Page 1-3 not required

Project Title

Development and Validation of a Direct ELISA for Quantitation of CP4 5-Enolpyruvylshikimate-3-Phosphate Synthase (CP4 EPSPS) Protein in Corn Tissues from Roundup Ready® Plants

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Project Completed

September 30, 1999

Performing Laboratory

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Laboratory Project ID

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Signatures of Approval

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TABLE OF CONTENTS

TABLE OF CONTENTS (cont'd.)

Tables

TABLE OF CONTENTS (cont'd.)

Page

Figures

ABBREVIATIONS

≈	approximately
CP4 EPSPS	CP4 5-enolpyruvylshikimate-3-phosphate synthase
CV	coefficient of variation
E. coli	*Escherichia coli*
ELISA	Enzyme-Linked ImmunoSorbent Assay
fwt	fresh weight of tissue
HRP	horseradish peroxidase
LOD	limit of detection
LOQ	limit of quantitation
MSL	Monsanto Technical Report, St. Louis
OD	optical density = absorbance
PBST	phosphate buffered saline, Tween 20
QC	quality control
R^2	coefficient of determination for line regression
RR	Roundup Ready®
SD	standard deviation
SOP	standard operating procedure
T:B	tissue to buffer
TBA	Tris-borate with L-ascorbic acid
TrisCl	Tris (hydroxymethyl) aminomethane hydrochloride

1. SUMMARY

A direct enzyme-linked immunosorbent assay (ELISA) was developed and validated to quantitate CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein levels in extracts from Roundup Ready® (RR) corn tissues. CP4 EPSPS protein confers tolerance to glyphosate (the active ingredient in Roundup™ herbicide) at the whole plant level. The double antibody sandwich ELISA utilizes a monoclonal anti-CP4 EPSPS antibody for capture and a goat polyclonal anti-CP4 EPSPS antibody conjugated to horseradish peroxidase (HRP) for detection of the CP4 EPSPS protein. Quantitation of the plant-expressed CP4 EPSPS was accomplished using purified *E. coli*-produced CP4 EPSPS protein as a reference standard. Forage was extracted using Tris-borate with L-ascorbic acid (TBA) buffer at 1:50 tissue to buffer ratio. Grain was extracted using phosphate buffered saline with Tween 20 (PBST) buffer at 1:100 tissue to buffer ratio.

The method was evaluated for accuracy (extraction efficiency and spike recovery) and precision. In addition, limits of detection (LOD) and quantitation (LOQ) for each matrix were defined. Extraction efficiency of CP4 EPSPS from forage ranged from 88.1 - 93.6% with a mean value of 90.6%. Extraction efficiency of CP4 EPSPS from grain ranged from 89.8 - 93.1% with a mean value of 91.1%. Average recoveries of CP4 EPSPS from forage ranged from 46.6 - 88.8% with an overall mean value of 63.1% at spike levels of 1.25, 5 and 20 ng/mL. Average recoveries of CP4 EPSPS from grain ranged from 72.1- 86.8% with an overall mean value of 79.1% at spike levels of 1.25, 5 and 20 ng/mL. The lower recovery of the CP4 EPSPS protein from forage and grain may be due to matrix effects caused by corn tissue which results in a bias in the estimation of CP4 EPSPS protein levels. Applications of the bias correction factors 0.57 and 0.72 for forage and grain, respectively, provides more accurate estimation of the true amount of protein present in the tissues. Based on dilution and conversion factors for each matrix, the LOQ was calculated to be 0.05 µg/g fwt for forage and 0.09 µg/g fwt for grain. The validated ELISA was shown to be accurate, precise and specific for the quantitation of the CP4 EPSPS protein levels in corn forage and grain tissues produced from RR plants.

2. INTRODUCTION

Monsanto Company has developed Roundup Ready® corn line NK603 which is tolerant to glyphosate (the active ingredient in Roundup™ herbicide) at the whole plant level. Corn line NK603 contains a 5-enolpyruvylshikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 (CP4 EPSPS). Corn plants that demonstrate commercial level tolerance to Roundup™ herbicide are called Roundup Ready® (RR). The CP4 EPSPS gene from *Agrobacterium* sp. strain CP4 has been completely sequenced and encodes a 47.6 kDa protein consisting of a single polypeptide of 455 amino acids (Padgette et al., 1996). The CP4 EPSPS protein is functionally similar to plant EPSPS enzymes but has a much reduced affinity for glyphosate (Padgette et al., 1993).

