTHOR / STUDY DIRECTOR: D. ROUQUIE

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STUDY COMPLETED ON: AUGUST 29, 2006

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) (1) (A), (B) or (C).

However, these data are the property of Bayer CropScience AG and, as such, are considered to be a trade secret and confidential for all purposes other than compliance with FIFRA 10.

Submission of these data in compliance with FIFRA does not constitute a waiver of any right to confidentiality which may exist under any other statute or in any country other than the USA.

Company: Bayer CropScience
Research and Development Department

Company Agent:

Title:

Signature:

Date:

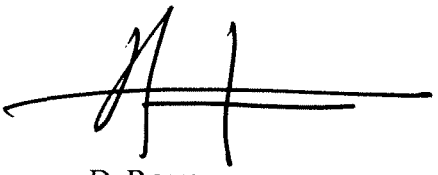
The above statement supersedes all other statements of confidentiality that may occur elsewhere in this report.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The study here reported was performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issue, with the exception of the dosing suspensions that were not analyzed for concentration, homogeneity or stability.

- O.E.C.D. (Organization for Economic Cooperation and Development) Principles of Good Laboratory Practice, 1997 (January 26, 1998)
- European Directive 2004/10/EC (February 11, 2004)
- U.S. E.P.A. (Environmental Protection Agency)
  . 40 CFR Part 160
  Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
  Good Laboratory Practice Standards: Final Rule, August 17, 1989.
- Good Laboratory Practice Standards for Toxicology studies on Agricultural chemicals, Ministry of Agriculture, Forestry and Fisheries (MAFF) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by 12 Nousan N°8628, December 06, 2000.
- French decree N°98-1312 regarding Good Laboratory Practice, December 31, 1998.

Author / Study Director:



Date: 28 August, 2006

D. ROUQUIE

Sponsor Representative:

Date: 29 August 2006

C. HEROUET-GUICHENEY

Study Submitter:

Date:

FLAGGING STATEMENTS

This page is reserved for flagging statements as may be required by E.P.A.

QUALITY ASSURANCE STATEMENT

The conduct of the study has been subjected to periodic inspections by the Bayer CropScience Sophia Antipolis Quality Assurance Unit. The types and dates of inspections and dates of reporting to Study Director and management are given below:

Type of Q.A. inspection	Date of Q.A. inspection	Date of reporting to Study Director	Date of reporting to Management
Protocol	June 21, 2006	June 21, 2006	June 21, 2006
Treatment	July 06, 2006	July 06, 2006	July 06, 2006
Report	August 02, 2006	August 02, 2006	August 29, 2006

This report has been audited by Quality Assurance personnel in accordance with the appropriate standardized operating methods. The reported results accurately reflect the original data of the study.

Quality Assurance Group Leader:

Date: August 29, 2006

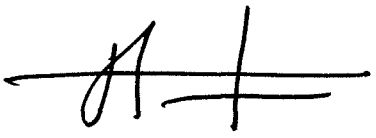
G. ODAGLIA

SIGNATURE

I, the undersigned, hereby declare that the work was performed under my supervision according to the procedures described and that this report provides a correct and faithful record of the results obtained.

There were no circumstances which affected the quality and integrity of the data.

Author / Study Director:



Date: 29 August, 2006

D. ROUQUIE

STUDY PROFESSIONALS

The following professionals were involved in the conduct of this study:

AUTHOR / STUDY DIRECTOR	:	D. ROUQUIE
LABORATORY ANIMAL RESOURCES	:	J.P. KOCWIN
TOXICOLOGY SUPERVISOR	:	D. SANTAMARIA
RESPONSIBLE TECHNICIAN	:	S. LEBAS
REPORT UNIT ASSISTANT	:	P. ALMERAS

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SUMMARY

The objective of this study was to assess the acute oral toxicity of 2mEPSPS (double mutated maize 5 enol pyruvylshikimate-3-phosphate synthase) protein in OF1 mice. In addition, the acute oral toxicity of bovine serum albumin (negative control) was also assessed.

Groups of 5 female OF1 mice were administered either 2mEPSPS protein or bovine serum albumin by oral gavage at dose levels of 2000 mg/kg body weight. All animals were observed for clinical signs daily for fifteen days whilst their body weights were measured weekly. At termination of the study period, animals were subjected to a necropsy including macroscopic examination.

There were no mortalities, no clinical signs or treatment-related effects on body weight in female OF1 mice after an acute oral administration of 2mEPSPS protein at 2000 mg/kg body weight.

In conclusion, treatment with 2mEPSPS protein at 2000 mg/kg body weight via the oral route did not produce signs of systemic toxicity in the OF1 female mouse.

INTRODUCTION

The objective of this study was to assess the potential for acute oral toxicity of 2mEPSPS (double mutated maize 5 enol pyruvylshikimate-3-phosphate synthase) protein in the OF1 mouse. In addition, the acute oral toxicity of bovine serum albumin (negative control) was also assessed.

The study protocol and its amendments are presented in Attachment 1.

The study time schedule was as follows:

Study initiation date *	June 21, 2006
Animal arrival date	June 21, 2006
Experimental starting date	June 21, 2006
Sponsor protocol approval date	June 21, 2006
Randomization date (Day -1)	July 04, 2006
Dosing date	July 06, 2006
Final sacrifice date	July 20, 2006
Experimental completion date	July 20, 2006

* Date of protocol approval by Study Director.

MATERIAL AND METHODS

1 - CONTROL AND TEST SUBSTANCE FORMULATION

The test substance 2mEPSPS protein (batch number LEJ5837, >99% purity) was used in this study. Information on the biochemical characterization of the test substance was documented by Bayer Bioscience NV, Gent, Belgium and is presented in Attachment 2. The test substance was stored in an air-tight, light resistant container at approximately -20°C.

The bovine serum albumin (reference number A7906, batch/lot number 114K2387, >97% purity) was used as a negative control. Biochemical information on the bovine serum albumin was documented by Sigma-Aldrich and is presented in Attachment 2. The control substance was stored in an air-tight, light-resistant container at approximately + 4°C.

The formulations were prepared by dissolving the substances in storage buffer (0.1 M Tris/HCl; 2.7 mM KCl; 137 mM NaCl; 1 mM DTT; pH 7.5) as described in the protocol to produce a suspension at the final concentration of 50 mg/ml (w/v). The formulations were placed in air-tight plastic tubes at +4°C and were used the day after preparation.

2 - ANIMALS, HOUSING, DIET AND WATER

2.1 Animals

The mouse was chosen because of its recommendation by regulatory authorities as an appropriate test species to assess acute oral toxicity. The Crl:OF1 strain was used since sufficient background toxicity data exist to support interpretation of results. A total of 15 female Crl:OF1 mice were obtained from Charles River Laboratories, Saint Germain sur l'Arbresle, France. Animals were acclimatized to laboratory conditions for 15 days prior to treatment and were 7 weeks old at the start of treatment.

a/Selection and randomization

All animals were examined for mortality and clinical signs during the acclimatization phase. Two days before test substance administration, all suitable animals were weighed. Ten female mice were selected for the study. An automatic randomization procedure was used to select animals for the study from the middle of the weight range of the available animals ensuring body weights were within ± 20% of the mean body weight at randomization. Selected animals were in a weight range from 21.69 to 23.98 g on the day of treatment.

b/Identification

Two days before treatment following randomization, animals were assigned permanent identification numbers within groups. Each animal was identified by a stainless steel ear tag bearing a unique animal number.

2.2 Housing

Mice were housed individually in suspended stainless steel wire mesh cages. Each cage was identified by a card specifying the study number, treatment group and dosage.

