

SUMMARY

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STUDY TITLE

Method Validation for the Determination of 5-Enol-Pyruvylshikimate-3-Phosphate Synthase
(2mEPSPS) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)

DATA REQUIREMENTS

N/A

AUTHOR(S)

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STUDY COMPLETED ON

22-Jun-2011

PERFORMING LABORATORY

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LABORATORY STUDY ID

101768

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The objective of this study was to validate Dow AgroSciences Method 101768, a sequential sandwich ELISA for the detection of 2mEPSPS protein extracted from transgenic soybean tissues. The validation study includes the evaluation of sensitivity and quantitative range, assay specificity, matrix effect, extraction efficiency, accuracy, equivalency, precision and ruggedness, and false positive/false negative rates. Soybean tissues included in the validation were as follows: grain, forage R3, leaf V5, leaf V10-12, and root R3. All phases of this study were conducted to meet the standards of Good Laboratory Practices (GLP). The method will be used to analyze soybean tissue samples for 2mEPSPS protein and support product registrations.

The results from this study are summarized below:

- The sensitivity of the method (limit of detection, LOD) was 4 ng/mg dry weight (DW) for all tissues. The lower limit of quantitation (LOQ) of the method was 8 ng/mg DW for all tissues. The validated standard curve range was from 4 ng/mL to 200 ng/mL in assay buffer.
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10,000 ng/mL were assessed for cross-reactivity. No significant cross-reactivity was observed for the other proteins tested.

- Matrix effects were evaluated by comparing standard curves that had not been fortified with matrix with those that had been fortified. Three different dilutions- 1X, 5X, and 10X- were tested for each matrix, which represent dilution levels commonly used in ELISA. Matrix effects were observed for grain, leaf V5, forage R3, and root R3 tissues at the 1X level. No matrix effects were observed at the 5X and 10X levels for all tissues. However, dilutions of 10X or greater are recommended for all tissues based on individual data points obtained during the study.
- The efficiency of the tissue extraction process was determined by comparison of five sequential extractions. The apparent extraction efficiency was based on the amount of 2mEPSPS protein in the first extract relative to the total 2mEPSPS in all five extracts. The mean extraction efficiency for soybean tissues ranged from 84.1% to 94.1%.
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- Equivalency of standard and test substance response in the 2mEPSPS ELISA was evaluated using serial dilutions of extracts of forage R3, leaf V10-12, leaf V5, grain, and root R3 positive soybean tissues. For each tissue extract, three to five of the seven dilutions fell within the quantitative range of the standard curve and the % CV of the quantified results were less than 5%.
- Precision and ruggedness of the ELISA method were demonstrated using fortified control forage R3 and root R3 tissues. Ruggedness results were compared within and across days. Data were interpolated from standard curves prepared by two analysts on at least two separate days. The intra-day variation (%CV) ranged from 2.36% to 8.67%, intra-

analyst variation ranged from 1.55% to 10.31%, and inter-assay variation across days and analysts ranged from 3.79% to 7.39%.

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Based on the results of this study, it is concluded that the 2mEPSPS ELISA method is suitable for quantitative measurement of the 2mEPSPS protein in soybean tissue.

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Compound: 5-Enol-Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS)

Title: Method Validation for the Determination of 5-Enol-Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)

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Company: Dow AgroSciences LLC

Company Agent: M. Krieger

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Signature: 

Date: 9 June 2011

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STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

Title: Method Validation for the Determination of 5-Enol-Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)







Study Initiation Date: 7-Dec-2010

This report represents data generated after the effective date of the EPA FIFRA Good Laboratory Practice Standards.

United States Environmental Protection Agency
Title 40 Code of Federal Regulations Part 160
FEDERAL REGISTER, August 17, 1989

Organisation for Economic Co-Operation and Development
ENV/MC/CHEM(98)17, Paris January 26, 1998

All aspects of this study were conducted in accordance with the requirements for Good Laboratory Practice Standards, 40 CFR 160.

 _____ M. Krieger Sponsor Dow AgroSciences LLC	 _____ 9 June 2011 Date
 _____ M. Krieger Submitter Dow AgroSciences LLC	 _____ 9 June 2011 Date
 _____ P.M. Maldonado Study Director/Author Dow AgroSciences LLC	 _____ 22-Jun-2011 Study Completion Date

Dow AgroSciences Quality Assurance Unit
Good Laboratory Practice Statement Page

Study ID: 101768

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GLP Quality Assurance Inspections

Date of GLP Inspection(s)	Date Reported to the Study Director and to Management	Phases of the Study which received a GLP Inspection by the Quality Assurance Unit
7-Dec-2010	7-Dec-2010	Protocol
15-Dec-2010	16-Dec-2010	Sample Analysis
13, 14, 16, 120-Jun-2011	22-Jun-2011	Final Report and Raw Data Review

QUALITY ASSURANCE STATEMENT:

The Quality Assurance Unit has reviewed the final study report and has determined that the report reflects the raw data generated during the conduct of this study.


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Based on the results of this study, it is concluded that the 2mEPSPS ELISA method is suitable for quantitative measurement of the 2mEPSPS protein in soybean tissue.

SCIENTIFIC TERMS AND ABBREVIATIONS

°C	degrees Celsius
µg	microgram (10^{-6} g)
µL	microliter (10^{-6} L)
2mEPSPS	5-Enol-Pyruvylshikimate-3-Phosphate Synthase
CV	coefficient of variation
DAS	Dow AgroSciences LLC
DW	dry weight
EE	extraction efficiency
ELISA	enzyme linked immunosorbent assay
g	gram
GLP	Good Laboratory Practices
LOD	limit of detection
LOQ	limit of quantitation
LLOQ	lower limit of quantitation
mg	milligram (10^{-3} g)
mL	milliliter (10^{-3} L)
M	molar
mM	millimolar
ng	nanogram (10^{-9} g)
OD	optical density
PBST	phosphate buffered saline with 0.05% Tween 20
QC	quality control
RSD	relative standard deviation (equivalent to CV)
SGN	sample group number
STD	standard
STDEV	standard deviation (or SD)
TSN	test substance number

INTRODUCTION

Soybean plants have been modified by the insertion of a gene encoding 5-Enol-Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS), which confers tolerance to the herbicide glyphosate. An enzyme-linked immunosorbent assay (ELISA) has been developed for the determination of 2mEPSPS protein expressed in soybean tissues.

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MATERIALS AND METHODS

Materials

Test Substances

The test substance in this study was transgenic 2mEPSPS protein. The presence or absence of transgenic 2mEPSPS protein was tested in 2mEPSPS transgenic soybean tissues and in control non-transgenic soybean tissue.

Reference Substances

The reference substance in this study was purified microbial 2mEPSPS protein TSN 033171-0001¹. Information regarding the source and identity of this 2mEPSPS protein as a test and reference substance were documented in the study records.

Name: 5-enol-pyruvylshikimate-3-phosphate synthase (2mEPSPS)
TSN number: 033171-0001
Concentration: 0.665 mg/mL
Reference: BIOT 10-255698

Additional reference substances were used to assess the detection of non-target proteins during Specificity testing. Information regarding these reference substances is included in the Study File.

Test System

The test system for this study was soybean tissues. The transgenic soybean tissues were genetically modified to express 2mEPSPS protein. The tissues, as listed below, were collected from plants grown from seeds in the greenhouse. Detailed information for seeds and sample collection were documented in the study file. Each sample was identified with a unique sample number (sample group number, SGN). These numbers were used to track the samples during collection, preparation, storage and analysis. The non-2mEPSPS soybean tissue was collected from plants grown in the greenhouse and used as the control tissue. Both 2mEPSPS transgenic and control tissues were lyophilized and ground under frozen conditions.

SGN	Tissue	Sample Description
101768-001-0001	Leaf V5	Control
101768-001-0002	Leaf V5	2mEPSPS Positive
101768-002-0001	Leaf V10-12	Control
101768-002-0002	Leaf V10-12	2mEPSPS Positive
101768-003-0001	Forage R3	Control
101768-003-0002	Forage R3	2mEPSPS Positive
101768-004-0001	Root R3	Control
101768-004-0002	Root R3	2mEPSPS Positive
101768-005-0001	Grain	Control
101768-005-0002	Grain	2mEPSPS Positive

Methods

Overview

A specific sandwich ELISA Kit (Catalog Number ABS-091) manufactured by Acadia BioScience, LLC (Portland, ME) was used to quantify levels of 2mEPSPS protein in genetically modified soybean tissues. An anti-2mEPSPS polyclonal antibody was immobilized onto a polystyrene surface of a microtiter plate. Samples were extracted with an appropriate assay buffer and incubated in specified wells of the coated plate. After a washing step, an enzyme-conjugated monoclonal antibody specific to the 2mEPSPS protein was also added to the microtiter plate. Both antibodies in the sandwich pair capture the 2mEPSPS protein in the sample. At the end of an incubation period, the unbound reagents were removed from the plate by washing with PBST. The presence of 2mEPSPS was detected by the addition of an enzyme substrate, generating a colored product. The resulting color intensity, measured as optical density (OD), is proportional to the concentration of 2mEPSPS in the sample (i.e., lower protein concentrations result in lower color development). Detailed operational procedures are described in Appendix A.

Sensitivity and Quantitative Range

The preliminary quantitative range for the method was established independently during method development and pre-validation studies at Dow AgroSciences, LLC Regulatory Sciences and Government Affairs laboratory.

