Study Title

Expression and Compositional Analyses of Roundup Ready™ Corn Lines MON 830, MON 831 and MON 832 in the 1995 U.S. Field Trials Following Treatment with Roundup® Herbicide

Authors

Patricia R. Sanders
Mark E. Groth
Bibiana E. Ledesma

Study Completed On

May 2, 1997

Performing Laboratories

Monsanto Company
CEREGEN
700 Chesterfield Parkway North
St. Louis, MO 63198

Corning Hazleton, Inc. (CHW)
Wisconsin Facility
3301 Kinsman Blvd.
Madison, WI 53704

Laboratory Project ID

Study 95-01-46-02
CHW Project 6103-187

Proprietary Information of
MONSANTO Company

Statement of Compliance

This study meets the requirements for 40 CFR Part 160 with the following exceptions:

1. The test and control substances for this study were not characterized at the molecular level of genetic elements to distinguish between corn lines until after their use in this study [160.105(a)].

2. The data contained in the Appendix was generated in another study conducted under GLP, not as part of this study.

3. Six leaf samples were processed prior to study initiation.

Jean-Noël Mabon — Applicant — 5-2-97 — Date

Ray Thuehs — Sponsor — 5/5/97 — Date

Patricia R Sanders — Study Director — 5-2-97 — Date

Quality Assurance Statement

Study Title: Expression and Compositional Analyses of Roundup Ready™ Corn Lines MON 830, MON 831 and MON 832 in the 1995 U.S. Field Trials Following Treatment with Roundup® Herbicide

Study Number: 95-01-46-02

Reviews conducted by the Quality Assurance Unit confirm that the final report reflects the raw data.

The following is a list of reviews conducted by Monsanto on the study reported herein. Additional reviews conducted by other Quality Assurance Units are presented in separate reports.

Dates of Audit/Inspection	Phase	Date Reported to Study Director	Date Reported to Management
Jul 25, 1995	Protocol Review	Jul 25, 1995	Jul 25, 1997
Sep 13, 1995	Inspection	Sep 13, 1995	Sep 13, 1995
Nov 21, 1995	Inspection	Nov 27, 1995	Nov 27, 1995
Apr 22-24, 1997	Data/Report Audit	Apr 24, 1997	Apr 24,1997
Apr 29, 1997	Data/Report Audit	Apr 29, 1997	Apr 29, 1997

DeAnn Holden
Quality Assurance Representative
Monsanto Company

May 2, 1997
Date

Signatures of Approval

Study Number: 95-01-46-02

Title: Expression and Compositional Analyses of Roundup Ready™ Corn Lines MON 830, MON 831 and MON 832 in the 1995 U.S. Field Trials Following Treatment with Roundup® Herbicide

Facilities: Monsanto Company
CEREGEN
700 Chesterfield Parkway North
St. Louis, Missouri 63198

Corning Hazleton, Inc.
Wisconsin Facility
3301 Kinsman Blvd.
Madison, WI 53704

Study Director: Patricia R. Sanders

Contributors: Monsanto (St. Louis): Mark E. Groth and Bibiana E. Ledesma
Corning Hazleton, Inc: Diane M. Henning

Study Initiation Date: July 27, 1995

Records Retention: All study specific raw data, protocols, final reports and facility records will be retained at Monsanto, St. Louis, except raw data and facility records for Corning Hazleton, Inc., Wisconsin Facility.

Specimen Storage: Any study samples that are to be retained will be stored at Monsanto, St. Louis.

Signatures of Approval:

Patricia R Sanders — 5-2-97
Study Director — Date

Ray Stucker — 5/5/97
Sponsor — Date

Table of Contents

Page

Statement of Compliance 2

Quality Assurance Statement 3

Signatures of Approval 4

Table of Contents 5

Abbreviations 7

I. Summary 9

II. Introduction
- A. Background 10
- B. Purpose 10

III. Materials
- A. Test substance 11
- B. Control substance 11
- C. Characterization of test and control substances 11
- D. Reference substance 11
- E. Test system 12

IV. Methods
- A. Summary of experimental design 12
- B. Field trials 12
- C. ELISA analytical methods 13
- D. Compositional analytical methods 14
- E. Control of bias 17
- F. Data reduction and statistical analyses 17
- G. Protocol amendment 18

V. Results and Discussion
- A. Field trial 18
  - 1. Test and control substance characterization 18
  - 2. Plant samples 18

Table of Contents (cont'd.)