The purpose of the experiments described herein was to develop and validate an ELISA for quantitation of the CP4 EPSPS protein. This report summarizes the development and validation of a direct ELISA using a monoclonal anti-CP4 EPSPS antibody for capture and a goat polyclonal anti-CP4 EPSPS antibody conjugated to HRP for detection of the CP4 EPSPS protein.

3. MATERIALS

The equipment and reagents needed for the extraction of CP4 EPSPS protein from corn tissues and for performing the CP4 EPSPS ELISA are specified in standard operating procedure (SOP) BR-ME-0197.

3.1 Protein Standard

The protein standard (lot #5199245) used in the ELISA development and validation was purified to a > 90% purity from *E. coli* expressing the *Agrobacterium* sp. strain CP4 EPSPS gene. Aliquots of standard were stored at approximately -20°C in 50 mM TrisCl, pH 7.5, 50% (v/v) glycerol, 2 mM DTT, 50 mM KCl at a concentration of 3.96 mg/mL. The protein standard has been previously characterized (Harrison et al.,1993).

3.2 Antibodies

Monoclonal anti-CP4 EPSPS antibody (lot #6199732) was purified to > 95% purity (TSD Bioservices, Newark, DE) from ascites produced from cell line 39B6.2 (Strategic Diagnostics, Newark, DE). The purified antibody was stored at approximately 4°C in 20 mM sodium phosphate, 150 mM sodium

chloride and 15 ppm ProClin 300, pH 7.2 at a concentration of 3.2 mg/mL. Polyclonal goat anti-CP4 EPSPS antibody was purified from goat sera HRB-G856 and HRB-G854 (Harlan Bioproducts for Science, Indianapolis, IN) using Protein-G techniques (TechServ Associates, St. Louis, MO). The purified antibodies (lot #6558603-A, 8.1 mg/mL and lot 6558603-C, 7.9 mg/mL, respectively) were each conjugated to horseradish peroxidase (HRP) using a modified periodate oxidation method (GEN-PRO-077). Equal amounts of the conjugated antibodies (lot #6558603-B, 6.0 mg/mL and lot #6558603-D, 4.8 mg/mL, respectively) were pooled (lot #6558618) and aliquots were stored at approximately 4°C in 0.02 M potassium phosphate, 0.15 M NaCl, pH 7.3 and 0.01% thimerosal at a concentration of 5.4 mg/mL. Monoclonal anti-CP4 EPSPS antibody was used for capture and goat polyclonal anti-CP4 EPSPS antibody conjugated to HRP was used for ELISA detection antibody. The optimum concentrations were 1 µg/mL for capture and 0.27 µg/mL for the HRP conjugated detection antibodies.

3.3 Test and Control Samples

The test samples were the forage and grain tissues collected from corn line NK603 which contain the CP4 EPSPS protein. All tissues were collected from field grown plants (Study 98-01-46-01) and used as test samples in the ELISA development and validation experiments. The control samples were the forage and grain tissues collected from corn line LH82 x B73 that do not contain the CP4 EPSPS protein. All tissues were also collected from field grown plants (Study 98-01-46-01) and used as control samples in the ELISA.

4. METHODS

4.1 Summary of Assay Procedure

The analytical method is based upon an ELISA technique as described in SOP #BR-ME-0197. It utilizes a double antibody sandwich which directly detects the CP4 EPSPS protein levels in extracts produced by homogenization of plant tissues.

Polystyrene microtiter plates (Nunc Maxisorp, Cat. #472230) are coated with monoclonal anti-CP4 EPSPS protein antibody. Coated plates are incubated overnight at ≈ 4°C prior to use. To each well is added an equivalent amount of goat anti-CP4 EPSPS IgG conjugated to HRP (Sigma Chemical, St. Louis, MO). Plant extracts are then added to antibody-coated wells and a concentration range of CP4 EPSPS protein standards are added to a separate