The limit of detection (LOD) and limit of quantitation (LOQ) for the determination of 2mEPSPS in soybean tissue were empirically defined on the basis of assay parameters (absorbance, background, and linear range), matrix interferences and/or doses constituting the standard curve. They were also supported by statistical approaches following the method of Keith et al.² and by testing each control sample fortified with 80 ng/mL (8 ng/mg DW) of 2mEPSPS protein. The target LLOQ was 8 ng/mg DW and the quantitation range was from 4 ng/mg to 200 ng/mg DW.

Specificity

The specificity of the 2mEPSPS ELISA to the non-target proteins PAT, AAD-1, AAD-12, Cry1F, Cry34Ab1, Cry35Ab1, and Cry1Ac were tested in this study. These proteins were prepared at a concentration range from 1 ng/mL to 10,000 ng/mL in PBST/ 2X Casein. On the same plate, a 2mEPSPS curve was generated as a reference. The OD response for the non-target proteins was interpolated from the 2mEPSPS standard curve and percent cross-reactivity was calculated using the following formula:

$$\% \text{ cross-reactivity} = 100 \times \frac{\text{measured concentration by 2mEPSPS standard curve}}{\text{theoretical concentration of non-target protein}}$$

Specificity to the CP4 EPSPS protein was tested by the kit manufacturer.

Matrix Effects

The matrix interference effects were tested by spiking the standard curve into different dilutions (1X, 5X, and 10X) of each soybean tissue extract (matrix) negative control. Briefly, the non-transgenic soybean tissue samples were extracted at 15 mg per 1.5 mL assay buffer as described in the method (Appendix A). The extracts were pooled and diluted with assay buffer to 1X, 5X, and 10X. Three standard curves were prepared by spiking the standard protein into the different matrix dilutions. The matrix-spiked standard curves were interpolated from a fourth, non-spiked standard curve that was run on the same plate. A difference in the observed OD greater than 15% between the non-spiked and the matrix-spiked standard curve concentrations was considered indicative of a potential matrix effect.

Extraction Efficiency

The extraction efficiency of 2mEPSPS protein from transgenic soybean tissues was evaluated by repeatedly extracting the soybean tissue samples up to five times and determining the amount of 2mEPSPS protein from each extraction. Briefly, 1.5 mL of buffer was added to the tissue sample (15 mg DW) and extracted as described in the method (Appendix A). Following each extraction, the supernatant was removed and a small volume of the assay buffer was added to the tissue sample pellet and centrifuged. The additional supernatant was combined with the first supernatant and the pooled supernatant was regarded as the first extraction solution. The extraction was repeated four more times and the amount of 2mEPSPS from each extraction was analyzed using the 2mEPSPS ELISA kit. The apparent extraction efficiency was expressed as the recovery percentage of 2mEPSPS protein in the first extract solution relative to the total 2mEPSPS in all five extracts. The extraction efficiency for all soybean tissues was assessed using five replicate samples.

Accuracy

Method accuracy was assessed by determining the recovery of 2mEPSPS protein that had been fortified into non-transgenic soybean tissues at three concentration levels, which approximated the lower (8 ng/mg), middle (32 ng/mg) and high (200 ng/mg) concentration levels of the standard curve after extract dilution. Briefly, assay buffer was fortified with different standard protein concentrations and was then used to extract non-transgenic negative soybean tissue samples. The amount of 2mEPSPS protein was interpolated from the standard calibration curve after extraction. The assay accuracy was indicated as percent of recovery:

$$\% \text{ recovery} = 100 \times \frac{\text{measured concentration by 2mEPSPS standard curve}}{\text{theoretical concentration of target protein}}$$

Equivalency

The purpose of the equivalency test was to evaluate if the standard and test substance in soybean tissue matrices exhibit a similar overall response in the 2mEPSPS ELISA. This was done by using serial dilutions of a single extract of transgenic soybean tissue sample. The amount of the 2mEPSPS protein was interpolated from the standard curve. The %CV was calculated for dilutions with OD readings within the quantitative range of the standard curve.

Precision and Ruggedness

Precision and ruggedness of the method were determined by spiking non-transgenic soybean samples with the 2mEPSPS protein at lower (8 ng/mg DW), middle (32 ng/mg DW) and high (200 ng/mg DW) points of the standard curve and measuring the recovery of the 2mEPSPS protein. The analysis was performed by two analysts across two separate days. The mean recovery concentration, standard deviation, and %CV were calculated. The %CV of intra- and inter-day analyses was calculated as indicative of the method precision and ruggedness.

False Positive and False Negative Rates

The false positive and false negative rates of the method were tested using unfortified control soybean samples and control soybean samples fortified with standard protein at the LOD level (4 ng/mg). A false positive result occurs when the measured 2mEPSPS protein from unfortified control soybean samples exceeds the LOD level. A false negative result occurs when no 2mEPSPS protein is detected from soybean samples fortified at the LOD level.

Data Analysis

ELISA readings were recorded from a Molecular Devices SpectraMax M2 Microplate Reader or a Grifols Triturus Automated Analyzer and the interpolated concentrations were calculated using the SoftMax Pro software program. Concentration data were transferred to Microsoft Excel for the calculation of relevant statistical parameters. Detailed calculations are provided in Appendix A. DAS-BRS Method 101768.

RESULTS AND DISCUSSION

Calculated Limits of Quantitation and Detection

The limit of detection (LOD) of an immunoassay is defined as the analyte concentration that gives a response which has a statistically significant difference from the response of analyte-free sample. Limits of quantitation (LOQ), or the working range of an assay, are generally defined as the highest and lowest concentrations which can be determined with an acceptable degree of precision. In this study, the targeted LOD and LOQ for the determination of 2mEPSPS in soybean tissue were empirically defined to be 4 ng/mg DW (LOD) and 8 ng/mg (LOQ) based on assay parameters (such as absorbance, background, signal-to-noise ratio, and

linear range), matrix interferences, and the standard curve concentrations. The LOD and LOQ were also verified by statistical approaches². Following established guidelines, the LOD and LLOQ were calculated using the standard deviation from the 8 ng/mg DW recovery results. The LLOQ was calculated as ten times the standard deviation (10X SD) and the LOD was calculated as three times the standard deviation (3X SD) of the results for at least 5 samples. As summarized in Table 1 and Tables 8-12, the validated standard curve quantitative range was from 4 ng/mg to 200 ng/mg DW, the calculated LOD results (3X SD) for all soybean tissues range from 1.08 ng/mg to 2.37 ng/mg, and the calculated LOQ (10X SD) for all soybean tissues range from 3.60 ng/mg to 7.88 ng/mg. The target LOD and LOQ were higher than the calculated values for all soybean tissues, which provide more conservative estimates of the working range of the assay (Table 1).

Specificity

This 2mEPSPS ELISA method is specific to 2mEPSPS and shows no significant cross-reactivity with other transgenic proteins such as PAT, AAD-1, AAD-12, Cry1F, Cry34Ab1, Cry35Ab1, Cry1Ac, and CP4 EPSPS at concentrations up to 10,000 ng/mL (Table 2).

Matrix Effect

Up to three matrix dilutions were tested in this study and the results are summarized in Table 3. A difference of greater than 15% between the observed and theoretical means of the standard curve OD readings was considered indicative of a matrix effect. Matrix effects were observed for grain, leaf V5, forage R3, and root R3 tissues at the 1X level. No matrix effects were observed at the 5X and 10X levels for all tissues. Although mean differences between the standard curve prepared in assay buffer and the standard curve prepared in 5X tissue extract did not vary by more than 15%, individual points on the standard curve from grain and root R3 5X extracts did exceed this limit. It is therefore recommended that dilutions of 10X or greater be used for all tissues.

Extraction Efficiency

Determining total 2mEPSPS protein levels in a sample is critical for examining extraction efficiency. Up to five 2mEPSPS transgenic samples were extracted with extraction buffer five consecutive times and the 2mEPSPS protein concentration in each extract was determined by ELISA. The apparent extraction efficiency was based on the amount of 2mEPSPS protein in the first extraction relative to the total amount of 2mEPSPS protein in all five extractions. The extraction efficiencies of the 2mEPSPS protein from all five matrices are summarized in Table 4. The mean extraction efficiencies for soybean tissue ranged from 84.1 – 94.1%.

Accuracy

The mean recovery levels of 2mEPSPS from soybean tissues fortified at the LOQ and at the middle and high points of the standard curve are summarized in Table 5. The mean recoveries ranged from 76.2% to 139.4% for samples spiked at the LOQ level or above. The %CV of mean recoveries at each level was equal to or less than 7.39%. All tissue types except for root R3 had mean recoveries between the target range of 70 - 120%. However, based on the overall consistency of the mean recoveries for root R3 fortified samples and on the robustness of the method across analysts and across days, mean recovery results for root R3 obtained during method validation are considered acceptable

False Positive and False Negative Rate

Twenty unfortified control samples (matrix blanks) and twenty samples fortified at target LOD levels (4 ng/mg DW) of each tissue type were analyzed to determine the false positive and false negative rate of the method, respectively. There were no false negatives or false positives observed in this study.

Precision and Ruggedness

The precision test results from two analysts across multiple days were summarized in Table 6. The intra-day variation (%CV) ranged from 2.36% to 8.67%, intra-analyst variation ranged from 1.55% to 10.31%, and inter-assay variation across days and analysts ranged from 3.79% to 7.39%.

Equivalency

Equivalence of standard and test substance response in the 2mEPSPS ELISA was demonstrated using up to seven serial dilutions of extracts from 2mEPSPS positive tissues. Between three to five out of the seven dilutions fell within the quantitative range of the standard curve. The %CV ranged from 1.38 to 4.84%.