The temperature, humidity and lighting in the animal room were constantly monitored by an automatic system.

The target specifications were:

* temperature: 20°C - 24°C
* humidity: 40% - 70%
* lighting: 12-hour light, 12-hour dark cycles (7 am-7 pm)

The ventilation system in the animal room was maintained to ensure adequate ventilation, with the performance of the system regularly checked for a target specification of 10 to 15 air changes per hour.

There were no deviations from target specifications which could have compromised the study. Housing data are placed in the study file.

2.3 Diet and water

Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animals Food and Engineering, Augy, France) was available *ad libitum*. Filtered and softened tap water from the municipal water supply was provided *ad libitum* using water bottles. Filters servicing the watering system were regularly changed and sterilization of the system was periodically performed. Certificates of analysis were provided by the diet manufacturer and the supplier. Additionally, quality control analytical reports of the physicochemical properties and concentration of specified contaminants were periodically obtained from independent consultant analysts. These routine analyses of feed and water indicated that there was no contamination which could have affected the integrity and outcome of this study.

3 - EXPERIMENTAL DESIGN

Due to the limitation of the solubility of the test material in the storage buffer, groups of 5 female mice were given the test or control substance by oral gavage in two doses, each dose at 1000 mg/kg body weight. Both doses were administered within a 4 hours period on the day of treatment. The test materials were orally administered in solution in storage buffer at a volume of 20 ml/kg (based on body weight on Day 1). Therefore, a dose of test or control substance at 2000 mg/kg body weight was administered to each mouse during the same day of treatment.

The oral route was selected as it is considered as one of the most important portals of entry of the digestive tract. Historically, the oral route of exposure has been used to investigate the toxicity of many novel proteins.

Details of group sizes and treatments:

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Bovine serum albumin	2000	5	QT1F2523 to 2527
2	Test substance: 2mEPSPS protein	2000	5	QT2F2528 to 2532

Clinical signs were recorded daily from Day 1 through Day 15. They were recorded approximately 30 minutes after each dosing, at least once more on Day 1 and at least once each day thereafter. The nature, onset, severity, reversibility and duration of all clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose feces. In addition, animals were checked twice daily for mortality, except on weekends and public holidays when they were checked once daily.

4 - BODY WEIGHT

Each animal was weighed on Days -6, -2, 1, 8 and 15.

5 - POST MORTEM PROCEDURES

At final sacrifice on Day 15, surviving animals were anesthetized by Isoflurane inhalation, then exsanguinated under deep anesthesia before necropsy. Necropsy included macroscopic examination of abdominal and thoracic cavities, major organs and tissues. Significant macroscopic abnormalities were recorded.

6 - CALCULATIONS

Means and standard deviations were calculated for body weights and absolute body weight gains.

7 - DATA STORAGE

All raw data, supporting documents, as well as protocol, protocol amendments and final report are maintained in the document archive room. A test substance reference sample is retained in the area of the products storeroom defined for the archiving of test substances. All of the above will be archived for at least 10 years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

RESULTS

1 - MORTALITY (Tab. 1)

Group mortality:

Groups	Control or Test substances	Dose levels (mg/kg)	Number of dead female animals
1	Negative control: bovine serum albumin	2000	0 / 5
2	2mEPSPS protein	2000	0 / 5

No mortality was observed during the study in bovine serum albumin or 2mEPSPS protein-treated animals at a dose of 2000 mg/kg body weight.

2 - DAILY OBSERVATIONS (Tab. 1)

No clinical signs were observed in bovine serum albumin or 2mEPSPS protein-treated animals throughout the study period.

3 - BODY WEIGHT (Tab. 2, 3)

Mean body weight gain over the entire study was similar between groups of animals that received the 2mEPSPS protein and the negative control protein.

Since no changes were observed in terms of body weight gain between negative control protein and 2mEPSPS test protein groups, it can be concluded that there is no adverse effect on body weight gain following treatment with 2mEPSPS protein.

4 - GROSS PATHOLOGY (Tab. 4)

The few macroscopic observations noted were those commonly observed in the untreated mice of this strain and age kept, under the laboratory conditions used, and were thus considered to have occurred spontaneously.

No 2mEPSPS-treatment related macroscopic findings were observed.

CONCLUSION

There were no mortalities, clinical signs or treatment-related effects on body weight in female OF1 mice after an acute oral administration of the 2mEPSPS protein at 2000 mg/kg body weight.

Animals treated at 2000 mg/kg body weight of bovine serum albumin (negative control animals) showed no visible signs of systemic toxicity.

In conclusion, treatment with 2mEPSPS protein at 2000 mg/kg body weight via the oral route did not produce signs of systemic toxicity in the female mouse.

PROTOCOL DEVIATIONS

1. Body weights were measured on study Days -6, -2, 1, 8 and 15.

2. A single batch of 2mEPSPS protein (batch number LEJ5837) was used in this study and not in combination with another one as stated in error in the protocol.

3. Between Day 2 and Day 4, all animals were observed and no clinical signs were observed but these clinical observation sessions were not recorded onto the PathTox data capture system in error.

It is the opinion of the Study Director that these deviations did not affect the quality and the integrity of the results of this study.

Author / Study Director:

Date: 29 August, 2006

D. ROUQUIE

ABBREVIATIONS

2mEPSPS	Double mutated maize 5 enol pyruvylshikimate-3-phosphate synthase
%	Percentage
°C	Degree Celsius
am	*ante meridiem*
Da	Daltons
E.E.C.	European Economic Communities
E.P.A.	Environmental Protection Agency
g	Gram
GLP	Good Laboratory Practice
M.A.F.F.	Ministry of Agriculture, Forestry and Fisheries
mg/kg	Milligram/kilogram
ml/kg	Milliliter/kilogram
mM	Millimolar
O.E.C.D.	Organization for Economic Cooperation and Development
pm	*post meridiem*
QA	Quality Assurance
SDEVS	Standard Deviation
Tab.	Table
USA	United States of America
v/v	Volume/volume
w/v	Weight/volume

TABLES

TABLE 1 - **INDIVIDUAL CLINICAL SIGNS AND DEAD ANIMAL STATUS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Bovine serum albumin	2000	5	QT1F2523 to 2527
2	Test substance: 2mEPSPS protein	2000	5	QT2F2528 to 2532

DOSAGE LEVEL IN: mg/kg

ANIMAL	OBSERVATION	DAYS OBSERVED
QT1F2523 2000.0	NORMAL THROUGHOUT INTERVAL	
QT1F2524 2000.0	NORMAL THROUGHOUT INTERVAL	
QT1F2525 2000.0	NORMAL THROUGHOUT INTERVAL	
QT1F2526 2000.0	NORMAL THROUGHOUT INTERVAL	
QT1F2527 2000.0	NORMAL THROUGHOUT INTERVAL	
QT2F2528 2000.0	NORMAL THROUGHOUT INTERVAL	
QT2F2529 2000.0	NORMAL THROUGHOUT INTERVAL	
QT2F2530 2000.0	NORMAL THROUGHOUT INTERVAL	
QT2F2531 2000.0	NORMAL THROUGHOUT INTERVAL	
QT2F2532 2000.0	NORMAL THROUGHOUT INTERVAL	

NOTE: DATA FOR Dosing phase 1

Animal Number	Grp	Sex	Study Phase	Date and Time Data was Entered	Oper. No.	Date of Death	Phase Day	Death Typ	Death Status	Term. Body Wt. (g)	Ow	Grs
QT1F2523	1	F	Dosing phase 1	20-Jul-06 09:19	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT1F2524	1	F	Dosing phase 1	20-Jul-06 09:19	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT1F2525	1	F	Dosing phase 1	20-Jul-06 09:20	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT1F2526	1	F	Dosing phase 1	20-Jul-06 09:21	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT1F2527	1	F	Dosing phase 1	20-Jul-06 09:21	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT2F2528	2	F	Dosing phase 1	20-Jul-06 09:22	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT2F2529	2	F	Dosing phase 1	20-Jul-06 09:22	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT2F2530	2	F	Dosing phase 1	20-Jul-06 09:23	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT2F2531	2	F	Dosing phase 1	20-Jul-06 09:23	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C
QT2F2532	2	F	Dosing phase 1	20-Jul-06 09:24	126	20-Jul-06	15	s	Final phase sacrifice	-----	-	C

Note: * = pretest animal no. P = partial data. C = complete data. - = no data.