set of wells. The plate is incubated for ≈1 hour at ≈ 37°C, allowing antigen capture by the surface bound antibodies and formation of an antibody sandwich. After washing the wells, an HRP substrate, 3,3', 5,5' tetramethylbenzidine (TMB, Kirkegaard & Perry, Gaithersburg, MD) is added to each well. Wells containing CP4 EPSPS protein will form an antibody-antigen-antibody-HRP sandwich, which reacts with the substrate to form a blue color. The reaction is stopped by the addition of 6 M phosphoric acid to each well which results in a color change from blue to yellow. Quantitation of CP4 EPSPS protein level in a sample is accomplished by comparison of sample absorbance (OD units at 450 nm, reference 650nm) to the absorbance curve produced by the range of CP4 EPSPS protein standards. The standard curve is generated using a 4-parameter transformation of CP4 EPSPS concentration vs absorbance. Four curve parameters a, b, c and d are generated and monitored along with the coefficient of determination (R^2). The standard curve encompasses the concentration range of 0.625 - 40.0 ng/mL. Data reduction calculations, converting the absorbance to a concentration of CP4 EPSPS were accomplished using Soft max Pro version 2.4.1 (Molecular Devices, Sunnyvale, CA) and Microsoft Excel™.

4.2 Assay Development

4.2.1 Extraction of CP4 EPSPS from Forage and Grain

Forage or grain tissue was frozen and then ground to a powder in a blender (Waring, model 34BL97). Excess heating was controlled by adding liquid nitrogen or dry ice to the blender prior to grinding. Samples were extracted using the appropriate buffer in a polytron (Brinkman Instruments model PT 3000 fitted with a PT-DA 3012/2TS generator) using two 15 second bursts. The insoluble fraction of the extract was pelleted by centrifugation (10,000 x g, 10 minutes) and the supernate was saved for analysis. Aliquots of the sample were transferred to microfuge tubes and stored in a -80°C freezer until needed.

Forage tissue was extracted using Tris-borate with L-Ascorbic acid (TBA) buffer, containing 100 mM Tris base, 100 mM sodium borate, 5 mM magnesium chloride, 0.05% (v/v) Tween 20 at pH 7.8 and 0.2% (w/v) L-Ascorbic acid at 1:50 tissue to buffer ratio. Grain was extracted using phosphate buffered saline with Tween 20 (PBST) buffer, containing 8.1 mM sodium phosphate, 138 mM sodium chloride, 1.5 mM potassium phosphate, 2.7 mM potassium chloride and 0.05% (v/v) Tween 20 at pH 7.4 at 1:100 tissue to buffer ratio.

4.2.2 Matrix Effects

The concentrations of the CP4 EPSPS protein measured by ELISA across different dilutions of plant extracts included in the standard curve were evaluated. These experiments establish the linear range and show possible matrix effects due to corn forage and grain tissues in the ELISA. Forage extract from control line LH82 x B73 was added to the assay buffer (PBST/0.1% BSA) used to prepare the standard curve at extract to buffer ratios of 1:16, 1:32 and 1:64. The calculated concentrations of the three standard curves containing the matrix were compared with the standard curve obtained in the presence of assay buffer alone. A regression analysis using a linear equation will produce a line with a slope approximately equal to one and a y-intercept value of about zero if there is no significant matrix effect. Only the regression analysis for the highest concentration of matrix is graphically shown. Grain extract from control line LH82 x B73 was added to the assay buffer at extract to buffer ratios of 1:4, 1:8 and 1:16 and the calculated concentrations were evaluated by ELISA as described above.

4.2.3 Parallelism

In addition to characterization of the analytical standard, ELISA analyses were also performed to evaluate the parallelism or immunological equivalence of the CP4 EPSPS protein standard and the plant-produced CP4 EPSPS protein. These experiments establish the appropriateness of using the *E. coli*-produced protein standard to quantitate the CP4 EPSPS protein in extracts from RR plants. ELISA response curves generated by dilutions of forage and grain tissue extracts were compared with dilutions of CP4 EPSPS protein standard. Serial dilutions of forage and grain extracts were analyzed for CP4 EPSPS protein by ELISA. The concentrations in ng/mL were plotted against the respective dilutions. A linear regression analysis will produce a line with a slope value close to zero if the two analytes are immunologically equivalent.

4.3 ELISA Validation

Tissues were homogenized with appropriate buffers and the extracts were evaluated for CP4 EPSPS protein by ELISA according to SOP #BR-ME-0197. The method was evaluated for accuracy and precision for quantitation of the CP4 EPSPS protein levels in forage and grain.

