

**Report Title**

**Assessment of CP4 EPSPS Protein Level in Corn Tissues Collected from  
MON 87427 Produced in U.S. Field Trials During 2008**

This report reflects data generated and reported in study REG-09-083.

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**Study Completed On**

December 09, 2009

**Report Completed On**

December 09, 2009

**Sponsor and Performing Laboratory**

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**Report Number**

MSL0022370

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

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

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### Statement of Compliance

This document describes the assessment of CP4 EPSPS protein level in corn tissues collected from MON 87427 Produced in U.S. Field Trials during 2008 performed under Monsanto study REG-09-083. The Statement of Compliance from Monsanto study REG-09-083 is provided below.

This study meets the U.S. EPA Good Laboratory Practice requirements as specified in 40 CFR Part 160 with the following exception: Storage stability has not been established for CP4 EPSPS protein in corn silk tissue.

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Study Director

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### Quality Assurance Statement

Reviews conducted by the Quality Assurance Unit confirm that the final report for REG-09-083 accurately describes the methods and standard operating procedures followed and accurately reflect the raw data of the study.

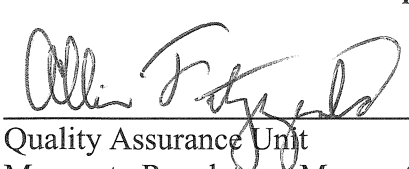
Following is a list of reviews conducted by the Monsanto Regulatory Quality Assurance Unit on the study REG-09-083.

Dates of Inspection /Audit	Phase	Date Reported to Study Director	Date Reported to Management
06/18/2009	ELISA	06/29/2009	06/26/2009
07/14/2009	Raw Data Audit	08/12/2009	08/12/2009
07/17/2009	Raw Data Audit	07/31/2009	07/31/2009
09/02/2009	Draft Report Review	09/03/2009	09/03/2009
09/18/2009	ELISA	09/22/2009	09/22/2009
12/9/2009	Draft Report Review	12/9/2009	12/9/2009

Additionally, the Quality Assurance Unit reviewed this report, MSL0022370, and confirmed that it accurately describes the portions of the final report for study REG-09-083.

Dates of Inspection /Audit	Phase	Date Reported to Study Director	Date Reported to Management
12/9/2009	Draft Report Review	12/9/2009	12/9/2009


  


 Quality Assurance Unit Monsanto Regulatory, Monsanto Company	<u>12/9/09</u> Date
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### Report Certification

This report is an accurate and complete representation of a portion of the work conducted in the study REG-09-083.

#### Signatures of Approval:

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Aster Beyene, Ph.D. Date  
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 12-09-2009  
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### **Report Information**

**Study Number:** REG-09-083

**Study Title:** Assessment of CP4 EPSPS, Cry1A.105, Cry2Ab2, and Cry3Bb1 Protein Levels in Corn Tissues Collected from MON 87427 and MON 87427 × MON 89034 × MON 88017 Produced in U.S. Field Trials During 2008

**Report Title:** Assessment of CP4 EPSPS Protein Level in Corn Tissues Collected from MON 87427 Produced in U.S. Field Trials During 2008

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**Study Initiation Date:** April 03, 2009

**Study Completion Date:** December 09, 2009

**Report Completion Date:** December 09, 2009

**Records Retention:** All study specific raw data (including rejected data), protocols, final reports, and facility records will be retained at Monsanto Company, St. Louis.