CONCLUSIONS

Dow AgroSciences, LLC analytical method 1017868, "Method Validation for the Determination of 5-Enol-Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)", has been demonstrated to be suitable for its intended purpose. The method was validated over the concentration range of 4 to 200 ng/mg dry weight (DW) and has a validated limit of quantitation (LOQ) in soybean tissues of 8 ng/mg DW and a limit of detection (LOD) of 4 ng/mg DW. The 2mEPSPS protein was recovered at acceptable levels from soybean tissues. Matrix interference was observed at the 1X extract dilution in all tested soybean tissues, except for leaf V10-12, and a 10X extract dilution is recommended for all soybean tissues. The 2mEPSPS protein was efficiently extracted from all tested soybean tissues. The assay was shown to have acceptable accuracy, precision and ruggedness, and no false positive or

false negative results were seen below the target LOD. This 2mEPSPS ELISA method has been demonstrated to be suitable for quantitative measurements of the 2mEPSPS protein in soybean tissue.

ARCHIVING

The protocol, raw data, and the original version of the final report are all filed in the Dow AgroSciences LLC archives at 9330 Zionsville Road in Indianapolis, Indiana 46268-1054.

REFERENCES

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2. Keith, L. H., Crummett, W., Deegan, J., Jr., Libby, R. A., Taylor, J. K., Wentler, G. 1983. Principles of Environmental Analysis, *Anal. Chem.*, 55, 2210-2218.

Table 1. Summary of LOD and LLOQ Determination for Soybean Tissue

Matrix	Fortified Level (ng/mg)	Average Recovery (ng/mg)	SD (ng/mg)	Target LOD (ng/mg)	3X SD (ng/mg)	Target LLOQ (ng/mg)	10X SD (ng/mg)
Grain	8.0	6.09	0.36	4.0	1.08	8.0	3.60
Leaf V5	8.0	7.11	0.40	4.0	1.20	8.0	4.00
Leaf V10-12	8.0	8.14	0.41	4.0	1.24	8.0	4.14
Forage R3	8.0	8.69	0.63	4.0	1.88	8.0	6.27
Root R3	8.0	11.15	0.79	4.0	2.37	8.0	7.88

Table 2. Summary of Specificity for 2mEPSPS Assay

Protein ^a	Lot # or TSN	Cross-reactivity ^{b,c} (%)
AAD-12	030732	0
AAD-1	105930	0
PAT	031116-1	0.00901
Cry1Ac	102337	0.00067
Cry35Ab1	030038	0
Cry34Ab1	104874	0.00031
Cry1F	104301	0

^a Cross-reactivity information for CP4 EPSPS provided by kit manufacturer.

^b Assessed at 10,000 ng/mL non-target protein concentration.

^c A cross-reactivity of 0 was assigned to proteins that resulted in negative concentration values.

Table 3. Summary of Soybean Tissue Matrix Effects

Matrix	Matrix Dilution ^a			Lowest Dilution w/o Matrix Effect
	1X	5X	10X	
Grain	Yes	No	No	5X
Leaf V5	Yes	No	No	5X
Leaf V10-12	No	No	No	1X
Forage R3	Yes	No	No	5X
Root R3	Yes	No	No	5X

^a “No” represents no matrix effects if the difference of the standard curves in the assay buffer and in the diluted matrices was less than 15%. “Yes” represents matrix effects if the difference of the standard curves in the assay buffer and in the diluted matrices was more than 15%.

Table 4. Summary of Extraction Efficiency Results for Soybean Tissue

Matrix	Mean Extraction Efficiency (%)	SD (%)	CV (%)	Extraction efficiency range (%)
Grain	84.1	3.2	3.8	79.8 – 87.2
Leaf V10-12	91.6	2.0	2.2	88.2 – 93.4
Leaf V5	94.1	0.5	0.6	93.3 – 94.6
Forage R3	88.5	1.0	1.2	87.4 – 89.8
Root R3	88.0	2.0	2.2	84.2 – 89.5

Table 5. Summary of Accuracy Results for Soybean Tissue

Matrix	Fortification Level (ng/mg)	Recovery Rate (%)		CV (%)	n
		Mean	Range		
Grain	200	82.0	79.2 – 85.9	3.34	5
	32	77.5	73.8 – 80.3	3.16	5
	8	76.2	70.9 – 83.3	5.91	5
Leaf V10-12	200	96.4	93.0 – 101.1	3.47	5
	32	99.9	95.8 – 107.1	4.01	6
	8	101.8	95.4 – 110.2	5.09	7
Leaf V5	200	88.0	85.3 – 92.6	3.17	5
	32	86.1	84.9 – 87.6	1.51	5
	8	88.9	83.4 – 96.8	5.61	5
Forage R3	200	107.2	94.0 – 118.8	5.49	20
	32	104.4	98.8 – 112.7	3.80	20
	8	108.6	96.7 – 130.4	7.23	20
Root R3	200	125.0	110.6 – 144.6	7.39	25
	32	125.6	107.3 – 135.2	4.80	25
	8	139.4	122.7 – 168.3	7.07	25

Table 6. Summary of Precision and Ruggedness Results for Soybean Tissue^a

Matrix	Spiked Protein Concentration		<u>Intra-day, Inter-analyst</u>		<u>Inter-day, Intra-analyst</u>		<u>Inter-day, Inter-analyst</u>
			Day 1	Day 2	Analyst 1	Analyst 2	
Forage R3	200 ng/mg	Mean	215.89	213.03	215.51	213.41	214.46
		SD	7.78	15.08	13.85	9.91	11.77
		CV%	3.60	7.08	6.43	4.64	5.49
		Min	201.37	187.94	187.94	200.29	187.94
		Max	227.30	237.56	237.56	227.30	237.56
	32 ng/mg	Mean	33.92	32.98	34.32	32.46	33.39
		SD	1.05	1.28	1.00	0.68	1.27
		CV%	3.10	3.89	2.91	2.09	3.80
		Min	32.86	31.63	32.88	31.63	31.63
		Max	36.05	35.19	36.05	33.48	36.05
	8 ng/mg	Mean	8.82	8.56	8.73	8.65	8.69
		SD	0.49	0.74	0.90	0.13	0.63
		CV%	5.59	8.67	10.31	1.55	7.25
		Min	8.07	7.73	7.73	8.49	7.73
		Max	9.73	10.43	10.43	8.90	10.43
Root R3	200 ng/mg	Mean	261.57	242.24	246.02	252.61	249.97
		SD	15.48	16.48	11.70	21.89	18.48
		CV%	5.92	6.80	4.76	8.66	7.39
		Min	237.66	221.15	229.12	221.15	221.15
		Max	289.27	289.72	262.87	289.27	289.27
	32 ng/mg	Mean	40.86	39.74	39.19	40.85	40.19
		SD	0.96	2.29	2.23	1.41	1.93
		CV%	2.36	5.76	5.68	3.46	4.80
		Min	39.63	34.35	34.35	38.18	34.35
		Max	42.26	43.27	41.93	43.27	43.27
	8 ng/mg	Mean	10.93	11.29	10.48	11.59	11.15
		SD	0.44	0.94	0.50	0.61	0.79
		CV%	4.03	8.33	4.78	5.26	7.07
		Min	10.31	9.81	9.81	11.03	9.81
		Max	11.52	13.46	11.23	13.46	13.46

^a Mean, standard deviation, minimum value, and maximum value are expressed as ng/mg. CV is expressed as a percent.

Table 7. Summary of Equivalency Results for Soybean Tissue

Matrix	# of Quantifiable Dilutions	Mean (ng/mg)	SD (ng/mg)	CV (%)	Range (ng/mg)
Grain	4	56.57	2.61	4.61	53.00 – 58.87
Leaf V10-12	5	1689.60	23.39	1.38	1656.24 – 1721.36
Leaf V5	5	1577.75	25.19	1.60	1550.45 – 1608.83
Forage R3	5	985.21	17.11	1.74	958.70 – 1004.56
Root R3	3	309.35	14.96	4.84	292.39 – 320.66

Table 8. Recovery of 2mEPSPS Protein from Soybean Grain

Sample	Date of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-005-0001 R59	17-Dec-2010	200	171.787	85.9	Mean = 163.90 ng/mg
101768-005-0001 R63	17-Dec-2010	200	164.980	82.5	SD = 5.50 ng/mg
101768-005-0001 R66	17-Dec-2010	200	165.387	82.7	CV = 3.36 %
101768-005-0001 R69	17-Dec-2010	200	158.439	79.2	Min = 158.40 ng/mg
101768-005-0001 R72	17-Dec-2010	200	158.867	79.5	Max = 171.80 ng/mg
101768-005-0001 R60	17-Dec-2010	32	24.884	77.8	Mean = 24.81 ng/mg
101768-005-0001 R64	17-Dec-2010	32	25.256	78.9	SD = 0.79 ng/mg
101768-005-0001 R67	17-Dec-2010	32	25.709	80.3	CV = 3.18 %
101768-005-0001 R70	17-Dec-2010	32	23.614	73.8	Min = 23.61 ng/mg
101768-005-0001 R73	17-Dec-2010	32	24.576	76.8	Max = 25.71 ng/mg
101768-005-0001 R62	17-Dec-2010	8	6.065	75.9	Mean = 6.09 ng/mg
101768-005-0001 R65	17-Dec-2010	8	5.955	74.5	SD = 0.36 ng/mg
101768-005-0001 R68	17-Dec-2010	8	6.658	83.3	CV = 5.91 %
101768-005-0001 R71	17-Dec-2010	8	6.096	76.3	Min = 5.67 ng/mg
101768-005-0001 R74	17-Dec-2010	8	5.669	70.9	Max = 6.66 ng/mg
				Mean =	78.6
				SD =	4.0
				CV =	5.1
				n =	15