4.4 Accuracy

The accuracy and bias of the ELISA for quantitation of CP4 EPSPS protein levels in corn tissue is defined by two components: extraction efficiency and spike recovery of the CP4 EPSPS protein from each tissue type.

4.4.1 Extraction Efficiency

The extraction efficiency experiments for corn forage and grain were conducted to estimate the amount of CP4 EPSPS protein that was extractable under optimized conditions. Three replicates of test samples from each tissue type were extracted with the appropriate extraction buffers. Three replicates of control samples from each tissue type were also extracted as a background control for the test samples. The insoluble fraction was pelleted by centrifugation (10,000 x g, 10 minutes) and the supernate was saved for analysis (S1). The pellet from the first extract was re-extracted a second time using identical conditions and the supernate was saved for analysis (S2). The pellet from the second extract was re-extracted a third time using identical conditions and the supernate was saved for analysis (S3). A final extraction of the third pellet was performed using extraction buffer and the final (S4) supernate was retained. Supernates S1, S2, S3 and S4 were analyzed by ELISA to determine CP4 EPSPS protein concentration. Percent extraction efficiency was calculated by determining the percent of CP4 EPSPS extracted in S1 compared to the total amount of CP4 EPSPS in all four extracts. The mean extraction efficiency, range and standard deviation (SD) were calculated for each tissue type.

4.4.2 Spike and Recovery

The other component of accuracy estimated for the measurement of CP4 EPSPS protein was spike and recovery. Known amounts of CP4 EPSPS protein were spiked into extraction buffers containing control forage or grain tissue. The spike levels were 1.25, 5 and 20 ng CP4 EPSPS/mL for both forage and grain. These mixtures were then subjected to homogenization and quantitation using the procedure previously described. The percent recovery of CP4 EPSPS protein was determined by dividing the amount of CP4 EPSPS recovered from the matrix by the amount of CP4 EPSPS recovered from the assay buffer control and then multiplying by 100. The average recovery, overall mean recovery and SD were calculated for each tissue type.

4.4.3 Assay Bias Correction

The overall assay bias was reported as a correction factor used to estimate the true amount of the CP4 EPSPS protein present in the tissue. The bias factor was calculated by multiplying the two components of accuracy (% extraction efficiency/100) x (% spike recovery/100). The amount of CP4 EPSPS protein measured by ELISA corrected for bias was calculated by dividing the ELISA value in µg/g by the bias factor determined for each tissue type.

4.5 Precision

4.5.1 Intra-Assay and Inter-Assay Precision

Intra-assay precision was based on the pooled variation (% CV) derived from OD values in standards from 10 independent assays performed over a period of 15 days. Inter-assay precision was based on the analysis of aliquots of frozen forage quality control (QC, lot #6178559) sample prepared from a pool of RR corn line NK603 field grown plants (Study 98-01-46-01) which were used for inter-assay controls. These data were obtained from 10 separate assays performed over a period of 44 days, when the assay was in routine use.

4.5.2 Limits of Detection and Quantitation

The limit of detection (LOD) was calculated as the mean absorbance for the control line plus 3 standard deviations. The lower limit of quantitation (LOQ) was defined as the lowest reference standard concentration (0.625 ng/mL). The upper LOQ was the highest standard concentration (40 ng/mL). Based on dilution and conversion factors for each matrix, the LOQ was calculated to be 0.05 µg/g fwt for forage and 0.09 µg/g fwt for grain.

4.5.3 Variability of the ELISA for Quantitation of CP4 EPSPS

Method variability (including extraction) was evaluated by extracting and analyzing by ELISA several replicates from the same tissue pool of corn line NK603 from each matrix. Forage was extracted at 1:50 tissue to buffer ratio in TBA and loaded at 1:32 dilution in the well for analysis. Grain was extracted at 1:100 tissue to buffer ratio in PBST and loaded at 1:8 dilution in the well for analysis. The % CV was calculated for each tissue type.

4.6 Storage Stability

The stability of the CP4 EPSPS protein in corn tissues has been previously evaluated (Elswick and Sanders, 1995a, b, c) to ensure that samples are properly stored and analyzed within the known limits of stability.