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## Table of Contents

Report Title .....	1
Statement of No Data Confidentiality Claim.....	2
Statement of Compliance.....	3
Quality Assurance Statement.....	4
Report Certification .....	5
Report Information.....	6
Table of Contents .....	7
Abbreviations and Definitions .....	9
1.0 Summary .....	10
2.0 Introduction.....	10
2.1 Background.....	10
2.2 Purpose.....	11
3.0 Materials .....	11
3.1 Test, Control, and Reference Substances.....	11
3.1.1 Test Substance .....	11
3.1.2 Control Substance .....	11
3.1.3 Reference Substance .....	11
3.1.4 Characterization of Test and Control Substances .....	11
3.1.5 Stability of Test and Control Substances.....	12
4.0 Methods.....	12
4.1 Generation of Plant Samples.....	12
4.1.1 Summary of Field Design .....	12
4.2 Tissue Processing and Protein Extraction Methods.....	12
4.2.1 Tissue Processing Method .....	12
4.2.2 Extraction Method .....	12
4.3 ELISA Reagents and Method .....	12
4.3.1 CP4 EPSPS Antibodies.....	12
4.3.2 CP4 EPSPS ELISA Method.....	13
4.3.3 CP4 EPSPS ELISA Validation .....	13
4.4 Control of Bias.....	13
4.5 Moisture Analysis .....	13
4.6 Data Analyses .....	14
4.7 Protocol Amendments.....	14
5.0 Results.....	15
5.1 CP4 EPSPS Protein Levels in Corn.....	15
6.0 Conclusions.....	16
7.0 Acknowledgments.....	16
8.0 References .....	16
Appendices.....	20

### **List of Tables**

Table 1. Summary of CP4 EPSPS Protein Levels in Leaf, Grain and Pollen Tissues from MON 87427 Grown in 2008 U.S. Field Trials .....	17
Table 2. Summary of CP4 EPSPS Protein Levels in Silk, Forage, Stover and Root Tissues from MON 87427 Grown in 2008 U.S. Field Trials.....	18
Table 3. Summary of CP4 EPSPS Protein Levels in Forage-Root, Senescent Root and Whole Plant Tissues from MON 87427 Grown in 2008 U.S. Field Trials.....	19

### **List of Appendices**

Appendix 1. Standard Operating Procedures.....	20
Appendix 2. Summary of the Validation Results for the CP4 EPSPS Protein ELISA in Corn Matrices.....	21



### Abbreviations<sup>1</sup> and Definitions

ANOVA	analysis of variance
BSA	bovine serum albumin
CP4 EPSPS	5-enolpyruvylshikimate-3-phosphate synthase derived from <i>Agrobacterium sp.</i> strain CP4
CV	coefficient of variation
DCA	deoxycholic acid
DWCF	dry weight conversion factor
dwt	dry weight of tissue
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
fw	fresh weight of tissue
HRP	horseradish peroxidase
IgG	immunoglobulin G
LOD	limit of detection
LOQ	limit of quantitation
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
PCR	polymerase chain reaction
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOP	standard operating procedure
TBA	Tris-borate buffer with L-ascorbic acid
TMB	3,3',5,5'-tetramethylbenzidine
Tris	tris(hydroxymethyl)aminomethane
VOI	verification of identity

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

## 1.0 Summary

Monsanto Company has developed MON 87427 for use in a Roundup® Hybridizing System (RHS) to facilitate the production of hybrid seed corn. MON 87427 has a glyphosate induced non-viable pollen phenotype. Appropriately timed glyphosate applications to MON 87427 will allow specific cross pollinations to be made in corn without using tradition methods to control self pollination. MON 87427 is tolerant to glyphosate during the vegetative stage and in female reproductive tissues. MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to limit expression of CP4 EPSPS in pollen microspore and tapetum cells. Therefore little to no CP4 EPSPS protein is expected to be produced in MON 87427 pollen, thus pollen from MON 87427 is susceptible to glyphosate.

This report determined the expression level of CP4 EPSPS protein in different corn tissues from MON 87427 produced in U.S. field trials during 2008. Tissues were produced in United States field trials during 2008. Tissue samples were collected from plants grown in the U.S. at five field sites in 2008 under Production Plan REG-08-069. In this report over-season leaf (OSL1-4), grain, pollen, silk, forage, stover, over-season root (OSR1-4), forage-root, senescent root and over-season whole plant (OSWP1-4) tissues were used for ELISA analysis. All protein levels for all tissue types were calculated on a microgram (µg) per gram (g) fresh weight (fwt) basis. Moisture content was then measured for all tissue types and all protein levels were converted and reported on a dry weight (dwt) basis.