Table 9. Recovery of 2mEPSPS Protein from Soybean Leaf V10-12

Sample	Dates of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-002-0001 R49	15-Dec-2010	200	202.153	101.1	Mean = 192.80 ng/mg
101768-002-0001 R65	16-Dec-2010	200	197.102	98.6	SD = 6.67 ng/mg
101768-002-0001 R71	16-Dec-2010	200	190.376	95.2	CV = 3.46 %
101768-002-0001 R74	16-Dec-2010	200	188.398	94.2	Min = 185.96 ng/mg
101768-002-0001 R77	16-Dec-2010	200	185.957	93.0	Max = 202.15 ng/mg
101768-002-0001 R53	15-Dec-2010	32	31.863	99.6	Mean = 31.97 ng/mg
101768-002-0001 R56	15-Dec-2010	32	30.649	95.8	SD = 1.28 ng/mg
101768-002-0001 R69	16-Dec-2010	32	34.264	107.1	CV = 4.00 %
101768-002-0001 R72	16-Dec-2010	32	32.440	101.4	Min = 30.65 ng/mg
101768-002-0001 R75	16-Dec-2010	32	31.426	98.2	Max = 34.26 ng/mg
101768-002-0001 R78	16-Dec-2010	32	31.178	97.4	
101768-002-0001 R54	15-Dec-2010	8	8.045	100.6	
101768-002-0001 R63	15-Dec-2010	8	7.633	95.4	Mean = 8.14 ng/mg
101768-002-0001 R67	16-Dec-2010	8	8.501	106.3	SD = 0.41 ng/mg
101768-002-0001 R70	16-Dec-2010	8	8.815	110.2	CV = 5.08 %
101768-002-0001 R73	16-Dec-2010	8	8.263	103.3	Min = 7.63 ng/mg
101768-002-0001 R76	16-Dec-2010	8	7.891	98.6	Max = 8.82 ng/mg
101768-002-0001 R79	16-Dec-2010	8	7.828	97.9	
			Mean =	99.7	
			SD =	4.7	
			CV =	4.7	
			n =	18	

Table 10. Recovery of 2mEPSPS Protein from Soybean Leaf V5

Sample	Date of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-001-0001 R11	14-Dec-2010	200	185.257	92.6	Mean = 176.07 ng/mg
101768-001-0001 R9	14-Dec-2010	200	176.144	88.1	SD = 5.61 ng/mg
101768-001-0001 R15	14-Dec-2010	200	170.521	85.3	CV = 3.19 %
101768-001-0001 R18	14-Dec-2010	200	175.563	87.8	Min = 170.52 ng/mg
101768-001-0001 R21	14-Dec-2010	200	172.860	86.4	Max = 185.26 ng/mg
101768-001-0001 R12	14-Dec-2010	32	27.154	84.9	Mean = 27.54 ng/mg
101768-001-0001 R10	14-Dec-2010	32	27.154	84.9	SD = 0.43 ng/mg
101768-001-0001 R16	14-Dec-2010	32	27.948	87.3	CV = 1.56 %
101768-001-0001 R19	14-Dec-2010	32	27.412	85.7	Min = 27.15 ng/mg
101768-001-0001 R22	14-Dec-2010	32	28.042	87.6	Max = 28.04 ng/mg
101768-001-0001 R13	14-Dec-2010	8	7.059	88.2	Mean = 7.11 ng/mg
101768-001-0001 R14	14-Dec-2010	8	7.175	89.7	SD = 0.40 ng/mg
101768-001-0001 R17	14-Dec-2010	8	7.744	96.8	CV = 5.63 %
101768-001-0001 R20	14-Dec-2010	8	6.918	86.5	Min = 6.67 ng/mg
101768-001-0001 R23	14-Dec-2010	8	6.670	83.4	Max = 7.74 ng/mg
				Mean =	87.7
				SD =	3.4
				CV =	3.9
				n =	15

Table 11. Recovery of 2mEPSPS Protein from Soybean Forage R3

Sample	Date of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-003-0001 R49	16-Dec-2010	200	224.197	112.1	Mean = 214.46 ng/mg SD = 11.77 ng/mg CV = 5.49 % Min = 187.94 ng/mg Max = 237.56 ng/mg
101768-003-0001 R52	16-Dec-2010	200	212.216	106.1	
101768-003-0001 R55	16-Dec-2010	200	201.370	100.7	
101768-003-0001 R58	16-Dec-2010	200	213.580	106.8	
101768-003-0001 R61	16-Dec-2010	200	216.234	108.1	
101768-003-0001 R81	17-Dec-2010	200	237.561	118.8	
101768-003-0001 R84	17-Dec-2010	200	228.603	114.3	
101768-003-0001 R87	17-Dec-2010	200	219.449	109.7	
101768-003-0001 R90	17-Dec-2010	200	187.940	94.0	
101768-003-0001 R93	17-Dec-2010	200	213.947	107.0	
101768-003-0001 R65	16-Dec-2010	200	213.539	106.8	
101768-003-0001 R68	16-Dec-2010	200	221.141	110.6	
101768-003-0001 R71	16-Dec-2010	200	227.304	113.7	
101768-003-0001 R74	16-Dec-2010	200	220.983	110.5	
101768-003-0001 R77	16-Dec-2010	200	208.356	104.2	
101768-003-0001 R99	20-Dec-2010	200	202.175	101.1	
101768-003-0001 R102	20-Dec-2010	200	218.160	109.1	
101768-003-0001 R105	20-Dec-2010	200	221.477	110.7	Mean = 33.39 ng/mg SD = 1.27 ng/mg CV = 3.80 % Min = 31.63 ng/mg Max = 36.05 ng/mg
101768-003-0001 R108	20-Dec-2010	200	200.286	100.1	
101768-003-0001 R111	20-Dec-2010	200	200.702	100.4	
101768-003-0001 R50	16-Dec-2010	32	33.821	105.7	
101768-003-0001 R53	16-Dec-2010	32	36.050	112.7	
101768-003-0001 R56	16-Dec-2010	32	34.599	108.1	
101768-003-0001 R59	16-Dec-2010	32	34.704	108.5	
101768-003-0001 R62	16-Dec-2010	32	34.654	108.3	
101768-003-0001 R82	17-Dec-2010	32	35.186	110.0	
101768-003-0001 R85	17-Dec-2010	32	34.813	108.8	
101768-003-0001 R88	17-Dec-2010	32	32.880	102.8	
101768-003-0001 R91	17-Dec-2010	32	33.394	104.4	
101768-003-0001 R94	17-Dec-2010	32	33.112	103.5	
101768-003-0001 R66	16-Dec-2010	32	33.475	104.6	
101768-003-0001 R69	16-Dec-2010	32	32.862	102.7	
101768-003-0001 R72	16-Dec-2010	32	33.151	103.6	
101768-003-0001 R75	16-Dec-2010	32	32.862	102.7	
101768-003-0001 R78	16-Dec-2010	32	33.042	103.3	
101768-003-0001 R100	20-Dec-2010	32	31.803	99.4	
101768-003-0001 R103	20-Dec-2010	32	31.940	99.8	
101768-003-0001 R106	20-Dec-2010	32	31.974	99.9	
101768-003-0001 R109	20-Dec-2010	32	31.631	98.8	
101768-003-0001 R112	20-Dec-2010	32	31.871	99.6	

Table 11. Recovery of 2mEPSPS Protein from Soybean Forage R3 (cont.)

Sample	Date of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-003-0001 R51	16-Dec-2010	8	8.065	100.8	Mean = 8.69 ng/mg SD = 0.63 ng/mg CV = 7.25 % Min = 7.73 ng/mg Max = 10.43 ng/mg
101768-003-0001 R54	16-Dec-2010	8	9.410	117.6	
101768-003-0001 R57	16-Dec-2010	8	9.729	121.6	
101768-003-0001 R60	16-Dec-2010	8	9.101	113.8	
101768-003-0001 R63	16-Dec-2010	8	8.446	105.6	
101768-003-0001 R83	17-Dec-2010	8	0.429	130.4	
101768-003-0001 R86	17-Dec-2010	8	8.427	105.3	
101768-003-0001 R89	17-Dec-2010	8	8.025	100.3	
101768-003-0001 R92	17-Dec-2010	8	7.888	98.6	
101768-003-0001 R95	17-Dec-2010	8	7.742	96.7	
101768-003-0001 R67	16-Dec-2010	8	8.904	111.3	
101768-003-0001 R70	16-Dec-2010	8	8.485	106.1	
101768-003-0001 R73	16-Dec-2010	8	8.520	106.5	
101768-003-0001 R76	16-Dec-2010	8	8.764	109.6	
101768-003-0001 R79	16-Dec-2010	8	8.729	109.1	
101768-003-0001 R101	20-Dec-2010	8	8.744	109.3	
101768-003-0001 R104	20-Dec-2010	8	8.548	106.9	
101768-003-0001 R107	20-Dec-2010	8	8.646	108.1	
101768-003-0001 R110	20-Dec-2010	8	8.580	107.3	
101768-003-0001 R113	20-Dec-2010	8	8.548	106.9	
				Mean =	106.7
				SD =	6.3
				CV =	5.9
				n =	60