5. RESULTS AND DISCUSSION

5.1 Assay Development

5.1.1 Matrix Effects

A linear regression analysis on the 1:16 (extract to buffer ratio) forage extract sample produced a slope value of 0.75 and a y- intercept value of 0.01. The matrix effects due to corn forage tissue in the CP4 EPSPS ELISA are shown in Figure 1. A linear regression analysis on the 1:4 (extract to buffer ratio) grain extract sample produced a slope value of 0.68 and a y- intercept value of 0.11. The matrix effects due to corn grain extracts in the CP4 EPSPS ELISA are shown in Figure 2. Corn forage and grain extracts showed slight to moderate matrix effects in the CP4 EPSPS ELISA. These matrix effects were accounted for by the bias correction factor in the analysis of study samples.

5.1.2 Parallelism

A linear regression analysis produced slope values of -0.04 and 0.10 for CP4 EPSPS standard and forage extracts, respectively. The parallelism curves for corn forage extracts obtained from RR corn line NK603 and CP4 EPSPS protein standard evaluated by ELISA are shown in Figure 3. A linear regression analysis produced slope values of -0.04 and 0.10 for CP4 EPSPS standard and grain extracts, respectively. The parallelism curves for corn grain extracts obtained from RR corn line NK603 and CP4 EPSPS protein standard are shown in Figure 4. CP4 EPSPS standard, corn forage and grain extracts have slope values close to zero and are immunologically equivalent.

5.2 ELISA Validation

5.3 Accuracy

5.3.1 Extraction Efficiency

Extraction efficiencies of CP4 EPSPS protein from forage ranged from 88.1 - 93.6% with a mean value of 90.6% and a SD of 2.7%. Extraction efficiencies

of CP4 EPSPS protein from grain ranged from 89.8 - 93.1% with a mean value of 91.1% and a SD of 1.7%. The results obtained from the extraction efficiency experiments are summarized in Table 1.

5.3.2 Spike and Recovery

Average recoveries of CP4 EPSPS protein from forage ranged from 46.6 - 88.8% with an overall mean value of 63.1% and a SD of 22.6% at spike levels of 1.25, 5 and 20 ng/mL. Average recoveries of CP4 EPSPS protein from grain ranged from 72.1- 86.8% with an overall mean value of 79.1% and a SD of 7.3% at spike levels of 1.25, 5 and 20 ng/mL. The results obtained from the recovery of spike experiments are summarized in Table 2.

5.3.3 Assay Bias Correction

Assay bias correction factor was calculated for each tissue type by mutiplying the two components of accuracy (% extraction efficiency/100) x (% spike recovery/100). The mean extraction efficiency for forage was 90.6% and the overall mean recovery was 63.1%. The mean extraction efficiency for grain was 91.1% and the overall mean recovery was 79.1%. The bias correction factors were 0.57 and 0.72 for forage and grain, respectively. The calculated bias correction results are summarized in Table 3.

5.4 Precision

5.4.1 Intra-Assay and Inter-Assay Precision

The intra-assay % CV (based on OD values in triplicates of standard curve in assay buffer) was about 10% or less for concentrations of CP4 EPSPS between 1.25 and 40 ng/mL. As expected, assay variation was much greater at the lowest concentration of CP4 EPSPS (0.625 ng/mL) but decreased to about 10% or less at concentrations above 1.25 ng/mL. The intra-assay precision results are summarized in Table 4. Inter-assay % CV (based on a QC sample run on every plate) was 14.9%. The inter-assay precision results are summarized in Table 5.

5.4.2 Variability of the ELISA for Quantitation of CP4 EPSPS

The variability of the method (including extraction) was evaluated for each matrix as previously described. The % CV were 8.7 and 9.4 for forage and grain, respectively. The data were taken from 3 and 6 different replicates evaluated by ELISA for forage and grain respectively. The variability of the ELISA results are summarized in Table 6.

6. CONCLUSIONS

A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was developed and validated to quantitate CP4 EPSPS protein levels in corn forage and grain tissues produced from RR plants. The method was shown to be accurate, precise and specific for the quantitation of CP4 EPSPS protein. CP4 EPSPS ELISA validation data and assay evaluation criteria are summarized in Table 7.

7. ACKNOWLEDGEMENTS

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Table 1. Extraction Efficiency[1] of CP4 EPSPS from Corn Forage and Grain

Tissue Type	T:B Ratio	Range % Extr'n. Efficiency	Mean % Extr'n. Efficiency	SD
Forage	1:50	88.1 - 93.6	90.6	2.7
Grain	1:100	89.8 - 93.1	91.1	1.7

[1]The values were calculated from 3 replicates of test samples.