ELISA results determined that the mean CP4 EPSPS protein level in MON 87427 across all sites were 4.2 µg/g dwt in grain, 100 µg/g dwt in silks, 120 µg/g dwt in forage, 43 µg/g dwt in stover, 72 µg/g dwt in forage-root, and 72 µg/g dwt in senescent root. In tissues harvested throughout the growing season, the mean CP4 EPSPS protein levels in MON 87427 across all sites ranged from 290 – 680 µg/g dwt in leaf, 73 – 140 µg/g dwt in root, and 240 – 500 µg/g dwt in whole plant. CP4 EPSPS protein levels in MON 87427 pollen across the sites were either < LOD µg/g dwt or were very low.

## 2.0 Introduction

### 2.1 Background

Monsanto Company has developed MON 87427 for use in a Roundup® Hybridizing System (RHS) to facilitate the production of hybrid seed corn. MON 87427 has a glyphosate induced non-viable pollen phenotype. Appropriately timed glyphosate applications to MON 87427 will allow specific cross pollinations to be made in corn without using tradition methods to control self pollination. MON 87427 is tolerant to glyphosate during the vegetative stage and in female reproductive tissues. MON 87427 utilizes a specific promoter and intron combination (*e35S-hsp70*) to limit expression of CP4 EPSPS in pollen microspore and tapetum cells. Therefore little to

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no CP4 EPSPS protein is expected to be produced in MON 87427 pollen, thus pollen from MON 87427 is susceptible to glyphosate.

CP4 EPSPS protein level was determined in corn plants produced in the U.S. at five field sites in 2008 under Production Plan REG-08-069. At each field site except one, corn was planted using a randomized complete block design consisting of three replicates.

## **2.2 Purpose**

The purpose of this report was to assess the level of CP4 EPSPS protein in different corn tissues from MON 87427 produced in U.S. field trials during 2008.

## **3.0 Materials**

### **3.1 Test, Control, and Reference Substances**

#### **3.1.1 Test Substance**

The test substances were MON 87427. Tissue samples were collected as outlined in Production Plan REG-08-069 from plants grown from starting seed lots 10001857.

#### **3.1.2 Control Substance**

The negative control substance was a non-transgenic, conventional corn with a similar genetic background to the test substances. Negative control tissue samples were collected as outlined in Production Plan REG-08-069 from plants grown from starting seed lot 10001859.

#### **3.1.3 Reference Substance**

An *E. coli*-produced protein standard were used in this study and a copy of the certificate of analysis was archived with the study data.

A CP4 EPSPS protein standard (lot 20-100015) was used as the reference substance for analysis of CP4 EPSPS protein levels. The purity was 97% as determined by SDS-PAGE and densitometric analysis. The purity-corrected protein concentration of the standard was 3.7 mg/ml as determined by amino acid composition analysis.

#### **3.1.4 Characterization of Test and Control Substances**

The identities of the test and control substances were confirmed by verifying the chain-of-custody documentation prior to analysis. To further confirm the identities of the test and control substances, event-specific polymerase chain reaction (PCR) analyses were conducted on the harvested grain from each site. The PCR analyses were archived by the Sponsor under the starting seed lot numbers described in sections 3.1.1 and 3.1.2. Any test or control substance, and its associated tissues that had three or more pools test unexpectedly positive or negative during the PCR verification were not used in this report.

### **3.1.5 Stability of Test and Control Substances**

All of the test and control substances were extracted and analyzed by ELISA within the time frame of verified tissue stability for the CP4 EPSPS protein except stability has not been evaluated for CP4 EPSPS in corn silk tissue.

## **4.0 Methods**

### **4.1 Generation of Plant Samples**

#### **4.1.1 Summary of Field Design**

Production Plan REG-08-069 was initiated during the 2008 planting season to generate test and control substances at various corn-growing locations in the U.S. The field sites were as follows: Jackson County, Arkansas (site code ARNE); Jefferson County, Iowa (site code IARL); Stark County, Illinois (site code ILWY); Parke County, Indiana (site code INRC); and York County Nebraska (site code NEYO). These field sites were representative of corn producing regions suitable for commercial production. At the ARNE, IARL and ILWY sites, three replicated plots of plants containing MON 87427 as well as the conventional control were planted using a randomized complete block field design. The NEYO site contained 3 replicated plots, but was not a randomized complete block field design. Tissues were collected from each replicated plot at all field sites. A detailed description of the field design and sample collection can be found in Production Report MSL0022386 (Rowland, 2009). However, at the ARNE site OSL1, OSR1 and OSWP1 which were to be collected at V2-V4, were actually collected at V5. This has no impact on the study.