Table 12. Recovery of 2mEPSPS Protein from Soybean Root R3

Sample	Date of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-004-0001 R9	16-Dec-2010	200	262.873	131.4	
101768-004-0001 R12	16-Dec-2010	200	255.312	127.7	
101768-004-0001 R15	16-Dec-2010	200	260.265	130.1	
101768-004-0001 R18	16-Dec-2010	200	245.140	122.6	
101768-004-0001 R21	16-Dec-2010	200	237.664	118.8	
101768-004-0001 R41	17-Dec-2010	200	245.239	122.6	
101768-004-0001 R44	17-Dec-2010	200	229.270	114.6	
101768-004-0001 R47	17-Dec-2010	200	229.122	114.6	
101768-004-0001 R50	17-Dec-2010	200	250.583	125.3	
101768-004-0001 R53	17-Dec-2010	200	244.766	122.4	
101768-004-0001 R25	16-Dec-2010	200	276.750	138.4	Mean = 249.97 ng/mg
101768-004-0001 R28	16-Dec-2010	200	258.003	129.0	SD = 18.48 ng/mg
101768-004-0001 R31	16-Dec-2010	200	254.395	127.2	CV = 7.39 %
101768-004-0001 R34	16-Dec-2010	200	276.053	138.0	Min = 221.15 ng/mg
101768-004-0001 R37	16-Dec-2010	200	289.265	144.6	Max = 289.27 ng/mg
101768-004-0001 R97	20-Dec-2010	200	221.154	110.6	
101768-004-0001 R100	20-Dec-2010	200	227.805	113.9	
101768-004-0001 R103	20-Dec-2010	200	281.172	140.6	
101768-004-0001 R106	20-Dec-2010	200	230.195	115.1	
101768-004-0001 R109	20-Dec-2010	200	225.634	112.8	
101768-004-0001 R113	27-Dec-2010	200	234.992	117.5	
101768-004-0001 R116	27-Dec-2010	200	266.466	133.2	
101768-004-0001 R122	27-Dec-2010	200	250.052	125.0	
101768-004-0001 R125	27-Dec-2010	200	246.689	123.3	
101768-004-0001 R128	27-Dec-2010	200	250.502	125.3	

Table 12. Recovery of 2mEPSPS Protein from Soybean Root R3 (cont.)

Sample	Date of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-004-0001 R10	16-Dec-2010	32	40.096	125.3	
101768-004-0001 R13	16-Dec-2010	32	41.926	131.0	
101768-004-0001 R16	16-Dec-2010	32	40.205	125.6	
101768-004-0001 R19	16-Dec-2010	32	39.628	123.8	
101768-004-0001 R22	16-Dec-2010	32	39.751	124.2	
101768-004-0001 R42	17-Dec-2010	32	40.060	125.2	
101768-004-0001 R45	17-Dec-2010	32	34.347	107.3	
101768-004-0001 R48	17-Dec-2010	32	36.381	113.7	
101768-004-0001 R51	17-Dec-2010	32	40.761	127.4	
101768-004-0001 R54	17-Dec-2010	32	38.740	121.1	
101768-004-0001 R26	16-Dec-2010	32	41.967	131.1	Mean = 40.19 ng/mg
101768-004-0001 R29	16-Dec-2010	32	42.259	132.1	SD = 1.93 ng/mg
101768-004-0001 R32	16-Dec-2010	32	41.238	128.9	CV = 4.80 %
101768-004-0001 R35	16-Dec-2010	32	40.545	126.7	Min = 34.35 ng/mg
101768-004-0001 R38	16-Dec-2010	32	40.982	128.1	Max = 43.27 ng/mg
101768-004-0001 R98	20-Dec-2010	32	42.531	132.9	
101768-004-0001 R101	20-Dec-2010	32	43.269	135.2	
101768-004-0001 R104	20-Dec-2010	32	41.688	130.3	
101768-004-0001 R107	20-Dec-2010	32	41.023	128.2	
101768-004-0001 R110	20-Dec-2010	32	40.532	126.7	
101768-004-0001 R114	27-Dec-2010	32	39.148	122.3	
101768-004-0001 R117	27-Dec-2010	32	38.175	119.3	
101768-004-0001 R123	27-Dec-2010	32	40.738	127.3	
101768-004-0001 R126	27-Dec-2010	32	39.761	124.3	
101768-004-0001 R129	27-Dec-2010	32	38.931	121.7	

Table 12. Recovery of 2mEPSPS Protein from Soybean Root R3 (cont.)

Sample	Date of Analysis	Spiked (ng/mg)	Recovery (ng/mg)	Percent Recovery (%)	Statistical Calculations
101768-004-0001 R11	16-Dec-2010	8	11.187	139.8	
101768-004-0001 R14	16-Dec-2010	8	10.307	128.8	
101768-004-0001 R17	16-Dec-2010	8	10.343	129.3	
101768-004-0001 R20	16-Dec-2010	8	10.895	136.2	
101768-004-0001 R23	16-Dec-2010	8	10.388	129.9	
101768-004-0001 R43	17-Dec-2010	8	10.000	125.0	
101768-004-0001 R46	17-Dec-2010	8	9.814	122.7	
101768-004-0001 R49	17-Dec-2010	8	11.234	140.4	
101768-004-0001 R52	17-Dec-2010	8	10.654	133.2	
101768-004-0001 R55	17-Dec-2010	8	9.978	124.7	
101768-004-0001 R27	16-Dec-2010	8	11.234	140.5	Mean = 11.15 ng/mg
101768-004-0001 R30	16-Dec-2010	8	11.523	144.0	SD = 0.79 ng/mg
101768-004-0001 R33	16-Dec-2010	8	11.033	137.9	CV = 7.09 %
101768-004-0001 R36	16-Dec-2010	8	11.348	141.9	Min = 9.81 ng/mg
101768-004-0001 R39	16-Dec-2010	8	11.068	138.4	Max = 13.46 ng/mg
101768-004-0001 R99	20-Dec-2010	8	11.988	149.9	
101768-004-0001 R102	20-Dec-2010	8	11.397	142.5	
101768-004-0001 R105	20-Dec-2010	8	11.824	147.8	
101768-004-0001 R108	20-Dec-2010	8	11.397	142.5	
101768-004-0001 R111	20-Dec-2010	8	11.956	149.5	
101768-004-0001 R115	27-Dec-2010	8	11.168	139.6	
101768-004-0001 R121	27-Dec-2010	8	11.099	138.7	
101768-004-0001 R124	27-Dec-2010	8	13.462	168.3	
101768-004-0001 R127	27-Dec-2010	8	11.515	143.9	
101768-004-0001 R130	27-Dec-2010	8	11.897	148.7	
			Mean =	130.0	
			SD =	10.8	
			CV =	8.3	
			n =	75	

APPENDIX A. DAS-BRS METHOD 101768

Determination of 5-Enol-Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein in
Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)

GRM: 101768
EFFECTIVE: N/A
SUPERSEDES: NEW

Determination of 5-Enol-Pyruvylshikimate-3-Phosphate Synthase (2mEPSPS) Protein in Soybean Tissues by Enzyme-Linked Immunosorbent Assay (ELISA)

P. M. Maldonado and E. Ma

1. SCOPE

This method is applicable for the quantitative determination of 5-enol-pyruvylshikimate-3-phosphate synthase (2mEPSPS) protein expressed in soybean tissues using an enzyme-linked immunosorbent assay (ELISA) kit. The calibration standard curve range is from 4 ng/mL to 200 ng/mL in assay buffer. The 2mEPSPS protein level in soybean grain, leaf V5, leaf V10-12, root R3, and forage R3 can be determined with a validated limit of quantitation (LOQ) of 8 ng/mg and a limit of detection (LOD) of 4 ng/mg dry tissue weight (DTW or DW).

2. PRINCIPLE

An analytical method has been developed for the determination of 2mEPSPS protein expressed in soybean plants. The 2mEPSPS protein is extracted from soybean samples with a phosphate buffered saline solution containing 0.05% Tween 20 (PBST) and 2X Casein (PBST/Casein). The extract is centrifuged; the aqueous supernatant is collected, diluted and assayed using a specific 2mEPSPS ELISA kit. A sequential sandwich ELISA format was applied in this assay. An aliquot of the diluted sample is incubated in the wells of an immobilized anti-2mEPSPS polyclonal antibody coated plate, and

then the unbound samples are removed from the plate by washing with PBST. An excess amount of enzyme-conjugated anti-2mEPSPS protein monoclonal antibody is added to the wells for incubation. These antibodies bind with 2mEPSPS protein in the wells and form a “sandwich” with 2mEPSPS protein bound between soluble and immobilized antibody. The presence of 2mEPSPS is detected by incubating the antibody-bound enzyme conjugate with an enzyme substrate, generating a colored product. Since the 2mEPSPS is bound in the antibody sandwich, the level of color development is proportional to the concentration of 2mEPSPS in the sample (i.e., lower protein concentrations result in lower color development). The absorbance at 450 nm minus absorbance at 650 or 620 nm is measured using a plate reader. A calibration curve is estimated from 7 or 8 standard concentrations using a quadratic regression equation.

3. SAFETY PRECAUTIONS

- 3.1. Each analyst must be acquainted with the potential hazards of the reagents, products, and solvents used in this method before commencing laboratory work. SOURCES OF INFORMATION INCLUDE: MATERIAL SAFETY DATA SHEETS, LITERATURE, AND OTHER RELATED DATA. Safety information on non-Dow AgroSciences LLC products should be obtained from the container label or from the supplier. Disposal of reagents, reactants, and solvents must be in compliance with local, state, and federal laws and regulations.
- 3.2. Avoid contact of Stopping Solution (0.5% sulfuric acid or 1N hydrochloric acid) with skin and mucous membranes. Wear protective clothing and proper eye protection when working with this material. If this reagent comes in contact with skin, flush the affected area with water.

- 3.3. It is imperative that proper eye and personal protective equipment be worn when handling these reagents.