Table 2. Spike and Recovery of CP4 EPSPS from Corn Forage and Grain

Tissue Type	T:B Ratio	Spike Level (ng)	Average % Recovery[1]	Overall Mean % Recovery	SD[2]
Forage	1:50	1.25	53.9	63.1	22.6
		5	46.6		
		20	88.8		
Grain	1:100	1.25	72.1	79.1	7.3
		5	78.4		
		20	86.8		

[1]The values were calculated from 3 replicates for each spike level.
[2]The values were calculated from the average % recovery for 3 spike levels.

Table 3. Overall Assay Bias for CP4 EPSPS ELISA in Corn Forage and Grain

Tissue Type	Mean % Extr'n. Efficiency	Overall Mean % Recovery	Bias Factor[1]
Forage	90.6	63.1	0.57
Grain	91.1	79.1	0.72

[1]Bias factor = (mean % extr'n. eff./100) x (overall mean % recovery/100)

Table 4. Intra-Assay Precision Profile for CP4 EPSPS ELISA

Standard No.	Concentration ng/mL	% CV (mean)[1]
1	40	3.0
2	20	4.0
3	10	5.2
4	5	3.9
5	2.5	10.1
6	1.25	8.5
7	0.625	26.2

[1]Mean % CV values were based on the pooled variation derived from OD values in standards from 10 separate assays performed on different days.

Table 5. Inter-Assay Precision Profile for CP4 EPSPS ELISA

Assay No.	QC Sample ng/mL
1	12.4
2	16.9
3	12.4
4	14.5
5	20.1
6	15.7
7	16.4
8	17.9
9	16.4
10	14.9
Mean	15.7
SD	2.4
% CV[1]	14.9

[1]% CV value was based on variation derived from QC sample (lot #6178559).

Table 6. Variability of the ELISA for Quantitation of CP4 EPSPS

Replicate No.	Forage Tissue (µg/g fwt)	Grain Tissue (µg/g fwt)
1	18.6	9.0
2	18.1	7.5
3	15.7	7.7
4	-	8.3
5	-	8.4
6	-	9.6
Mean	17.5	8.4
SD	1.5	0.8
% CV	8.7	9.4

Table 7. CP4 EPSPS ELISA Validation Summary

I. Accuracy

Tissue Type	T:B Ratio	Extraction Buffer	Extraction Efficiency (%)	Spike and Recovery (%)	Bias Correction Factor
Forage	1:50	TBA	90.6	63.1	0.57
Grain	1:100	PBST	91.1	79.1	0.72

II. Precision

Intra-Assay Variability: Std. Curve Precision	3.0 - 26.2% CV
Inter-Assay Variability: QC[1] sample	14.9% CV
CP4 EPSPS ELISA Variability:	8.7% CV for forage 9.4% CV for grain

III. Range of Quantitation:

	0.625 - 40 ng/mL CP4 EPSPS
Limit of Quantitation (LOQ):	0.05 µg/g fwt for forage 0.09 µg/g fwt for grain

IV. Assay Evaluation Criteria:

Quality Control (QC)[1] lot #6178559:	7.4 - 23.6 ng/mL CP4 EPSPS
OD of buffer blank:	< 0.17 OD
% CV for triplicate sample wells:	10%
Method for curve fit:	4-Parameter (Molecular Devices Soft max Pro)

[1]Quality control sample is a corn line NK603 forage extract which expresses CP4 EPSPS protein.