### **4.2 Tissue Processing and Protein Extraction Methods**

#### **4.2.1 Tissue Processing Method**

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

#### **4.2.2 Extraction Method**

The CP4 EPSPS protein was extracted from corn tissues as described in Monsanto Standard Operating Procedure (SOP) BR-ME-0197-06. Extraction parameters for CP4 EPSPS in each tissue type are described in Appendices 2. The extracts were aliquoted and stored in a -80°C freezer until analysis.

### **4.3 ELISA Reagents and Method**

#### **4.3.1 CP4 EPSPS Antibodies**

Mouse monoclonal antibody clone 39B6.1 (IgG2a isotype, kappa light chain; lot 7022111) specific for the CP4 EPSPS protein was purified from mouse ascites

fluid using Protein-A Sepharose affinity chromatography and was used as the capture antibody in the CP4 EPSPS ELISA. The concentration of the purified IgG was determined to be 2.3 mg/ml by spectrophotometric methods. Production of the 39B6.1 monoclonal antibody was performed by Strategic Biosolutions (Newark, DE). The purified antibody was stored in a buffer (pH 7.2) containing 20mM sodium phosphate, 150 mM sodium chloride, and 15 ppm Proclin 300 (Sigma-Aldrich, St. Louis, MO).

The detection reagent was goat anti-CP4 EPSPS antibody, otherwise known as anti-protein 4 (Sigma-Aldrich, catalog number P-5867) conjugated to HRP.

#### **4.3.2 CP4 EPSPS ELISA Method**

The CP4 EPSPS ELISA was performed manually according to SOP BR-ME-0197-06. Mouse anti-CP4 EPSPS antibody was diluted in coating buffer (15 mM Na<sub>2</sub>CO<sub>3</sub>, 35 mM NaHCO<sub>3</sub>, and 150 mM NaCl, pH 9.6) to a final concentration of 2.0 µg/ml, and immobilized onto 96-well microtiter plates followed by incubation in a 4°C refrigerator for ≥8 h. Prior to each step in the assay, plates were washed with 1X PBST. CP4 EPSPS protein standard or sample extract was added at 100 µl per well and incubated for 1 h at 37°C. The captured CP4 EPSPS protein was detected by the addition of 100 µl per well of anti-CP4 EPSPS peroxidase conjugate. Plates were developed by adding 100 µl per well of TMB. The enzymatic reaction was terminated by the addition of 100 µl per well of 6 M H<sub>3</sub>PO<sub>4</sub>. Quantification of the CP4 EPSPS protein was accomplished by interpolation from a CP4 EPSPS protein standard curve that ranged from 0.456 – 14.6 ng/ml.

#### **4.3.3 CP4 EPSPS ELISA Validation**

Appendices 2 summarize the result of validation of the ELISA used to assess the CP4 EPSPS protein level in corn tissues.

### **4.4 Control of Bias**

At each site three replicated plots of the test and control substances were planted. The substances were randomly assigned, except for the NEYO site, to the plots to prevent any experimental bias. Representative tissues from each plot were collected as described in the production plan. All tissues, except pollen were processed by thoroughly grinding to produce a homogeneous sample before extraction to minimize sampling bias. The ELISA method used was optimized to minimize method bias. Protein extracts from the test and control substances were analyzed by ELISA with the appropriate protein standards and inter-assay negative and positive controls.