Page

B. Protein expression in corn plant samples 19
1. CP4 EPSPS protein levels in corn tissues 20
2. GOX protein levels in corn tissues 21
C. Compositional analyses of grain and forage samples 22
1. Proximate analysis of corn grain 22
2. Amino acid composition of corn grain 24
3. Fatty acid profile of corn grain 26
4. Tocopherol, calcium and phosphorus analysis of corn grain 27
5. Proximate analysis of forage 28

VI. Conclusions 29

VII. Acknowledgments 29

VIII. References 30

Tables

1 Levels of CP4 EPSPS Protein in Leaf, Forage and Grain Samples 20
2 Levels of GOX Protein in Leaf, Forage and Grain Samples 21
3 Summary of Proximate Analysis of Corn Grain 23
4 Amino Acid Composition of Corn Grain 25
5 Fatty Acid Composition of Corn Grain 26
6 Analysis of Tocopherol and Inorganic Components of Corn Grain 27
7 Summary of Analysis of Forage 28

Attachment

Protocol 35

Abbreviations

ADF	Acid detergent fiber
a	acre
ai	active ingredient
AOAC	Association of Official Analytical Chemists
AP	Alkaline phosphatase
≈	approximately
B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
BSA	Bovine serum albumin
°C	degree Celsius
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium* species strain designated CP4
cryIA(b)	Class I (Lepidoptera-specific) crystal protein gene
CryIA(b)	Class I (Lepidoptera-specific) crystal protein
CV	Coefficient of variance
DTT	Dithiothreitol
ECB	European corn borer
ECL	Enhanced chemiluminescence
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
Exp	Experiment
fwt	Fresh weight of tissue
g	Gram
GOX	Glyphosate oxidoreductase protein
gox	Glyphosate oxidoreductase gene
IPC	Insect Protected Corn
IP/RR	Insect Protected Roundup Ready™ Corn
KCl	potassium chloride
kg	Kilogram
l	liter
LOD	Limit of Detection
lb	pound
M	Molar
mg	Milligram
$MgCl_2$	Magnesium chloride
mL	Milliliter

Abbreviations (cont'd.)

mM	Millimolar
N.A.	Not applicable
NaCl	sodium chloride
N.D.	Not detected
NDF or NDFE	Neutral detergent fiber
ng	Nanogram
NPTII	Neomycin phosphotransferase II protein
nptII	Neomycin phosphotransferase II gene
O.D. (OD)	Optical density
PBST	Phosphate-buffered saline, Tween
PBSTO	Phosphate-buffered saline, Tween, ovalbumin
PMSF	Phenylmethylsulfonyl fluoride
pNPP	para-Nitrophenyl phosphate
RR	Roundup Ready™
SDS	Sodium dodecyl sulfate
SOP	Standard operating procedure
TBA	Tris borate ascorbic extraction buffer
TMB	(3,3',5,5' Tetramethylbenzidene) peroxidase substrate
Tris	tris(hydroxymethyl)-aminomethane
subsp.	subspecies
µg	Microgram
µL or µl	Microliter
wt	weight

I. Summary

Corn lines MON 830, MON 831 and MON 832, have been modified to express the genes encoding CP4 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4 EPSPS) (Padgette *et al.*, 1996) and glyphosate oxidoreductase (GOX) (Padgette *et al.*, 1996). These proteins confer tolerance to glyphosate (the active ingredient in Roundup® herbicide) at the whole plant level. Corn lines tolerant to glyphosate are called Roundup Ready (RR). The corn transformation plasmids used to produce these corn lines included the *cryIA(b)* gene (Höfte and Whitely, 1989) which was not integrated into these corn lines. The plasmids also included a gene cassette containing a bacterial specific promoter and the coding region for neomycin phosphotransferase, NPTII. The NPTII protein allows selection of bacteria containing the plasmid in media containing kanamycin. The *nptII* gene is under the control of a bacterial-specific promoter and therefore, does not produce the NPTII protein in plant cells. The control line, MON 822, has background genetics representative of the test lines, but has not been genetically modified and therefore, does not express the CP4 EPSPS or GOX proteins.