Figure 1. Matrix Effects in CP4 EPSPS ELISA Due to Corn Forage Tissue

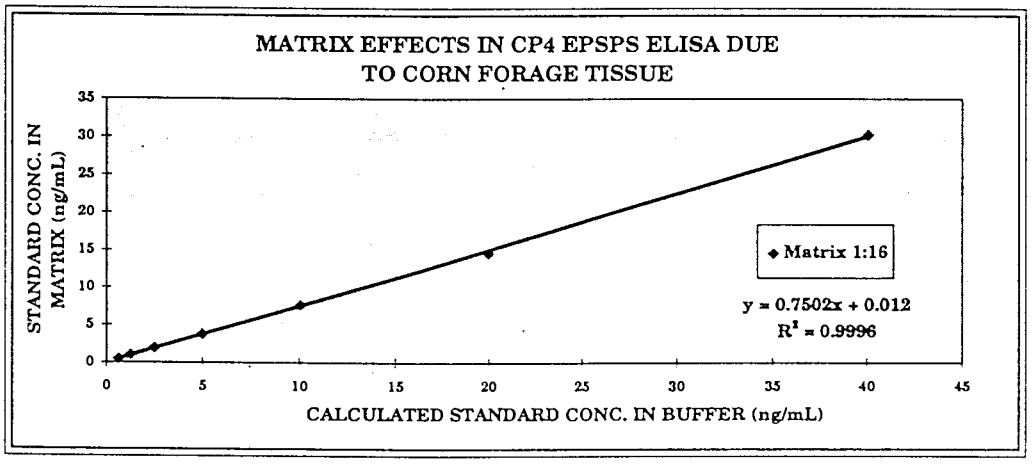

Figure 2. Matrix Effects in CP4 EPSPS ELISA Due to Corn Grain Tissue

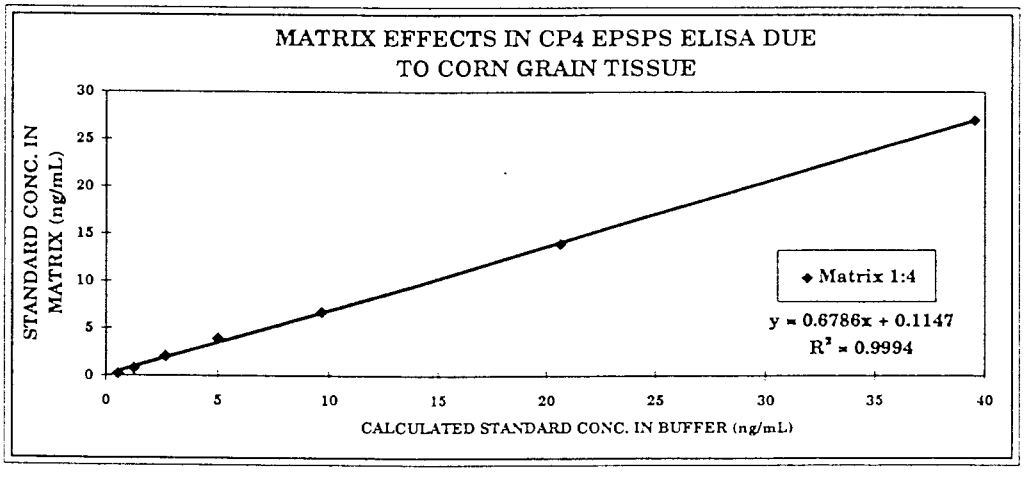

Figure 3. Parallelism of Corn Forage Extracts in the CP4 EPSPS ELISA

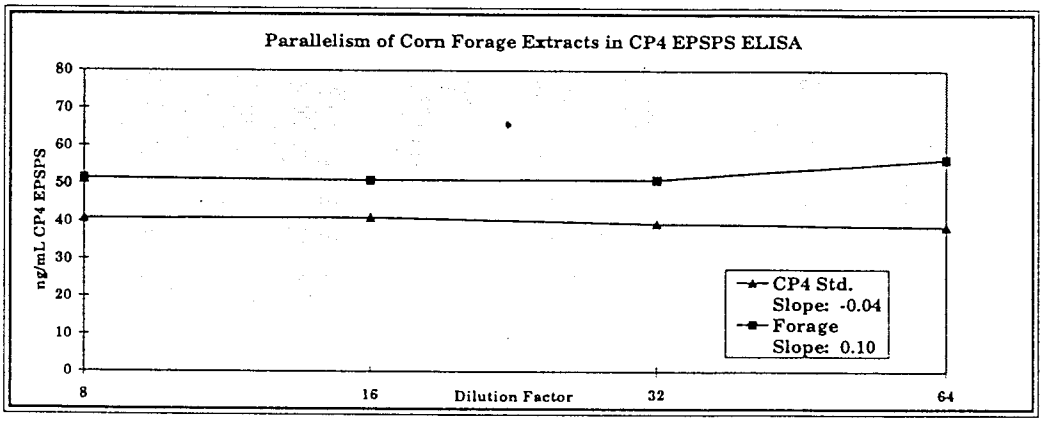

Figure 4. Parallelism of Corn Grain Extracts in the CP4 EPSPS ELISA