### **4.5 Moisture Analysis**

Tissues were analyzed for moisture content using an IR-200 Moisture Analyzer (Denver Instrument Company, Arvada, CO) according to SOP BR-ME-1238-01. A homogeneous tissue-specific site pool (TSSP) was prepared consisting of at least

three test and control samples of a given tissue type grown at a given site. These pools were prepared for all tissue types in this study. The average percent moisture for each TSSP was calculated from triplicate analyses. A TSSP Dry Weight Conversion Factor (DWCF) was calculated as follows:

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

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

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

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

#### **4.6 Data Analyses**

All CP4 EPSPS ELISA plates were analyzed on a SPECTRAmax Plus 384 or a SPECTRAmax Plus Molecular Devices, Sunnyvale, CA) microplate spectrophotometer, using a dual wavelength detection method. All protein concentrations were determined by optical absorbance at a wavelength of 450 nm with a simultaneous reference reading of 620-655 nm. Data reduction analyses were performed using Molecular Devices SOFTmax PRO GXP version 5.0.1. Absorbance readings and protein standard concentrations were fitted with a four-parameter logistic curve fit. Following the interpolation from the standard curve, the amount of protein (ng/ml) in the tissue was reported on a “µg/g fwt” basis for data that were greater than or equal to the LOQ. This conversion utilized a sample dilution factor and a tissue-to-buffer ratio. The protein values in “µg/g fwt” were also converted to “µg/g dwt” by applying the DWCF. Microsoft Excel 2007 (Version (12.0.6504.5001) SP1 MSO (12.0.6320.5000) Microsoft, Redmond, WA) was used to calculate the CP4 EPSPS protein levels in corn tissues. The sample means, standard deviations, and ranges were also calculated by Microsoft Excel 2007.

Any test or control substance extract that resulted in unexpectedly negative or positive results by ELISA analysis were re-extracted twice for the protein of interest and re-analyzed by ELISA to confirm the results. Samples with confirmed unexpected results were omitted from all calculations. Samples that were not confirmed to be either positive or negative were reported as inconclusive and omitted from all calculations.

#### **4.7 Protocol Amendments**

The protocol was amended to drop the analysis of forage-root samples from Arkansas site. The forage-root samples from the Arkansas site were not cleaned properly; therefore all samples were omitted. The impact of this change was no data of forage-

root was collected for Arkansas site in the study; however, there were sufficient sites for representative data.

The protocol was also amended to state that the tissue-specific site pools (TSSPs) would consist of at least three test and control substances instead of four due to unexpected TCR results in the INRC site. There was no impact on data and results, as sufficient representative tissues were added to generate TSSPs.

In addition, the protocol was amended to include the method that was used to determine CP4 EPSPS protein level in silk. There was a positive impact on the study as the data supporting the validation was generated and method to be used in the study was listed in the protocol.

## **5.0 Results**

The across-site mean, standard deviation (SD), and range are reported for CP4 EPSPS protein levels on a µg/g fwt and µg/g dwt basis in corn tissues collected from five U.S. field sites in 2008 in Tables 1-3.

### **5.1 CP4 EPSPS Protein Levels in Corn**

The CP4 EPSPS protein levels for MON 87427 are presented in Table 1–3.

ELISA results showed that the mean CP4 EPSPS protein levels in MON 87427 across all sites were 4.2 µg/g dwt in grain, 100 µg/g dwt in silks, 120 µg/g dwt in forage, 43 µg/g dwt in stover, 72 µg/g dwt in forage-root, and 72 µg/g dwt in senescent root. In tissues harvested throughout the growing season, the mean CP4 EPSPS protein levels in MON 87427 across all sites ranged from 290 – 680 µg/g dwt in leaf, 73 – 140 µg/g dwt in root, and 240 – 500 µg/g dwt in whole plant.

As stated in section 2.1, MON 87427 has a glyphosate inducible non-viable pollen phenotype, and little to no CP4 EPSPS is expected to be produced in pollen from MON 87427. The CP4 EPSPS expression data from MON 87427 pollen reported in this study is consistent with the MON 87427 product concept.

Six pollen samples across three of the sites had CP4 EPSPS protein levels in pollen that were < LOD µg/g dwt in pollen.

Six pollen samples from four of the sites had CP4 EPSPS expression levels > LOQ. The mean CP4 EPSPS protein level from these pollen samples was 0.87 µg/g dwt. The small amount of CP4 EPSPS protein found in these pollen samples could be due to the presence of anther tissue mistakenly collected with the pollen from MON 87427 (previous research with MON 87427 has demonstrated that this product produces CP4 EPSPS protein in anthers (Monsanto proprietary data). Alternatively, a low amount of CP4 EPSPS in MON 87427 may be inherent to this product due to the use of the *e35S* promoter (CaJacob et al, 2004).