4. EQUIPMENT AND MATERIALS (Note 12.1)

4.1. Equipment

- 4.1.1. Balance, analytical, Model AE50, Mettler-Toledo Inc., Columbus, OH 43240.
- 4.1.2. Centrifuge, refrigerated, capable of holding 96-well plates, Model GR4-22, Jouan Inc., Winchester, VA 22602.
- 4.1.3. Centrifuge rotor, RTR M4 Hz 4 place, Jouan Inc.
- 4.1.4. Centrifuge, capable of holding 2-mL Eppendorf tubes, Eppendorf-5417C, Brinkmann Instruments. Inc., Westbury, NY 11590.
- 4.1.5. Freezer, capable of maintaining -80 °C, Ultima II Series, Model ULT2586-9, Revco, Asheville, NC 28806.
- 4.1.6. Incubator, Precision Economy, Model 3EG, Thermo Electron Corp., Franklin MA 02038.
- 4.1.7. Microplate Reader, Maxline V Max microplate reader with Softmax PRO software, capable of reading 450 and 650 nm, Molecular Devices, Sunnyvale, CA 94089.
- 4.1.8. Microplate Washer, 96-well microplate, Model ELx 405, Bio-Tek Instruments, Inc., Winooski, VT 05404.
- 4.1.9. Pipettor, Eppendorf Research 2100, 500-5000 µL, catalog number 22 47 215 1, Brinkman.

- 4.1.10. Pipettor, Eppendorf Research 2100, 100-1000 μ L, catalog number 22 47 210 1, Brinkman.
- 4.1.11. Pipettor, Eppendorf Research 2100, 20-200 μ L, catalog number 22 47 205 4, Brinkman.
- 4.1.12. Pipettor, Eppendorf Research 2100, 2-20 μ L, catalog number 22 47 195 3, Brinkman.
- 4.1.13. Pipettor, Eppendorf Research 2100, 0.5-10 μ L, catalog number 22 47 190 2, Brinkman.
- 4.1.14. Pipettor, Eppendorf Research 12 channel, 30-300 μ L, catalog number 22 45 210-0, Brinkman.
- 4.1.15. Pipet-Aid, Eppendorf motorized pipet filler/dispenser, catalog number 2223020-4, Brinkman.
- 4.1.16. Refrigerator, capable of maintaining 4 °C, general purpose, Revco.
- 4.1.17. Shaker/Grinder, Geno/Grinder Model 2000-115, Certiprep, Metuchen, NJ 08840.
- 4.1.18. Stir plate, Corning Magnetic Stirrer, Model 6795 410, Corning Inc., Acton, MA 01720.
- 4.1.19. Titer plate shaker, Model 4625, Barnstead/Thermolyne, Dubuque, IA 52001.
- 4.1.20. Ultracentrifuge, TL-100, Serial Number TA716, Catalog number 346457. Beckman Instruments Inc., Palo Alto, CA 94304.
- 4.1.21. Vortex, Model Genie-2, catalog number 12-812, Fisher Scientific, Pittsburgh, PA 15275.
- 4.1.22. Water purification system, Model Milli-Q UV Plus, Millipore Corp., Milford, MA 01757.
- 4.1.23. Enzyme immunoassay automated analyzer, Triturus Analyzer, Diagnostic Grifols, S.A., Parets del Valles, Spain.

4.2. Materials

- 4.2.1. Basin, reagent, non-sterile, catalog number 730-001, Labcor Inc., Fredrick, MD 21704.
- 4.2.2. Bead, 1/8" chrome steel, catalog number BS-0125-C, Small Parts Inc., Miami Lakes, FL 33014.
- 4.2.3. Cap, for 2.0-mL conical tube, catalog number 02-681-361, Fisher Scientific.
- 4.2.4. Pipet, 10-mL disposable serological, catalog number 13-678-36C, Fisher Scientific.
- 4.2.5. Pipet tip, Eppendorf epTIPS, 100-5000 μ L catalog number 22 49 138-5, Brinkman.
- 4.2.6. Pipet tip, Eppendorf epTIPS, 50-1000 μ L, catalog number 22 49 145-8, Brinkman.
- 4.2.7. Pipet tip, Eppendorf epTIPS, 2-200 μ L, catalog number 22 49 143-1, Brinkman.
- 4.2.8. Pipet tip, Eppendorf epTIPS, 0.5-20 μ L, catalog number 22 49 142-3, Brinkman.
- 4.2.9. Plate, 96-well, non-binding for sample transfer, catalog number 9205, Thermo Electron Corp.
- 4.2.10. Plate sealer, 96-well, catalog number 3095, Corning Inc.
- 4.2.11. Plate stand, 96-well, catalog Z36, 335-9, Sigma, St. Louis, MO 63178.
- 4.2.12. Rack, 24-position for sample extraction, catalog number 373661, Beckman Coulter, Inc., Fullerton, CA 92834.
- 4.2.13. Rack inserts, 11-mm diameter for 2.0-mL tubes, catalog number 373696, Beckman Coulter Inc.
- 4.2.14. Tube, 2.0-mL conical microcentrifuge, catalog number 02-681-344, Fisher Scientific.

- 4.2.15. Tube, 5-mL polypropylene with cap, catalog number 14-956-1D, Fisher Scientific.
- 4.2.16. Tube, 15-mL polypropylene centrifuge with cap, catalog number 05-539-12, Fisher Scientific.
- 4.2.17. Tube, 50-mL polypropylene centrifuge with cap, catalog number 06-443-18, Fisher Scientific.

5. REAGENTS, STANDARDS AND PREPARATION

5.1. Reagents (Note 12.1)

- 5.1.1. Acadia 2mEPSPS ELISA Kit, catalog number ABS-091, Acadia BioScience LLC, Portland, ME 04103 (Note 12.2). Store at 2-8 °C. Contents:
 - a. Rabbit anti-2mEPSPS polyclonal antibody coated plates
 - b. Mouse anti-2mEPSPS/HRP enzyme conjugate
 - c. Substrate
 - d. Stop solution
- 5.1.2. Phosphate buffered saline solution containing 0.05% Tween 20 (PBST), pH 7.4, catalog number P-3563, Sigma. Store at 2-8 °C.
- 5.1.3. Phosphate Buffered Saline/Casein Block and Diluent- 5X Concentrate, catalog number PBSC-1000-01, SurModics. Store at 2-8 °C.

5.2. Standards

5.2.1. Obtain 2mEPSPS microbial protein from Test Substance Coordinator, Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268.

5.2.2. If needed, quality control samples (positive and negative) may be obtained from Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268.

5.3. Reagent and Standard Preparations

5.3.1. Phosphate Buffered Saline, pH 7.4, with 0.05% Tween 20 (PBST) (Note 12.3)

- a. Add one packet of PBST to 1.0 liter of de-ionized water.
- b. Add a stir bar and mix to dissolve on the stir plate.
- c. Store at 20-25 °C for up to 2-3 months or at 2-8 °C for a maximum of 6 months.
Discard if any visible contamination is observed.

5.3.2. Phosphate Buffered Saline, pH 7.4, with 0.05% Tween 20 (PBST) plus 2X Casein (PBST/Casein):

- a. Add 40 mL of 5X Casein concentrate to 60 mL of PBST (Section 5.3.1).
- b. Add a stir bar and mix on stir plate.
- c. Store at 2-8 °C for up to 24 hours.

5.3.3. Preparation of 2mEPSPS Working Stock Solutions

- a. The starting 2mEPSPS standard may be lyophilized powder or aliquoted liquid stock solutions. For example, one stock solution used in DAS Regulatory Laboratories is a 0.665-mg/mL solution (TSN 033171-0001).
- b. Vortex the stock solution and then add 10 µL of the 0.665-mg/mL 2mEPSPS stock solution into 990 µL of PBST/Casein and mix well to make a 6650-ng/mL working

stock solution. Similarly, add 100 µL of the 6650-ng/mL working stock solution into 565 µL of PBST/Casein and mix well to make a 1000-ng/mL working stock solution. Keep the stock solutions on ice. Discard if any visible contamination is observed.

5.3.4. Fortification Solutions

Dilute appropriate aliquots of the stock solution to volume with PBST/Casein in 15-mL tubes to obtain the desired concentrations for the fortification of recovery samples, as shown in the table below^a:

Initial Stock Soln. Conc.	Aliquot of Stock Soln.	Buffer Vol. Added	Final Soln. Volume	Spiking Soln. Final Conc.	Equivalent Sample Conc. ^b
ng/mL	mL	mL	mL	ng/mL	ng/mg
6650	4.0	9.3	13.3	2000	200
2000	1.6	8.4	10.0	320	32
320	2.5	7.5	10.0	80	8

^a Spiking solutions can be stored on ice for up to 2 hours after preparation.

^b The equivalent sample concentrations are based on fortifying the 15-mg samples with 1.5 mL of spiking solutions.

6. INSTRUMENT SETTINGS

To obtain results from the 2mEPSPS ELISA kit, use the following parameter settings on Microplate Reader:

Parameter	Reader Abbreviation	Setting
Read Mode	Read Mode	Endpoint
Data Reduction	Data Reduction	quadratic
Number of Standard Replicate		3
Number of Standards ^a		7
Control	Ctl	0
Standard #1 Concentration	Std01	200
Standard #2 Concentration	Std02	128
Standard #3 Concentration	Std03	64
Standard #4 Concentration	Std04	32
Standard #5 Concentration	Std05	16
Standard #6 Concentration	Std06	8
Standard #7 Concentration	Std07	4
Minimum Correlation	Correlation Flag	0.990
Wavelength	Dual Wavelength	450 nm - 650 (or 620) nm
Data Mode	Data Mode	Absorbance
Units	Units	ng/mL
Precision of Standards	Rep %CV Flag	15

^a Analysis can also be done using an 8 point calibration curve, in which case the Control would serve as the 0 ng/mL standard concentration.