TABLE 2 - **MEAN AND INDIVIDUAL BODY WEIGHTS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Bovine serum albumin	2000	5	QT1F2523 to 2527
2	Test substance: 2mEPSPS protein	2000	5	QT2F2528 to 2532

Bayer CropScience
Center of Toxicology
Sophia-Antipolis
Mouse/OF 1

ANIMAL BODY WEIGHTS IN (G)
Study number: sa 06175
Study start date: 06-Jul-06

Printed: 20-Jul-06
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Acute Toxicity/Oral limit test

DOSAGE IN mg/kg / ANIMAL	SEX	DAY OF PHASE 1	DAY OF PHASE 8	DAY OF PHASE 15
		FEMALE ANIMALS		
QT1F2523 2000.0	F	21.69	26.68	26.68
QT1F2524		21.14	27.11	26.65
QT1F2525		22.20	29.40	31.66
QT1F2526		23.98	27.95	30.69
QT1F2527		22.88	28.10	30.76
	(n)	5	5	5
	MEANS	22.38	27.85	29.29
	SDEVS	1.10	1.05	2.42
QT2F2528 2000.0	F	22.05	26.96	29.22
QT2F2529		22.57	29.82	29.50
QT2F2530		22.82	27.67	29.22
QT2F2531		21.71	26.62	27.23
QT2F2532		23.41	28.24	30.61
	(n)	5	5	5
	MEANS	22.51	27.86	29.16
	SDEVS	0.66	1.26	1.22

NOTE: DATA FOR Dosing phase 1

TABLE 3 - **MEAN AND INDIVIDUAL ABSOLUTE WEIGHT GAINS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Bovine serum albumin	2000	5	QT1F2523 to 2527
2	Test substance: 2mEPSPS protein	2000	5	QT2F2528 to 2532

Bayer CropScience — ANIMAL ABSOLUTE WEIGHT GAINS IN (G) — Printed: 20-Jul-06
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Sophia-Antipolis — ABSOLUTE WEIGHT GAINS REFERENCED TO Dosing phase 1 (DAY 1)
Mouse/OF 1 — Study start date: 06-Jul-06 — Acute Toxicity/Oral limit test

DOSAGE IN mg/kg / ANIMAL	SEX	DAY OF PHASE 8	DAY OF PHASE 15
		FEMALE ANIMALS	
QT1F2523 2000.0	F	4.99	4.99
QT1F2524		5.97	5.51
QT1F2525		7.20	9.46
QT1F2526		3.97	6.71
QT1F2527		5.22	7.88
	(n)	5	5
	MEANS	5.47	6.91
	SDEVS	1.20	1.81
QT2F2528 2000.0	F	4.91	7.17
QT2F2529		7.25	6.93
QT2F2530		4.85	6.40
QT2F2531		4.91	5.52
QT2F2532		4.83	7.20
	(n)	5	5
	MEANS	5.35	6.64
	SDEVS	1.06	0.71

NOTE: DATA FOR Dosing phase 1

TABLE 4 - **INDIVIDUAL GROSS FINDINGS**

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Bovine serum albumin	2000	5	QT1F2523 to 2527
2	Test substance: 2mEPSPS protein	2000	5	QT2F2528 to 2532

Animal number	Sex	Group/ Subgroup	Date and time data was entered	Date data taken	Opr #	Tissue / Observation(s) Locator, Severity, Other, Distribution, Shape/Attachments, Texture
QT1F2523	F	1/1	20-Jul-06 09:45	20-Jul-06	75	GENERAL COMMENT All organs, no abnormality, , , , ,
QT1F2524	F	1/1	20-Jul-06 10:06	20-Jul-06	75	GENERAL COMMENT All organs, no abnormality, , , , ,
QT1F2525	F	1/1	20-Jul-06 10:22	20-Jul-06	75	GENERAL COMMENT All organs, no abnormality, , , , ,
QT1F2526	F	1/1	20-Jul-06 10:29	20-Jul-06	75	GENERAL COMMENT All organs, no abnormality, , , , ,
QT1F2527	F	1/1	20-Jul-06 10:38	20-Jul-06	75	KIDNEY(S) Pale, , Slight, Bilateral, Diffuse, Not sampled.
QT2F2528	F	2/1	20-Jul-06 09:53	20-Jul-06	75	SPLEEN Enlarged, , Minimal, , , Not sampled.
QT2F2529	F	2/1	20-Jul-06 10:46	20-Jul-06	75	KIDNEY(S) Pale, , Slight, Bilateral, Diffuse, Not sampled.
QT2F2530	F	2/1	20-Jul-06 10:51	20-Jul-06	75	KIDNEY(S) Pale, , Minimal, Bilateral, Diffuse, Not sampled.
QT2F2531	F	2/1	20-Jul-06 10:57	20-Jul-06	75	SPLEEN Enlarged, , Minimal, , , Not sampled.
QT2F2532	F	2/1	20-Jul-06 11:08	20-Jul-06	75	KIDNEY(S) Pale, , Minimal, Bilateral, Diffuse, Not sampled.

ATTACHMENTS

ATTACHMENT 1 – **PROTOCOL AND AMENDMENTS**

2mEPSPS

ACUTE TOXICITY BY ORAL GAVAGE IN MICE

TESTING FACILITY:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

SPONSOR:

Bayer AG
Bayer CropScience
Alfred Nobel
40789 Monheim
Germany

1 - GENERAL

1.1 PURPOSE OF STUDY

The objective of this study is to investigate the acute toxicity of the 2mEPSPS protein after a single oral administration in mice. The potential acute toxicity of the test protein will be compared to the oral acute toxicity of Bovine serum albumin, a negative control protein.

1.2 GOOD LABORATORY PRACTICE COMPLIANCE

This study will be performed in accordance with the principles of Good Laboratory Practice ("Bonnes Pratiques de Laboratoire") described in the following issues:

-O.E.C.D. (Organization for Economic Cooperation and Development) Principles of Good Laboratory Practice, 1997 (January 26, 1998)

-European Directive 2004/10/EC (February 11, 2004)

-U.S. E.P.A. (Environmental Protection Agency)

.40 CFR Part 160
Federal Insecticide, Fungicide and Rodenticide Act (FIFRA);
Good Laboratory Practice Standards: Final Rule, August 17, 1989.

-Good Laboratory Practice Standards for Toxicology studies on Agricultural Chemicals, Ministry of Agriculture, Forestry and Fisheries (MAFF) in Japan, notification 11 Nousan N°6283, October 01, 1999, modified by 12 Nousan N°8628, December 06, 2000.

-French decree N°98-1312 regarding Good Laboratory Practice, December 31, 1998.

1.3 REGULATORY GUIDELINES

This study is based on the U.S. E.P.A. Health Effects Test Guidelines OPPTS 870.1100, adopted in 2002 (1) and on the O.E.C.D. Test Guideline 425, adopted in 2001 (2).