Two MON 87427 pollen samples were reported as inconclusive because when each were re-extracted twice to confirm the positive result, one extraction gave a > LOQ

result while the other was < LOQ. Results such as this typically indicate that the sample is non-homogeneous. Therefore, this result further supports that there may have been some presence of anther tissue in these pollen samples, resulting in a non-homogeneous sample.

The levels of CP4 EPSPS in tissue samples from the conventional corn control were below the CP4 EPSPS LOQ or LOD for each tissue type with the exception seven samples. One silk conventional control sample was inconclusive because when the sample was re-extracted twice to confirm the positive result, one extraction gave a > LOQ result while the other was < LOQ.

## **6.0 Conclusions**

MON 87247 was grown in the U.S. at five field sites during the 2008 growing season. Tissue samples were collected at various growth stages throughout the growing season and analyzed for CP4 EPSPS protein levels in MON 87247 using validated ELISA method. These data provide an estimation of the CP4 EPSPS protein levels on a fresh weight and dry weight basis in 19 tissue types of MON 87247 collected at different growth stages in the U.S. during the 2008 growing season.

## **7.0 Acknowledgments**

The authors would like to acknowledge Michelle Starke, and the Quality Assurance Unit for their critical reviews of this report. We also thank Andre Van Oyen, Jr. and the Agronomy & Sample Processing Team for processing and sample distributions.

## **8.0 References**

Rowland, M. 2009. Amended Report for MSL0021755: Field Production of Corn Tissues from MON 87427, MON 87441, MON 88017, MON 89034, NK603, Conventional Crosses MON 87427 × NK603, MON 87441 × NK603, MON 87427 × MON 88017 × MON 89034, MON 87441 × MON 88017 × MON 89034, and a Conventional Control Grown in the U.S. during 2008. Monsanto Technical Report, St. Louis, MO. MSL0022386.

CaJacob, C.A., P.C.C. Feng, G.R. Heck, M.F. Alibhai, R.D. Sammons, and S.R. Padgett. 2004. Engineering Resistance To Herbicides. *In Handbook of Plant Biotechnology.* (eds) P. Christon and H. Klee. John Wiley & Sons, pages 365-367.



**Table 1. Summary of CP4 EPSPS Protein Levels in Leaf, Grain and Pollen Tissues from MON 87427 Grown in 2008 U.S. Field Trials**

Tissue Type <sup>1</sup>	MON 87427	
	Mean (SD) <sup>4</sup> Range <sup>5</sup> (µg/g fwt) <sup>2</sup>	Mean (SD) Range (µg/g dwt) <sup>3</sup>
OSL1	100 (21) 75 – 140	680 (170) 400 – 940
OSL2	83 (25) 30 – 110	410 (130) 130 – 560
OSL3	61 (19) 35 – 95	290 (74) 210 – 410
OSL4	95 (30) 17 – 140	370 (120) 70 – 520
Grain	3.6 (0.73) 2.6 – 5.3	4.2 (0.89) 2.8 – 6.2
Pollen <sup>6</sup>	< LOD (NA) NA	< LOD (NA) NA
	0.49 (0.36) 0.18 – 1.1	0.87 (0.70) 0.25 – 2.2

- Tissues were collected at the following growth stages:
  - OSL1: V2 – V5
  - OSL2: V6 – V8
  - OSL3: V10 – V12
  - OSL4: VT
  - Grain: R6
  - Pollen: During pollination
- Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
- Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a dry weight (dwt) basis. The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factors obtained from moisture analysis data.
- The means and standard deviations were calculated for each tissue type across all sites (n=14). The “n” values for the calculated mean and standard deviations represent the number of samples figured into the calculation.
- Minimum and maximum values were determined each tissue type across all sites.
- CP4 EPSPS protein levels in MON 87427 pollen across the sites were either < LOD µg/g dwt (n=6), or had a very low level of CP4 EPSPS (n=6).