The purpose of this study was to evaluate the Roundup Ready corn lines following treatment with Roundup. This study was designed to estimate the levels of CP4 EPSPS and GOX proteins in leaf, forage and grain samples from several corn lines. Compositional analyses were performed on forage and grain samples.

Plant samples were collected from Roundup Ready and control corn plants grown in the 1995 U.S. field trials following treatment with Roundup, as representative of commercially grown corn. Therefore, data collected on protein expression levels and compositional components was representative of the levels expected in the commercial crop of these corn lines.

Expression levels of CP4 EPSPS and GOX proteins varied for each corn line analyzed and are sufficient to confer glyphosate tolerance at the whole plant level. The CP4 EPSPS and GOX protein levels measured in samples collected from Roundup treated plants were similar to levels in samples from unsprayed plants of the same lines.

The levels of the major components of corn grain (protein, fat, ash, neutral detergent fiber, acid detergent fiber, carbohydrates, moisture, alpha tocopherol, calcium, phosphorus, amino acids and fatty acids) were similar

between the test and control samples, and typical of the values published (Watson, 1982; Jugenheimer, 1976) and previously observed for control lines with similar genetic backgrounds (Sanders and Patzer, 1995; and Sanders *et al.*, 1996a,b; 1997a,b). The major components of forage (protein, fat, ash, neutral detergent fiber, acid detergent fiber, carbohydrate, moisture, calcium and phosphorus) were similar between the corn test lines and the control line, MON 822 and within the published literature ranges (Watson, 1982) and observed ranges for control lines with similar genetic backgrounds (Sanders *et al.*, 1996b; 1997b). It was concluded that these corn lines are substantially equivalent in composition to the control corn line and representative of corn grain currently in commerce.

II. Introduction

A. Background

Corn lines MON 830, MON 831 and MON 832, have been modified to express the genes encoding CP4 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4 EPSPS) (Padgette *et al.*, 1996) and glyphosate oxidoreductase (GOX) (Padgette *et al.*, 1996). These proteins confer tolerance to glyphosate (the active ingredient in Roundup® herbicide) at the whole plant level. Corn lines tolerant to glyphosate are called Roundup Ready (RR). The corn transformation plasmids used to produce these corn lines included the *cryIA(b)* gene (Höfte and Whitely, 1989) which was not integrated into these corn lines. The plasmids also included a gene cassette containing a bacterial specific promoter and the coding region for neomycin phosphotransferase, NPTII. The NPTII protein allows selection of bacteria containing the plasmid in media containing kanamycin. The *nptII* gene is under the control of a bacterial-specific promoter and therefore, does not produce the NPTII protein in plant cells. The control line, MON 822, has background genetics representative of the test lines, but has not been genetically modified and therefore, does not express the CP4 EPSPS or GOX proteins.

B. Purpose

The purpose of this study was to evaluate the Roundup Ready corn lines following treatment with Roundup. This study was designed to estimate the levels of CP4 EPSPS and GOX proteins in leaf, forage and grain samples from several corn lines. Compositional analyses were performed on forage and grain samples.

III. Materials

A. Test substance

The test substances for this study were the Roundup Ready (RR) corn lines MON 830, MON 831 and MON 832. The test substance seed were BC2F3 progeny, derived from crossing BC2F3 plants containing the Roundup Ready genes, by a non-genetically modified tester Mo17.

B. Control substance

The control substance for this study, MON 822 has not been genetically modified, but has background genetics representative of the test substances. The MON 822 seed planted were BC2F1 progeny, derived from crossing BC2F1 plants by a non-genetically modified tester, Mo17. The MON 822 seed was hybrid material. Two plots of MON 822 were planted at each site: one was sprayed with Roundup and the other plot remained unsprayed.

C. Characterization of test and control substances

The identity of the test and control substances was verified by the Study Director prior to their use in the study by verifying the chain-of-custody documentation supplied with the samples.

Southern blot analysis was performed to confirm corn line identity of the plants from which the samples were collected as part of Study 95-01-50-01 and has been archived with the study files (Groth and Sanders, 1997).

D. Reference substance

There was no reference substance for this study.

Appropriate standards were used in each assay as reference standards for the analytical procedures. The analytical standards used for compositional analyses are listed in the Analytical Subreport (Method Summaries), archived with the raw study data.