1.4 QUALITY ASSURANCE

The Quality Assurance Unit of Bayer CropScience, 355 rue Dostoïevski, BP 153, 06903 Sophia Antipolis Cedex, France, will undertake and document inspections while the study is in progress and will audit the study report.

2 - STUDY PERSONNEL

2.1 STUDY DIRECTOR:

Date: 21/06/06

D. ROUQUIE

2.2 SPONSOR REPRESENTATIVE:

Date: 21/06/06

p.o. C. HEROUET-GUICHENEY

2.3 OTHER STUDY PERSONNEL

Responsibility	**Name**
In-life Supervisor	SANTAMARIA Daniel
Responsible Technician	LEBAS Stéphane

Other study personnel will be identified as appropriate in the study file.

3 - PROPOSED DATES

Arrival of animals	: June 21, 2006
Experimental starting date	: June 21, 2006
Randomization	: July 04, 2006
Start of treatment	: July 05, 2006
Final sacrifice	: July 19, 2006
Experimental completion date	: August 31, 2006 (estimated)

4 - OVERVIEW OF THE STUDY DESIGN

Groups of five female mice will be treated by oral gavage at the limit dose level of 2000 mg protein/kg body weight in order to identify any acute toxic effects of the test protein.

Animals will be observed for clinical signs daily for 15 days. Body weight will be recorded at weekly intervals and on the day of necropsy. All mice, including those found dead or killed for humane reasons during the study, will be subjected to macroscopic observations and, when deemed appropriate by the Study Director or the Sponsor Representative, tissues will be retained for possible microscopic examinations.

5 - SPECIES SPECIFICATIONS

5.1 TEST SYSTEM

Species: Mouse
Sex: Female
Strain: Crl:OF1
Body weight at study start: 20-30 g
Age at start of study: 7-8 weeks

5.2 ANIMAL SUPPLIER

Charles River France Laboratories (Saint Germain sur l'Arbresle, France).
Fifteen female mice will be ordered.

5.3 REASON FOR SELECTION OF SPECIES

The mouse has been chosen because of its acceptance by Regulatory Authorities as a test species to assess acute toxicity.

The OF1 strain has been used extensively in toxicity evaluation studies, hence sufficient background data exist to support interpretation of results.

5.4 ACCLIMATIZATION PHASE AND RANDOMIZATION

The duration of the acclimatization phase will be at least 5 days.

Animals will be checked twice daily for moribundity and mortality except on weekends and public holidays when they will be checked once daily.

All animals will be weighed at least weekly during the acclimatization phase.

The acceptable body weight range will be ± 20% of the mean body weight on the day of randomization.

Any animal deemed unsuitable for the study based on weight or clinical signs will not be used in the study.

Remaining animals will be allocated to the dosage group by using a computerized randomization procedure that ensures a similar body weight distribution within this group.

5.5 IDENTIFICATION

At the time of randomization each animal will be identified by a stainless steel ear tag bearing a unique animal number.

6 - DIET INFORMATION

6.1 FEED

Certified rodent pelleted and irradiated diet A04C-10 from S.A.F.E. (Scientific Animals Food and Engineering, Augy, France) will be available *ad libitum*.

Feed will be stored in an identified room controlled for temperature and humidity.

Diet will be used only until date of expiry.

6.2 WATER

Filtered and softened tap water from the municipal water supply will be provided *ad libitum* using automatic watering system. Filters servicing the watering system are changed regularly and sterilization of the system is periodically performed.

6.3 ANALYSES

Analytical data will be provided by the manufacturer for each batch of diet including the size of pellets and concentration of nutritional components, selected heavy metals, pesticides, mycotoxins, microorganisms and nitroso compounds. Batches of diet will be only released for use after confirmation they meet specification.

Certificates of water analysis will be provided by the "Laboratoire Municipal d'Hygiène de la Ville de Nice" (France) and "Institut d'Hygiène Alimentaire de Longjumeau" (France).

6.4 RECORDS

Records of certificates of feed and water analyses will be retained in the archives.

7 - ENVIRONMENTAL CONDITIONS

7.1 ROOM

From June 21, 2006 to July 04, 2006 in the animal room number: 1448
From July 04, 2006 to July 19, 2006 in the animal room number: 1463

The animal rooms are within a barrier maintained unit with restricted entry.

7.2 HOUSING

Animals will be housed individually in suspended, stainless steel, wire mesh cages. The cage of each animal will be identified by a card bearing a unique identification number.

7.3 TEMPERATURE, HUMIDITY AND VENTILATION

Air temperature will be controlled to ensure:

- a temperature of 20°C - 24°C
- a relative humidity of 40% - 70%

with a target of 15 air changes per hour.

7.4 LIGHTING

Twelve-hour light/dark cycles will be provided by automatically controlled fluorescent-tube lighting (7am - 7pm).

7.5 RECORDS

The temperature, humidity and lighting in the animal room are constantly monitored by computer and the performance of the ventilation system is regularly checked. Records of all deviations from specifications will be placed in the study file.

8 - CONTROL AND TEST SUBSTANCE

8.1 SUBSTANCE CHARACTERISTICS

Negative control name: Bovine serum albumin

Supplier: Sigma

Batch number: 114K2387

Purity: > 98 %

Test substance name: 2mEPSPS protein encoded by *2mepsps* gene.

The test substance will be supplied by the Sponsor (Bayer BioScience NV, Gent, Belgium). The storage of a retention sample is under the responsibility of the Sponsor.

Batch numbers: LEJ5837 and LEJ5838

8.1.1 Storage

The control and the test substances will be stored frozen in an air-tight, light-resistant container at approximately minus 20°C or according to the conditions described in the test substance specifications when available. The storage stability is certified through at least 6 months at -20°C for the control and test substances.

8.1.2 Safety handling and requirements

Information on the appropriate safety precautions when handling the test substance will be given by the supplier or the Sponsor Representative.

In the absence of information on the potential toxic effects of the test substance, safety precautions will be applied according to the relevant standard operating procedures.

8.1.3 Analyses

The confirmation of the identity, purity and activity of the control and the test substances will be provided by the Sponsor Representative. The certificate of analysis of the test substance will be placed in the final study report.

8.2 VEHICLE AND TEST SUBSTANCE FORMULATION

8.2.1 Preparation

The control and the test substances will be received from the Sponsor as a lyophilized powder.

The appropriate amount of test substance will be dissolved in distilled water. The formulation will be prepared on the day of dosing and will be mixed thoroughly immediately before administration. The unused residue of the formulation will be stored at -20°C at the end of the administration period.

8.2.2 Storage

The control and the test formulations will be placed in glass air-tight bottles, at room temperature and used as quickly as practicable after preparation.

8.2.3 Analyses

Stability and homogeneity of the formulated test substance will be confirmed analytically. If the test substance is soluble in the used formulation, homogeneity will not be confirmed analytically. Stability and homogeneity of the formulated test substance will not be confirmed analytically if test substance is known to be stable and homogenous in both undiluted and in-ready-to-use dilution with distilled water (e.g., commercial product).

9 - TREATMENT GROUPS AND DOSAGES

9.1 CHOICE OF DOSE

A starting limit dose level of 2 000 mg 2mEPSPS protein/ kg body weight was selected after discussion with the Sponsor Representative.
This choice was based on the preliminary safety assessment of the test substance which led to the conclusion with a high degree of certainty of the lack of harmful effects caused by the 2mEPSPS protein after oral administration to mammals, for the following reasons:

1- EPSPS proteins are ubiquitous in nature, including in food and feed products (e.g., soybean, tomato, maize) and no adverse health effects have been related to these substances. Since the 2mEPSPS protein is very similar to the other EPSPS proteins, its safety profile is expected to be similar.