**Table 2. Summary of CP4 EPSPS Protein Levels in Silk, Forage, Stover and Root Tissues from MON 87427 Grown in 2008 U.S. Field Trials**

Tissue Type <sup>1</sup>	MON 87427	
	Mean (SD) <sup>4</sup> Range <sup>5</sup> (µg/g fwt) <sup>2</sup>	Mean (SD) Range (µg/g dwt) <sup>3</sup>
Silk	9.4 (0.97) 8.1 – 11	100 (12) 90 – 120
Forage	38 (14) 8.3 – 57	120 (48) 21 – 200
Stover	14 (6.3) 5.9 – 26	43 (27) 13 – 98
OSR1	18 (5.3) 8.1 – 27	140 (46) 58 – 210
OSR2	16 (6.8) 8.3 – 29	110 (62) 48 – 240
OSR3	12 (4.3) 4.9 – 19	73 (28) 22 – 110
OSR4	15 (5.7) 5.6 – 23	83 (36) 23 – 140

- Tissue were collected at the following growth stages:
  - Silk: During pollination
  - Forage: R5, early dent stage
  - Stover: R6
  - OSR1: V2 – V5
  - OSR2: V6 – V8
  - OSR3: V10 – V12
  - OSR4: VT
- Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
- Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a dry weight (dwt) basis. The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factors obtained from moisture analysis data.
- The means and standard deviations were calculated for each tissue type across all sites (n=14). The “n” values for the calculated mean and standard deviations represent the number of samples figured into the calculation.
- Minimum and maximum values were determined each tissue type across all sites.

**Table 3. Summary of CP4 EPSPS Protein Levels in Forage-Root, Senescent Root and Whole Plant Tissues from MON 87427 Grown in 2008 U.S. Field Trials**

Tissue Type <sup>1</sup>	MON 87427	
	Mean (SD) <sup>4</sup> Range <sup>5</sup> (µg/g fwt) <sup>2</sup>	Mean (SD) Range (µg/g dwt) <sup>3</sup>
Forage-Root	15 (5.2) 8.6 – 24	72 (23) 39 – 100
Senescent Root	16 (8.3) 5.9 – 29	72 (37) 26 – 130
OSWP1	50 (8.3) 37 – 66	500 (190) 310 – 840
OSWP2	46 (7.6) 33 – 58	360 (42) 300 – 420
OSWP3	43 (7.1) 28 – 56	380 (78) 230 – 500
OSWP4	37 (6.3) 23 – 47	240 (42) 160 – 340

- Tissues were collected at the following growth stages:
  - Forage-root: R5, early dent
  - Senescent root: R6
  - OSWP1: V2 – V5
  - OSWP2: V6 – V8
  - OSWP3: V10 – V12
  - OSWP4: VT
- Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a fresh weight (fwt) basis.
- Protein levels are expressed as microgram (µg) of protein per gram (g) of tissue on a dry weight (dwt) basis. The dry weight values were calculated by dividing the µg/g fwt by the dry weight conversion factors obtained from moisture analysis data.
- The means and standard deviations were calculated for each tissue type except forage-root across all sites (n=14). For forage-root n=11. The “n” values for the calculated mean and standard deviations represent the number of samples figured into the calculation.
- Minimum and maximum values were determined each tissue type across all sites.

## **Appendices**

### **Appendix 1. Standard Operating Procedures**

BR-ME-0197-06	Extraction and Direct ELISA Analysis of CP4 EPSPS in Corn Tissues
BR-ME-1238-01	Analysis of Moisture Content Using the Denver Instrument IR-200 Moisture Analyzer

## Appendix 2. Summary of the Validation Results for the CP4 EPSPS Protein ELISA in Corn Matrices

### 1.0 Accuracy

#### 1.1. Extraction Efficiency and Spike and Recovery

Extraction Efficiency (Leaf, Pollen, Root, Forage) acceptance criteria = 70 – 100%.

Extraction Efficiency (Grain) acceptance criteria = 60 – 100%.

Spike and Recovery acceptance criteria = 70 – 130%.