7. DETERMINATION OF RECOVERY OF 2mEPSPS PROTEIN IN SOYBEAN TISSUES

7.1. Preparation of Recovery Samples

- 7.1.1. Extract the recovery samples at the same time and manner as the unknowns. Store the recovery extracts in the same manner as the unknown samples.
- 7.1.2. For all sample types, weigh 15-mg (\pm 0.5 mg) portions of the prepared control soybean tissue samples and dispense into 2-mL polypropylene tubes. Add two or three metal beads to each tube. For laboratory recovery samples, add 1.5 mL of the appropriate spiking/extraction solution from Step 5.3.3. A control should be carried through the

method with each sample set. Non-fortified PBST/Casein buffer is used for extraction as a control.

- 7.1.3. Cap all of the tubes. Extract the samples using the Geno/Grinder automatic shaker/grinder at a dial setting of 500 and the toggle switch at the 1X setting (approximately 1500 strokes per minute) for 3 minutes as one cycle.
- 7.1.4. Centrifuge the samples at 3,000 (or greater) rpm for 5 minutes or until separated (no visible particles in the supernatant). The supernatant can be transferred to a separate tube or aliquoted for analysis as described in Section 7.2. Keep the extract on ice and assay it within 2 hours.
- 7.1.5. Assay each sample according to the procedure described in Section 7.2.

7.2. Assay Procedure

7.2.1. 2mEPSPS ELISA Kit Preparation (Note 12.2)

Bring the ELISA kit reagents and plate to 20-25 °C by removing them from the refrigerator at least 30 minutes prior to performing the assay.

7.2.2. Standard Calibration

Prepare standard calibration solutions in 5-mL polypropylene tubes by diluting the 1000-ng/mL stock solution from Section 5.3.3 with PBST/Casein as described below. The following example preparation provides enough standard for 1 plate. Adjust volumes as necessary for additional plates. Store tubes on ice.

Conc. of Stock Soln. (ng/mL)	Aliquot of Stock Soln (μ L)	Buffer Volume (μ L)	Final Soln. Volume (μ L)	Final Standard Conc. (ng/mL)	Remaining Volume after Aliquot (μ L)
1000	228	912	1140	200	500
200	640	360	1000	128	500
128	500	500	1000	64	500
64	500	500	1000	32	500
32	500	500	1000	16	500
16	500	500	1000	8	500
8	500	500	1000	4	1000
N/A	N/A	500	500	0	500

7.2.3. ELISA Analysis

- 7.2.3.1. Conduct each test in an individual microtiter plate. Multiple tests can be included in the same plate. The average of replicate analyses of a sample or standard constitutes a single result. A calibration curve and the appropriate control must be included in each plate.
- 7.2.3.2. Transfer the ELISA standard calibration solutions from Step 7.2.2 in triplicate to a non-binding 96-well microtiter plate (~150 μ L/well) and record the location on the 96-well assay template sheet (Figure 1).
- 7.2.3.3. Prepare sample dilutions as needed and transfer the samples in 2 or 3 replicates to the non-binding 96-well microtiter plate (~150 μ L/well) containing the standard calibration solutions and record the location on the 96-well assay template sheet (Figure 1).

- 7.2.3.4. Add 100 μ L of the ELISA standard solutions and samples from the non-binding 96-well microtiter plate to the antibody coated 96-well microtiter plate, keeping the same orientation as the 96-well assay template. Change pipet tips with each sample.
- 7.2.3.5. Cover the plate with an adhesive plate sealer. Allow the microtiter plate to incubate at room temperature (20-30 °C) on an orbital shaker set to approximately 130 rpm for 60 minutes.
- 7.2.3.6. Wash the plate five times with 350 μ L/well PBST using an automatic plate washer. Tap out the excess liquid on a paper towel.
- 7.2.3.7. Dispense approximately 12 mL of the 2mEPSPS antibody conjugate per plate into a reagent basin.
- 7.2.3.8. Pipet 100 μ L of the 2mEPSPS antibody conjugate from the reagent basin to each well of the antibody coated 96-well microtiter plate. **Discard any unused 2mEPSPS antibody conjugate solution.**
- 7.2.3.9. Cover the plate with an adhesive plate sealer. Allow the microtiter plate to incubate at room temperature (20-30 °C) on an orbital shaker set to approximately 130 rpm for 30 minutes.
- 7.2.3.10. Wash the plate five times with 350 μ L/well PBST using an automatic plate washer. Tap out excess liquid onto a paper towel.
- 7.2.3.11. Dispense approximately 12 mL of the Substrate Solution per plate into a reagent basin.
- 7.2.3.12. Pipet 100 μ L of the Substrate Solution from the reagent basin to each well of the antibody coated 96-well microtiter plate. **Discard any unused Substrate Solution.**
- 7.2.3.13. Cover the plate with an adhesive plate sealer and then cover with foil to protect the plate from light. Allow the microtiter plate to incubate at room temperature (20-30 °C) on an orbital shaker set to approximately 130 rpm for 30 minutes.
- 7.2.3.14. Dispense approximately 12 mL per plate of the Stop Solution into a reagent basin.

- 7.2.3.15. Add 100 μ L of Stop Solution to each well to stop the reaction. Mix the plate gently. The addition of stop solution should be completed without interruption. Protect the microtiter plate from sunlight; otherwise, color intensity is influenced.
- 7.2.3.16. Read the absorbance at 450 nm minus 650 or 620 nm using a 96-well microtiter plate reader. All readings should be completed within 30 minutes of adding the stop solution.

8. DETERMINATION OF 2mEPSPS PROTEIN IN SOYBEAN TISSUES

- 8.1. Prepare the samples as described in Step 7.1.2.
- 8.2. For all sample types, weigh 15-mg (\pm 0.5 mg) portions of the prepared unknown soybean samples and dispense into 2-mL polypropylene tube. Add two or three metal beads to each tube. Add 1.5 mL of PBST/Casein extraction solution.
- 8.3. Extract the samples as described in Steps 7.1.3 and 7.1.4, substituting the spiking/extraction solutions for PBST/Casein.
- 8.4. Assay each sample according to the procedure described in Section 7.2. If the sample contains more than 200 ng/mL of 2mEPSPS protein, perform an additional dilution of the sample from Step 7.2.3.3 prior to assay (e.g., for a 1:10 or a 10X dilution, pipet 135 μ L of PBST/Casein onto a non-binding dilution plate, add 15 μ L of sample from Step 8.3, and mix with the pipettor). Assay the diluted aliquot as described in Section 7.2.3.

9. DATA ANALYSIS AND CALCULATIONS

9.1. Calibration Curve

- 9.1.1. Microplate analysis software, such as SoftMax Pro or the Triturus instrument software, allows for the creation of electronic data files containing all of the parameters required for acquiring and analyzing data from the microplate reader. The calibration curve for the 2mEPSPS ELISA kit is constructed using a quadratic curve regression of the known concentration of the standard calibration solutions and their respective absorbance (optical density).

- 9.1.2. The equation fits the best parabola to the standard curve based on the equation:

$$y = A + Bx + Cx^2$$

Where:

y = mean absorbance value (OD)

x = reference standard concentration

An example of a 2mEPSPS calibration curve is presented in Figure 2.

9.2. Calculation of 2mEPSPS in Unknown Samples

- 9.2.1. The SOFTmax PRO software will calculate the concentration of 2mEPSPS in each sample as noted in Section 9.1 above. The absorbance value and calculated concentration as well as individual well results, mean sample result, standard deviation and the percent coefficient of variation are reported on the SOFTmax PRO data report.

9.3. Example Calculations

$$\text{Method Factor (MF)} = \frac{\text{weight of tissue (mg)}}{\text{extraction volume (mL)}}$$

Note: For simplicity, a 15 mg tissue weight will be used in the calculation for all tissue samples with an acceptable variation of $\pm 3.3\%$ to reflect the actual weight range of 15 ± 0.5 mg.