2 -The 2mEPSPS protein has no amino acid sequence similarity to known toxins. As expected, it has only a high similarity with non-toxic proteins of the same functional family.

3- The 2mEPSPS protein has a very well known biochemical role in plants. The metabolic effect of its expression is conferring tolerance to glyphosate herbicides. The 2mEPSPS protein is a highly specific enzyme and shares very similar molecular weight and functional properties with non-toxic proteins belonging to the safe class of shikimate synthase proteins.

4- The EPSPS protein has been reported to be rapidly and completely degraded in simulated gastric fluids. This maximizes the likelihood that this protein could be degraded in the digestive tract and be bioavailable.

9.2 CHOICE OF ROUTE

The 2mEPSPS protein will be potentially present in food and feed and the oral route is one of the most important portals of entry of the digestive tract.

9.3 NUMBER OF ANIMALS

Five female mice will be administered with the control substance or the test substance.

9.4 CONDITIONS OF ADMINISTRATION

All animals used in the study will be diet fasted prior to dosing. All animals will receive the appropriate concentration of test substance in distilled water at approximately the same time at a volume of 20 ml/kg body weight. After dosing, food will be withheld for approximately a further 3 hours.

9.5 DOSES

GROUP	CONTROL OR TEST SUBSTANCE(S)	DOSE LEVELS mg/kg	NUMBER OF FEMALE ANIMALS PER GROUP	ANIMALS IDENTITY FEMALES
1	Negative control: Bovine serum albumin	2000	5	QT1F2523 to 2527
2	Test substance: 2mEPSPS protein	2000	5	QT2F2528 to 2532

9.6 ANIMAL WELFARE

During the study the care and use of animals will be conducted in accordance with the regulations of the Guide for the Care and Use of Laboratory Animals (Public Health Service, National Institute of Health, NIH, publication N° 86-23, revised 1985) and "Le Guide du Journal Officiel des Communautés Européennes L358, 18 Décembre 1986, n° 86/609/CEE du 24 Novembre 1986".

10 - LABORATORY DETERMINATIONS AND SCHEDULES

All animal data will be recorded using a dedicated computer system (Path/Tox system, version 4.2.2, protocol number RP-0639).

10.1 CLINICAL EXAMINATION

10.1.1 Clinical signs and mortality

Clinical signs will be recorded individually starting on Day -1, at least once during the first 30 minutes after dosing, periodically during the first 24 hours post-dosing, with special attention given during the first 4 hours, and every day thereafter through Day 15. Additional observations will be necessary if the animals continue to display signs of toxicity. The nature, onset, severity, reversibility and duration of clinical signs will be recorded.

During the acclimatization phase and throughout the study, animals will be checked twice daily for moribundity and mortality (once daily except on weekends and public holidays). Any animal suffering from severe distress, in a moribund condition or considered unlikely to survive will be humanely killed, and will be considered in the interpretation of the test results in the same way as animals that died on test.

10.1.2 Body weight

Body weights will be measured on study Days -6, -1, 1 (shortly before the test substance is administered), 8 and 15. Additionally, animals will be weighed when killed for humane reasons or when found dead.

10.2 POST MORTEM EXAMINATION

10.2.1 Necropsy procedures

Animals found dead:
Any animal found dead during the study will be necropsied as soon as possible but within 24 hours of the time of discovery. If necropsy cannot be performed immediately after a dead animal is discovered, the animal should be refrigerated (not frozen).

Scheduled sacrifice and moribund animals:
Animals surviving to the end of the study and animals sent to necropsy for humane reasons will be deeply anesthetized at the end of the study by Isoflurane inhalation, then exsanguinated before necropsy.

10.2.2 Necropsy

The necropsy of animals will include the macroscopic examination of the external surface, all orifices and all major body cavities and organs.

Significant macroscopic findings will be recorded. Tissues may be sampled at the discretion of the Study Director or the Sponsor Representative.

10.2.3 Microscopic evaluation

Microscopic examination of organs showing evidence of gross pathology in animals surviving at least 24 hours may be considered.

11 - CALCULATIONS

For body weights, means and standard deviations will be calculated when survival exceeds 1 day.
The LD50 will be estimated according to the O.E.C.D Test Guideline document (2).

12 - REPORTING

12.1 INTERIM REPORTS

Any unexpected findings during the course of the study will be reported to the Sponsor Representative.

12.2 FINAL REPORT

A copy of the draft report will be submitted to the Sponsor Representative and the Quality Assurance Unit for review. With the exception of the dated signature of scientists and other professional personnel, the draft report will contain all information and data to be included into the final report. The final report will include the information and data required by the referenced guidance documents (1, 2).

13 - ARCHIVING

All raw data, supporting documents, as well as protocol, protocol amendments and the final report will be maintained in the archive room. An aliquot of the control and test substance reference samples and will be maintained in the archived sample room.

All of the above will be saved for at least ten years in the designated areas at:

Bayer CropScience
355, rue Dostoïevski
BP 153
06903 Sophia Antipolis Cedex
France

14 - REFERENCES

1- U.S. E.P.A. (United States Environmental Protection Agency), 1998. Prevention, Pesticides and Toxic Substances (7101), Health Effects Test Guidelines OPPTS 870.1100, Acute Oral Toxicology, EPA 712-C-98-190, December 2002, 35 pages.

2- O.E.C.D. (Organization for Economic Co-operation and Development), 2001. O.E.C.D. Guideline for Testing of Chemicals, Test Guideline N°425: Acute Oral Toxicity – Up-and-Down Procedure. December 17, 2001, 26 pages.

PROTOCOL AMENDMENT

Protocol SA 06175

2mEPSPS
ACUTE TOXICITY BY ORAL GAVAGE IN MICE

Protocol amendment: N°1

Reason:

Due to a problem of solubility of the 2mEPSPS protein in the storage buffer, the 2mEPSPS protein will be liquidized by using an UltraTurax. The administration of the protein solutions (test and control) to mice will therefore be delayed by one day. The starting date of treatment will therefore be July 06, 2006 and the final sacrifice date will be July 20, 2006.
The formulation will be prepared on the previous day of dosing (July 05, 2006) and will be stored at +4°C overnight.

Study Director:

Date: July 05, 2006

D. ROUQUIE

PROTOCOL AMENDMENT

Protocol SA 06175

2mEPSPS
ACUTE TOXICITY BY ORAL GAVAGE IN MICE

Protocol amendment: N°2

Reason: The 2mEPSPS and bovine serum albumin proteins will be put in suspension in storage buffer instead of distilled water as stated in the protocol. The two proteins will be formulated at 50 mg/ml. To administer the dose of 2000 mg/kg of 2mEPSPS or bovine serum albumin proteins to the mice, two administrations each at 1000 mg/kg will be performed during the same day (July 06, 2006).

Study Director:

Date: July 06, 2006

D. ROUQUIE

PROTOCOL AMENDMENT

Protocol SA 06175

2mEPSPS PROTEIN
ACUTE TOXICITY BY ORAL GAVAGE IN MICE

Protocol amendment: N°3

Reason: Change in study title.

Due to an editing error, the study title should be 2mEPSPS protein: Acute Toxicity by Oral Gavage in Mice and not 2mEPSPS: Acute Toxicity by Oral Gavage in Mice.

Study Director:

Date: July 26, 2006

D. ROUQUIE

ATTACHMENT 2 - **CERTIFICATES OF ANALYSIS**

Title

Certificate of analysis for the 2mEPSPS protein produced in *E. coli*

Batch n°: LEJ5837

Author

Nadine Bautsoens
Koen Hendrickx, Ph.D.