Sample Type	Tissue-To-Buffer Ratio	Extraction Efficiency <sup>1</sup>	Spike and Recovery <sup>2</sup>
Leaf	1:100	92 %	86 %
Grain	1:100	78 %	105 %
Pollen	1:100	98 %	80 %
Silk	1:100	90.5 %	80 %
Root	1:50	83 %	80 %
Forage	1:100	85 %	76 %

1. Extraction efficiency for each tissue type was determined by successive extraction of three replicates, where the last extraction employed a harsh buffer (e.g., 1X Laemmli buffer).

2. To evaluate the analytical accuracy of the ELISA, extracts prepared from each tissue type of conventional corn plants were spiked with known quantities of CP4 EPSPS protein at three concentrations spanning the range of the standard curve.

#### 1.2. Matrix Effects

No matrix interferences (non-specific binding) were noted when sample extracts were analyzed at matrix dilutions stated below.

Matrix Effects acceptance criteria = 70 – 130%.

Sample Type	Matrix Dilutions	% Recovery
Leaf	1:3	94 %
Grain	1:5	116 %
Pollen	1:3	94 %
Silk	1:3	95 %
Root	1:3	94 %
Forage	1:3	97 %

### 1.3. Parallelism

Parallelism is defined as the plant-produced CP4 EPSPS protein being immunologically equivalent to the *E. coli*-produced CP4 EPSPS protein standard. Parallelism acceptance criteria = 70 – 130%.

Sample Type	Average % Recovery
Leaf	95 %
Grain	90 %
Pollen	95 %
Silk	100 %
Root	90 %
Forage	95 %

### 2.0 Precision<sup>3</sup>

Range of Quantitation: 0.456 – 14.6 ng/ml

#### Leaf, Pollen, Root and Forage:

Standard Curve Precision Range (CV): 4.1 – 10.0 %  
Intra-Assay Precision (CV) 8.6%  
Inter-Assay Precision (CV) 14.6 %

#### Grain:

Standard Curve Precision Range (CV): 7.0 – 14.7 %  
Intra-Assay Precision (CV) 9.7%  
Inter-Assay Precision (CV) 16.9 %

3. The inter- and intra-assay precision were assessed by determining the CV of the concentration of CP4 EPSPS protein measured for the positive control sample from 24 independent ELISAs using one-way analysis of variance (ANOVA).

### 3.0 Sensitivity

#### 3.1. Limits of Detection

Sample Type	Dilution	LOD (ng/mL)	LOD <sup>4</sup> (µg/g fwt.)
Leaf	1:3	0.23	0.069
Grain	1:5	0.31	0.16
Pollen	1:3	0.33	0.099
Silk	1:3	0.40	0.121
Root	1:3	0.22	0.033
Forage	1:3	0.23	0.069

4. The limit of detection (LOD) was calculated as the mean value plus three SD using the data generated with conventional sample extracts for each tissue type. The LOD value in “ng/mL” value was converted to “µg/g fwt.” using the respective dilution factor and tissue-to-buffer ratio.

### 3.2. Limits of Quantitation

Sample Type	Dilution	LOQ (ng/mL)	LOQ <sup>5</sup> (µg/g fwt.)
Leaf	1:3	0.456	0.137
Grain	1:5	0.456	0.228
Pollen	1:3	0.456	0.137
Silk	1:3	0.456	0.137
Root	1:3	0.456	0.068
Forage	1:3	0.456	0.137

5. The limit of quantitation (LOQ) was calculated based on the lowest standard concentration. The “ng/mL” value was converted to “µg/g fwt.” using the respective dilution factor and tissue-to-buffer ratio.

### 4.0 Extraction Parameters<sup>6</sup>

Sample Type	Tissue-to-Buffer Ratio	Extraction Buffer
Leaf	1:100	1X PBST with 0.1% (w/v) BSA
Grain	1:100	1X TBA with 10 mM DCA
Pollen	1:100	1X PBST with 0.1% (w/v) BSA
Silk	1:100	1X PBST with 0.1% (w/v) BSA
Root	1:50	1X PBST with 0.1% (w/v) BSA
Forage	1:100	1X PBST with 0.1% (w/v) BSA

6. The CP4 EPSPS protein was extracted from each tissue by adding the appropriate volume of CP4 EPSPS Extraction Buffer, and shaking in a Harbil mixer. The extracted sample was clarified using a serum filter.