Final concentration calculation:

$$\begin{array}{ccc} \text{2mEPSPS Concentration} & = & \text{Mean Result} / \text{MF} \\ (\text{ng/mg}) & & (\text{ng/mL}) \end{array}$$

Example: For a 15.0-mg sample extracted with 1.5 mL of buffer, the mean results of two dilutions of the sample were 5.574 and 5.451 ng/mL.

$$\begin{aligned} \text{2mEPSPS concentration} &= \frac{[(5.574 + 5.451)/2] \text{ ng/mL}}{15.0 \text{ mg}/1.5\text{mL}} \\ &= 0.551 \text{ ng/mg} \end{aligned}$$

9.4. Calculation of Percent Recovery

The percent recovery is calculated as the average of all replicate (well) concentrations divided by the fortification concentration.

$$\begin{aligned} \text{Mean \% recovery} &= \frac{\text{the average of protein concentration found}}{\text{fortification concentration}} \times 100\% \\ \text{Recovery} &= \frac{0.551 \text{ ng/mg}}{0.60 \text{ ng/mg}} \times 100\% \\ \text{Recovery} &= 92 \% \end{aligned}$$

9.5. Predicted Concentration (predconc)

9.5.1. The predicted concentration is the basis for the mean percent error calculation. The predicted concentration is determined using the coefficients of the curve and optical density (OD) readings in the quadratic formula. The regression equation was applied as follows:

$$y = C_1x^2 + C_2x + C_3$$

(where x = predicted concentration and y = OD)

$$\text{predicted concentration} = \frac{-C_2 + \sqrt{C_2^2 - 4C_1(C_3 - OD)}}{2C_1}$$

For example, given equation parameters of $C_1 = -0.01$, $C_2 = 0.24$, $C_3 = 0.016$, and $OD = 1.039$

$$\text{Predconc} = \frac{-0.24 + \sqrt{0.24^2 - 4(-0.01)(0.016 - 1.039)}}{2(-0.01)} = 5.542$$

9.6. Mean Percent Errors

9.6.1. Mean percent errors are determined for each standard concentration of the database curves. The percent error is calculated from the predicted concentration and the theoretical concentration (tconc).

$$\text{Mean percent error} = \left| \frac{\text{predconc} - \text{tconc}}{\text{tconc}} \right| \times 100\%$$

For example, given the following predicted concentration for the 1.20-ng/mL standard:

$$\text{Predconc} = 1.209 \text{ ng/mL}$$

$$\text{Mean percent error} = \left| \frac{1.209 - 1.20}{1.20} \right| \times 100\% = 0.75\% \text{ error}$$

9.7. Standard Deviation

$$\text{Standard deviation} = \sqrt{\frac{\sum_{i=1}^N (y - \bar{y})^2}{N - 1}}$$

Where: y = individual data values,
 N = number of data values

9.8. Coefficient of Variation

$$\% \text{ coefficient of variation (CV)} = \frac{\text{standard deviation}}{\text{mean}} \times 100\%$$

10. QUALITY CONTROL

10.1. Analytical Batch Definition

An analytical batch of samples is defined as a group of 96 wells. The size of the batch is based on the capacity of the unit (1 solid microplate) of the 2mEPSPS ELISA test kit. An analytical batch of less than 96 wells can be analyzed. The first 24 wells (well positions in columns 1, 2, and 3: A1-H1, A2-H2, A3-H3) are used for triplicate analysis of the seven concentrations of the standard and a reagent blank (buffer) control. The Quality Control (QC) Sample should be included in each batch if available. Following the Quality Control Sample, up to 35 samples may be analyzed in duplicate (two wells). If more samples are to be analyzed than can be accommodated in one plate, the remaining samples should be analyzed as a different analytical batch with a new standard curve.

10.2. Study Samples

All study samples should be assayed at least in duplicate. If the concentration of 2mEPSPS in the sample exceeds the range of the assay, dilute the sample with PBST/Casein and then assay the diluted sample aliquot. Multiply the result by the appropriate method factor and dilution factor to obtain the final result.

10.3. Criteria for Acceptance of an Analytical Batch

Each run shall meet the acceptance criteria in the procedure to be valid as listed below. If the data fail to meet these performance criteria, the analyst should evaluate the results, determine the potential source of the variation, and repeat the analysis if necessary.

Assay Buffer Blank	Absorbance (450 nm-650 nm) < 0.150
200 ng/mL standard	Absorbance (450 nm-650 nm) \geq 0.900
Calibration curve	r^2 (Correlation of determination) > 0.990
All positive reference standards	CV (OD) of triplicates \leq 15%
Unknown or QC samples	CV (OD) of replicates \leq 20%
Quality control sample (if applicable)	Measured value within \pm 20% expected value

10.4. Specificity

This 2mEPSPS ELISA method is specific to 2mEPSPS and shows no cross-reactivity with other transgenic proteins including AAD-1, AAD-12, Cry1Ac, Cry1F, Cry34Ab1, Cry35Ab1, CP4 EPSPS, and PAT.

10.5. Matrix Effect

Tissue matrix effects have been evaluated by comparing standard curves that had not been fortified with matrix to those that have been fortified. Three different matrix dilutions (1X, 5X, and 10X) were tested, representing dilution levels commonly used in the ELISA. A difference of greater than 15% between the observed and theoretical means for any of the seven standard concentration levels was considered indicative of a matrix effect. No matrix effects were found at the 5X and 10X spiked matrix levels for all tissues. However, matrix effects were found in root R3, forage R3, leaf V5, and grain at the 1X level. A 10X dilution or greater is recommended for all matrices on the basis of experimental observations.

10.6. Modifications and Uses

Modifications to the assay procedure are not recommended. This procedure is for use with soybean tissue samples (grain, leaf V5, leaf V10-12, forage R3, and root R3). Analysis of other sample matrices requires that a method validation be performed prior to implementation.

11. RESULTS AND DISCUSSION

11.1. Confirmatory Method

If needed, the detection of 2mEPSPS protein in soybean tissue samples can be confirmed by Western Blotting, using Dow AgroSciences, LLC SOP ECL-27 “SDS-PAGE and Western Blotting”.

11.2. Assay Time

The time required to analyze a typical batch (35 samples or recoveries in duplicate, 7 or 8 standards in triplicate), including the sample extraction, is estimated to be 4-5 hours.

11.3. Limitation of the Method

This ELISA method is limited to samples where the amount of 2mEPSPS protein can be correlated with the level of 2mEPSPS present in the microbial standard or reference material used.

The 2mEPSPS ELISA test kit is designed to give optimum performance at ambient temperatures between 20 °C and 30 °C. The absorbance of the highest reference material should be equal to or greater than 0.900 optical density (OD) and should not fall outside the linear range of the spectrometer. At temperatures greater than 30 °C, OD values will rise more rapidly, and a reduced substrate incubation time may be necessary. At low temperatures (less than 20 °C) the substrate incubation time should be increased.

12. NOTES

- 12.1. Equipment, glassware, materials, reagents, and chemicals considered to be equivalent to those specified may be substituted with the understanding that their performance must be confirmed by the appropriate tests. Common laboratory supplies are assumed to be readily available and are therefore not listed. If running assay on an automated analyzer, refer to the equipment User Manual for any additional reagents or materials.

- 12.2. The analytical method was validated using a 2mEPSPS ELISA Kit available from Acadia BioScience, LLC. Equivalent kits may be used provided that their performance is confirmed by the appropriate tests.
- 12.3. PBST may be made from individual ingredients or can be prepared from a concentrated solution to achieve the same final concentration.

13. REFERENCES

- 13.1. Keith, L. H.; Crummett, W.; Deegan, J., Jr.; Libby, R. A.; Taylor, J. K.; Wentler, G. 1983 Principles of environmental analysis, *Anal. Chem.*, 55, 2210-2218.

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Template Sheet

Analyst: _____
 Date: _____
 Protein Assay: _____
 Standard TSN: _____
 Standard Lot: _____
 Sample Matrix: _____
 Sample ID: _____

Protocol Number: _____
 Experimental Purpose: _____

ELISA Kit Information

Lot # _____
 Kit: _____
 Plate: _____
 Conjugate: _____
 Substrate: _____
 Stop Solution: _____
 Buffer: _____

STDs Buffer: _____
 Sample Buffer/Extraction: _____
 Sample Buffer/Dilution: _____

Plate Identification: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Comments: _____

Figure 1. Sample 96-well ELISA Plate Template

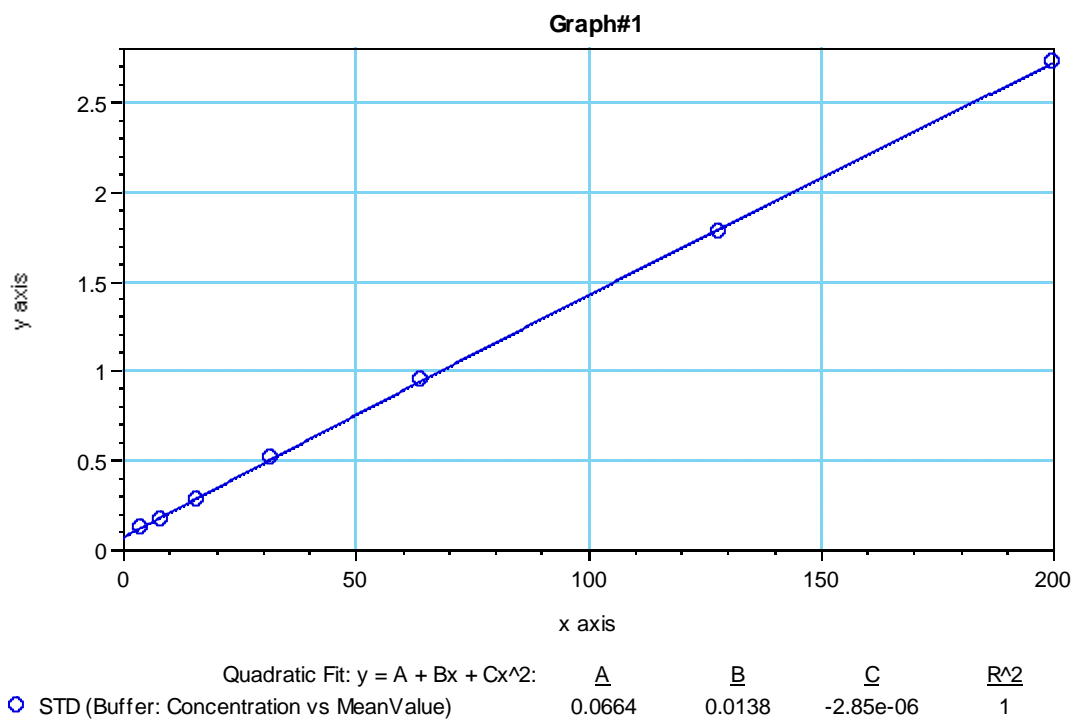


Figure 2. Sample Standard Curve for 2mEPSPS