Completed On
June 16, 2006

Testing Facility

Molecular & Biochemical Analytical Services
Expression and Protein Characterization
Bayer BioScience N.V.
Technologiepark 38
B-9052 Gent
Belgium

Study number

BBS06-003

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

This report is confidential. No part of the report or any information contained herein may be disclosed to any third party without the written prior authorisation of Bayer BioScience N.V.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The undersigned hereby declare that the work to which this report refers was performed according to the procedures herein described and this report provides an accurate record of the results obtained. The study was conducted in accordance with the Good Laboratory Practice Standards as specified in the OECD/EU principles of Good Laboratory Practice except for the following:

1. No validation of computerized systems attached to: multifunctional monochromator Tecan Safire², and autoclave was performed.

Study Director

Koen Hendrickx
Expression and Protein Characterization
Molecular and Biochemical Analytical Services

12/06/06
Date

STUDY IDENTIFICATION PAGE

Study start date:	April 3th, 2006
Experimental start date:	April 10th, 2006
Experimental Termination date:	May 5th, 2006
Study Completion date:	June 16, 2006
Test Facility Address:	Bayer BioScience N.V. Molecular & Biochemical Analytical Services GLP Test Facility Technologiepark 38 9052 Gent – Belgium Tel: +32 9-243 04 11 Fax: +32 9-224 06 94
Test Facility Manager:	Elizabeth Bates Address see Test Facility Tel: +32 9-243 04 25 Fax: +32 9-224 06 94 e-mail: elizabeth.bates@bayercropscience.com
Study Director:	Koen Hendrickx Address see Test Facility Tel: +32 9-243 04 40 Fax: +32 9-224 06 94 e-mail: koen.hendrickx@bayercropscience.com
Study Personnel:	Nadine Bautsoens Address see Test Facility Tel: +32 9-243 05 86 Fax: +32 9-224 06 94 e-mail: nadine.bautsoens@bayercropscience.com
Sponsor Representative:	Dominique Rouan Global Regulatory Affairs Manager Regulatory Affairs Address see Test Facility Tel: +32 9-243 04 21 Fax: +32 9-233 19 83 e-mail: dominique.rouan@bayercropscience.com
Study Director in Training:	Nadine Bautsoens Address see Test Facility Tel: +32 9-243 05 86 Fax: +32 9-224 06 94 e-mail: nadine.bautsoens@bayercropscience.com

QUALITY ASSURANCE STATEMENT

Report **BBS06-003** Date: 12 JUN 2006
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Quality Assurance (GLP)

Quality Assurance Statement

Title: **Certificate of analysis for the 2mEPSPS protein produced in *E. coli*.**

Study: BBS06-003

This study was periodically inspected and properly signed records of these inspections were submitted to testing facility management and the study director as shown below.
This report has been audited by the GLP Quality Assurance. The reported results accurately reflect the original data of the study.

Phase of Study	Inspection	Report
Study plan	06 APR 2006	06 APR 2006
Study plan amendment	07 APR 2006	07 APR 2006
Study conduct	10 APR 2006	10 APR 2006
	24 APR 2006	25 APR 2006
	04 MAY 2006	05 MAY 2006
Draft report	01 JUN 2006	02 JUN 2006
Final report	12 JUN 2006	12 JUN 2006

S. Berghman
GLP Quality Assurance

Berghman
12/06/06

APPROVALS PAGE

Study Director / Author	Koen Hendrickx	Date 16/06/06
Test facility management	Elizabeth Bates	Date 13/06/06
Sponsor representative	Dominique Rouan	Date 13 June 2006
Head of MBAS	Henk Joos	Date 16 June 2006 HJ RC 16 June 2006

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SUMMARY

This study was undertaken to confirm the identity of the bacterially produced 2mEPSPS to be used in subsequent studies and to determine the concentration and purity of the produced protein.

Five tests were performed in this study: (1) The concentration of the protein was determined by the Bradford technique. (2) The molecular weight of the protein was estimated by SDS-PAGE. (3) The same SDS gel was used to estimate the purity of the test item. (4) The immunological relationship with 2mEPSPS was tested by western blotting. (5) The activity of the protein was determined in an activity assay.

The identity of the 2mEPSPS protein was confirmed based on the observed molecular weight, cross-reactivity with the antibodies previously raised towards 2mEPSPS and the activity of the protein. The concentration of the protein was estimated to be 0.93 mg/ml and the purity was estimated to be 99.52%.

1.OBJECTIVE

In this study, the purified 2mEPSPS protein produced in *Escherichia coli* was analyzed for its concentration, identity and purity.

2. OVERVIEW OF EXPERIMENTAL DESIGN

Five analyses were performed on test item T09-01 produced in *E. coli*. (1) The concentration of the protein was determined with the Bradford method (1976). (2) The molecular weight of the protein was determined after sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). (3) The purity was estimated by qualitative comparison of the protein bands in the SDS gel. (4) The identity of the protein was confirmed by western blotting. (5) The activity of the protein was determined with the malachite green dye method (Lanzetta *et al.*, 1979) with minor modifications.

3. TEST ITEM AND REFERENCE ITEM

3.1 Test item

The test item was purified protein produced in *Escherichia coli*. The protein was produced at Bayer HealthCare AG (Wuppertal, Germany) according to the purification protocol described in Priestman *et al.* (2005). One aliquot of protein was received. Upon arrival at the Bayer BioScience N.V. test facility, the frozen aliquot was stored in the ultrafreezer (112UF – position 8-03) until the analysis took place.
Protein stored in the ultrafreezer is stable for at least 2 years.
To perform the analysis an aliquot was taken from the test item stock solution. This aliquot received the identification number PR003-01

Test Item ID:	T09-01
Test Item Identity:	2mEPSPS protein
Origin:	*Escherichia coli*
Batch n°:	LEJ5837
Expiry date:	April 3th, 2008
Buffer solution	100 mM Tris (pH 7.5), 2.7 mM KCl, 137 mM NaCl, 1 mM DTT

3.2 Reference items

No reference items were used in this study.

3.3 Standards

The Precision Plus Protein™ Standard Dual Color Marker was supplied by BioRad. The stock solution of the Precision Plus Protein™ Standard Dual Color Marker was stored in freezer 90FZ located in room L79. After opening the Precision Plus Protein™ Standard Dual Color Marker was stored in refrigerator 91RF in room L79.

4. ANALYSIS OF THE TEST ITEM T09-01

The concentration of the protein was estimated and the purity plus the identity of the protein was confirmed on the basis of five techniques. The concentration of the protein was determined according to the method of Bradford (1976). The molecular weight is a distinctive characteristic of a protein and was estimated to confirm the identity by performing a denaturing SDS-PAGE analysis. The same gel was used to estimate the purity of the produced protein. Western blotting is the fourth technique used to confirm the identity of the protein. This technique demonstrates the immunological relationship of the investigated protein to 2mEPSPS proteins by the reactivity of available antibodies directed towards 2mEPSPS. The activity of the purified protein was also assayed and indicates that the protein has the correct enzymatic nature.

4.1 Quantification of the test item T09-01

The Coomassie Plus™ kit (Pierce) is a ready-to-use Bradford assay for protein quantification. When the coomassie dye of the kit binds protein in an acidic medium, an immediate shift in absorption maximum occurs from 465 nm to 595 nm with a concomitant color change from brown to blue. To perform the assay in either test tube or microplate format: a small amount of protein sample is combined with the assay reagent, mixed well, incubated briefly and the absorbance at 595 nm is measured. Protein concentrations are estimated by reference to absorbances obtained from a series of standard protein dilutions, which are assayed alongside the unknown samples.

A dilution series of the test item protein was made to determine the concentration of the protein according to SOP BBS 07/60/00. The dilution series samples were mixed with the assay reagents of the Coomassie Plus™ kit. With the Magellan software, the concentration of the protein samples was estimated by fitting the measured absorbances to a standard curve of known dilutions of bovine serum albumin. A correlation coefficient of 0.99 was obtained for the standard curve. The average of the concentration and the standard deviation was calculated with the Microsoft Excel software (Table 1). The average protein concentration was estimated from the observed three data points. A large standard deviation was introduced by the dilution factor of the sample. A small error in the highest dilution gets multiplied by 400 and becomes much bigger. Therefore we consider the 100 fold dilution to be the most accurate. The 50 fold dilution would give us a smaller deviation but is situated in a less accurate part of the standard curve. The most accurate estimation is 0.93 mg/ml.

4.2 Molecular weight determination of the test item T09-01

SDS-PAGE is an analytical technique used to separate proteins through an acrylamide gel based on their relative molecular weight. The migration distance of a sample is directly proportional to the molecular size of the protein.

The test item protein was separated from side products/protein trace amounts with the SDS-PAGE technique according to SOP BBS 07/63/00 and the obtained gel (041106A, fig. 1) was stained according to SOP BBS 07/66/00.

The logarithm of the molecular weight of the standards was plotted versus the distance of migration with the Microsoft Excel software. A standard curve based on 6 data points was made covering the molecular weight range of the test item. The correlation coefficient of this standard curve was 0.99 (fig. 2). The molecular weight of the test item was extrapolated from the graph by using the equation $y = -0.684x + 3.7023$. The obtained x-value is 4.68. The molecular weight of the test item corresponds to the inverse logarithm of the x-value and is estimated to be 48 kDa. The molecular weight deduced from the amino acid sequence was 47 kDa. Since the error margin of the technique in the molecular weight region of the protein is calculated to be 3 kDa, the deduced molecular weight is confirmed within the limitation of the analysis.

4.3 Purity analysis of test item T09-01

The purity of the test item was estimated by quantification of the bands visible on the SDS gel and calculating the percentage of each band compared to the total density observed.

The purity of the test item was analyzed on the stained protein gel 041106A with the FluorS™ MultiImager according SOP BBS 04/40/00. A representative digitized image was made and an analysis report was prepared. Based on the relative density of each individual band, the purity of the test item was estimated to be 99.52%.

4.4 Electrotransfer of proteins to a membrane

SDS-PAGE was used to separate the proteins through an acrylamide gel in an electrical field based on molecular weight. Afterwards the separated proteins were transferred to a PVDF membrane to make them accessible for the detection with antibodies.

The test item protein was separated from side products/protein trace amounts with the SDS-PAGE technique according to SOP BBS 07/63/00 and the obtained PAGE gel (041106E) was further processed according to SOP BBS 07/64/00. Since the prestained molecular weight markers were visible on the membrane (041106F, fig. 3) we concluded that the transfer to the PVDF membrane was successful.

4.5 Western blotting

Rabbit antiserum anti 2mEPSPS
The rabbit anti 2mEPSPS was produced by and tested at Bayer Bioscience N.V. An aliquot of the antibody was stored in freezer 90FZ located in room L79 until used.

Goat antiserum raised against rabbit IgG coupled with Alkaline Phosphatase
The goat anti-rabbit antiserum coupled with alkaline phosphate was supplied by Sigma. The antibody was stored in the refrigerator 91RF in room L79 until used

The immobilization of proteins to a membrane (041106C) makes them accessible for the reaction with a specific antibody. The membrane (041106C), obtained by electrotransfer of the protein was treated according to SOP BBS 07/65/00.

Protein-free areas on the membrane were blocked overnight by incubation with StartingBlock™ (PBS) Blocking buffer (Pierce). After washing the membrane was incubated with a 1:10000 dilution of rabbit anti 2mEPSPS (Bayer Bioscience NV). After washing the reaction was incubated with a goat anti-rabbit antibody coupled to alkaline phosphatase (1:7000). Addition of substrate caused two bands to become visible. This result is in conflict with the SDS-PAGE data where only one band became visible after staining. The experiment was repeated and a membrane 041106F was generated. After staining only one major band became visible (fig. 3).

4.6 EPSPS activity assay

In the shikimate pathway, phosphoenolpyruvate and erythro-4-phosphate are converted to chorismate through seven enzymatic steps. EPSPS catalyzes the transfer of the enolpyruvyl moiety of PEP to the 5-OH hydroxyl group of shikimate 3-phosphate and releases inorganic phosphate. The EPSPS activity assay was measured according to a colorimetric method described by Forlani et al. (1994) and described in SOP BBS 07/74/00. The inorganic phosphate release is measured using the malachite green dye method (Lanzetta et al., 1979) with minor modifications described in SOP BBS 07/74/00.

Reaction mixtures were made according to SOP BBS 07/74/00 and placed at 37°C for 20 minutes. Reactions were made visible by a colorimetric solution and stopped with the addition of 34% sodiumcitrate after 60 seconds. Samples were transferred to a microtiterplate and the absorbance was read at 660 nm. The absorbance of the duplicates of the buffer control sample was 0.0537 and 0.0536. The absorbance of the duplicates of the test item sample was 0.1562 and 0.1597.

5. RESULTS AND DISCUSSION

The protein concentration of the test item T09-01 was estimated to be 0.93 mg/ml and the purity is estimated to be 99.52%. Based on the migration of the protein in the SDS-PAGE we confirmed the experimental molecular weight of the protein to be comparable to the deduced molecular weight from the amino acid composition, taking into account the error margin of the technique. As expected the test item reacts with an antibody directed towards 2mEPSPS. This reaction demonstrates the immunological relationship of the test item with 2mEPSPS. An activity assay confirmed the activity of the test item.

6. CONCLUSION

Biochemical analyses were performed to confirm the identity of test item T09-01. Based on the analyses we identified the T09-01 test item to be 2mEPSPS. The concentration of the protein in the test item T09-01 was estimated at 0.93 mg/ml with an estimate purity of 99.52%.

7. ARCHIVING

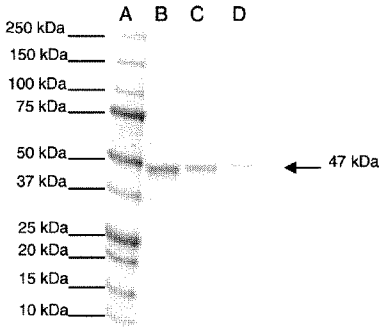
The study plan, the final report and the study data are archived under study number BBS06-003 in the GLP Test Facility Archives of Bayer BioScience N.V., Technologiepark 38, 9052 Gent, Belgium.

A sample of test item (T09-01) was archived in the archive ultrafreezer 113UF of the GLP Test Facility Archives of Bayer BioScience N.V., Technologiepark 38, 9052 Gent, Belgium (room number L57).

Table 1: Protein concentration of test item T09-01

Subsample	Dilution factor	Measured concentration µg/ml	Concentration of test item mg/ml
PR003-1A	400	4.54	1.82
PR003-1B	100	9.31	0.93
PR003-1C	50	14.74	0.74

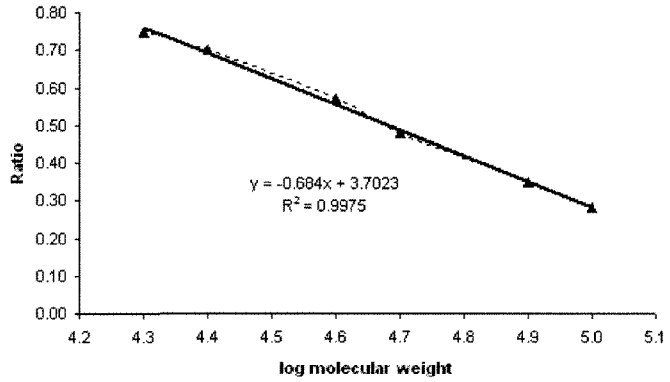
Figure 1: SDS-PAGE gel of test item T09-01(gel ID# 041106A)



Legend : A molecular weight marker
B 2 µg test item T09-01
C 1 µg test item T09-01
D 0.5 µg test item T09-01

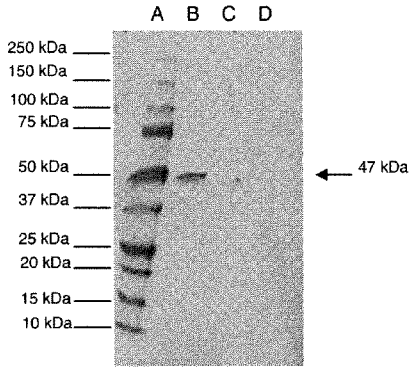
Figure 2: Plot standard curve molecular weight of test item T09-01

A	B	C	D	E
Molecular weight kDa	log Molecular weight	Migrated distance cm	Total distance cm	Ratio C/D
100	5.0	1.7	6.0	0.28
75	4.9	2.1	6.0	0.35
50	4.7	2.9	6.0	0.48
37	4.6	3.4	6.0	0.57
25	4.4	4.2	6.0	0.70
20	4.3	4.5	6.0	0.75



Legend : Plot of the logarithm of molecular weight of the standard proteins plotted against the ratio of the migrated distance and the total distance.

Figure3: Western blot of test item T09-01 (blot ID# 041106F)



Legend : A molecular weight marker
B 0.6 µg test item T09-01
C 0.3 µg test item T09-01
D 0.1 µg test item T09-01

REFERENCES

No	Doc No	Author(s), year, title, source, edition, pages
1.	M-268549-01-1	Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Analytical Biochemistry 72, 248-254
2.	M-268612-01-1	Lanzetta P.A., Alvarez L.J., Reinach P.S. and Candia O.A. (1979) An improved assay for nanomole amounts of inorganic phosphate. Analytical Biochemistry 100, 95-97
3.	M-269790-01-1	Priestman M.A., Funke T., Singh I.M., Crupper S.S. and Schonbrunn E. (2005) 5-Enolpyruvylshikimate-3-phosphate synthase from Staphylococcus aureus is sensitive to glyphosate. FEBS Letters 579, 728-732
4.	M-268551-01-1	Forlani G., Parisi B. and Nielsen E. (1994) 5-enol-Pyruvyl-Shikimate-3-Phosphate Synthase from Zea mays cultured cells. Plant Physiol. 105, 1107-1114

Study data index

Bayer BioScience N.V. Study number: BBS06-003

Study Title: Certificate of analysis for the 2mEPSPS protein produced in *E. coli*

Total number of study data pages: 71 pages

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Albumin from bovine serum, pH ~7 (1% in 0.15 M NaCl), ≥98% (agarose gel electrophoresis), lyophilized powder
Product Number	A7906
Product Brand	Sigma-Aldrich
CAS Number	9048-46-8
Storage Temp	2-8°C

TEST	SPECIFICATION	LOT 114K2387 RESULTS
APPEARANCE	WHITE TO LIGHT TAN POWDER	PASS
SOLUBILITY	CLEAR TO SLIGHTLY HAZY FAINT YELLOW TO YELLOW-GREEN SOLUTION AT 1GM IN 25ML IN WATER	PASS
LOSS ON DRYING	NMT 5%	0.8 %
NITROGEN	14.5 TO 16.5%	15.2 %
PH TEST	6.5 TO 7.5 (1% IN 0.15MSODIUM CHLORIDE)	7.0
IDENTITY	OF BOVINE ORIGIN	CONFORMS
AGAROSE ELECTROPHORESIS	NLT 98%	100 %
SHELF LIFE	5 YEARS	NOVEMBER 2009
QC ACCEPTANCE DATE		NOVEMBER 2004

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

ATTACHMENT 3 - **GLP COMPLIANCE CERTIFICATES (AND ENGLISH TRANSLATION)**

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

GROUPE INTERMINISTERIEL DES PRODUITS CHIMIQUES

Paris, le 13 DEC. 2005

OBJET : Evaluation de la conformité aux B.P.L. selon la directive 2004/10/CE du 11 février 2004.

Consécutivement à votre engagement vis à vis du GIPC et du COFRAC et en application du décret n° 81-278 du 25 mars 1981 portant création d'un Groupe Interministériel des Produits Chimiques (GIPC), modifié notamment par le décret 90-206 du 7 mars 1990 et par le décret n° 98-1312 du 31 décembre 1998 concernant les bonnes pratiques de laboratoires, je vous confirme que le GIPC, au vu des résultats du contrôle exercé par le Comité français d'accréditation (COFRAC) - Section Laboratoires a décidé pour votre installation du statut suivant :

Respect des principes de B.P.L.
Domaines de reconnaissance :

- ☐ **2 - études de toxicité**
- ☐ **3 - études de mutagénicité**
- ☐ **4 - études écotoxicologiques sur les organismes aquatiques et terrestres**
- ☐ **8 - méthodes de chimie analytique et clinique**
- ☐ **9 - autres études (métabolisme animal)**

Date d'inspection : 26 & 27 octobre 2005 ☐ **inspection de renouvellement (i.r)**

Date de décision du G.I.P.C. : 9 décembre 2005
Date de prise d'effet : 27 octobre 2005
Année de première conformité : 1992
Durée de validité : 18 mois

P. O.
Le Président,

Pierre CREYSSEL
Conseiller d'Etat h.

BAYER CROPSCIENCE
355 rue Dostoievski
BP 153
06903 Sophia Antipolis Cedex

Secrétariat général du GIPC - DGE- Simap - 12, rue Villiot - 75572 Paris cedex 12
Téléphone : 01 53 44 96 10 – Télécopie : 01 53 44 91 72

REPUBLIC OF FRANCE

INTERMINISTERIAL GROUP FOR CHEMICAL PRODUCTS

Paris, the 13 Dec. 2005

SUBJECT: Evaluation of G.L.P conformity to directive 2004/10/CE of 11 February 2004

Following your commitment vis-a-vis GIPC and COFRAC and by applying for decree n°. 81-278 of 25 March 1981, supporting the creation of an Interministerial Group for Chemical Products (GIPC), modified, among others, by decree 90-206 of 7th March, 1990 and by decree n° 98-1312 of 31 December 1998 concerning good laboratory practice, I confirm that the GIPC, in view of the results of the inspection carried out by the French Accreditation Committee (COFRAC) - Tests Section has decided on the following status for your test installation:

Observing the G.L.P principles

Areas of recognition

☐ **2 - toxicity studies**
☐ **3 - mutagenicity studies**
☐ **4 - ecotoxicology studies on aquatic and terrestrial organisms**
☐ **8 - analytical and clinical chemistry methods**
☐ **9 - other studies (animal metabolism)**

Inspection date: 26 & 27 October 2005 ☐ renewal inspection (i.r)

Date of GIPC decision: 9 December 2005
Date of taking effect: 27 October 2005
Year of first conformity: 1992
Validity duration: 18 months

The President
Pierre CREYSSEL
State Advisor

Bayer CropScience
355 rue Dostoievski
BP153
06903 SOPHIA ANTIPOLIS CEDEX

General Secretary of GIPC – DGE – Simap – 12, rue Villiot – 75572 Paris cedex 12
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