CP4 EPSPS protein standard for ELISA. CP4 EPSPS protein standard (lot #5192245, prepared 12-12-92) was purified to >90% purity from *E. coli* expressing an *Agrobacterium* species strain CP4 EPSPS gene (Harrison *et al.*, 1993). The aliquots of standard were stored at approximately -20°C in 50 mM Tris-HCl pH 7.5, 50% glycerol, 2 mM DTT and 50 mM KCl at 2.9 mg/mL.

GOX protein standard for ELISA. The reference substance was *E. coli* produced GOX protein, lot #LAH4/13/92 #8 characterized previously (Harrison *et al.*, 1994). The GOX standard was determined to be approximately 85% pure by gel densitometry of a Coomassie stained gel. The specific activity of the enzyme was 2.4 U/mg and was stored and used as a solution (0.63 mg/mL) in 40% sucrose and maintained at approximately -20°C.

E. Test system

The test system for this study was a panel of analytical biochemical methods. Validated Enzyme Linked Immunosorbent Assays (ELISA) were performed to estimate the CP4 EPSPS and GOX protein levels in the leaf, forage and grain samples. Compositional analyses were performed by published methods (Association of Official Analytical Chemists, AOAC, 1990) which are currently used to evaluate nutritional quality of corn.

IV. Methods

A. Summary of experimental design

Test and control corn plants were grown at five U.S. sites under conditions typical for corn in each region. These sites provided a variety of environmental conditions which were representative of regions where Roundup Ready corn lines would be grown as commercial products.

Glyphosate (Roundup-2139) was applied once at the rate of 0.56 lb ai/a when the corn plants were approximately at the V2 stage (Peters *et al.*, 1996).

Young leaf, forage and grain samples were collected from these plants as described in the Study Protocol (Attachment). These tissues were evaluated for CP4 EPSPS and GOX protein levels using sensitive and specific ELISA assays developed and validated for each protein. The compositional analyses of forage included measurements of moisture, protein, fat, ash, carbohydrates, fiber, calcium and phosphorus. The compositional analyses of grain included measurements of protein, moisture, fat, ash, carbohydrates, fiber, amino acid analysis, fatty acid profile, alpha tocopherol, calcium and phosphorus.

B. Field trials

Test lines MON 830, MON 831, and MON 832 and control line MON 822 were sprayed with glyphosate (Roundup-2139) at 0.56 lb ai/a when corn

plants were approximately at the V2 stage. The control line MON 822 was sprayed with Roundup-2139 to ensure that the rate of application was sufficient to kill nontransgenic corn. A second MON 822 plot was left unsprayed in this study to provide plant samples.

Samples were collected from plants at the following locations: Jerseyville, IL; VanHorne, IA; Ames, IA; Mead, NE; and Monmouth IL. The locations encompass a range of environmental conditions. Twenty-five to thirty-five seed of each corn line were planted at each site. All field sites were managed in a manner such that the identity and integrity of all samples was maintained. Line purity was maintained by bagging the tassels and ear shoots at anthesis and self-pollinating each plant. All samples from the corn plants were shipped promptly to Monsanto facilities, St. Louis, Missouri and stored according to the protocol (Attachment).

C. ELISA analytical methods

Extraction of protein from corn tissues. Corn tissues were processed and extracts prepared according to SOPs. Tissue was ground to a fine powder on dry ice or liquid nitrogen in a blender or vertical cutter mixer. All tissue powders were kept on dry ice during extract preparation. The tissue was extracted in the appropriate extraction buffer (as specified in the SOP) using a Polytron tissue homogenizer (Brinkman, Inc., Westbury, NY) at approximately 17,000 rpm for ≈ 30 seconds. Insoluble material was removed by centrifugation at ≈ 8,000 x g for 10-15 minutes at ≈ 4°C. The supernatant was removed and stored frozen at approximately -80°C until assayed.

CP4 EPSPS ELISA. An enzyme-linked immunosorbent assay, ELISA, has been developed and validated to quantitate the levels of CP4 EPSPS protein in genetically modified corn plants (Elswick, 1995a,b). CP4 EPSPS protein levels in corn tissue protein extracts were measured by a direct double antibody sandwich ELISA. The extraction buffer for CP4 EPSPS protein was PBST (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, 0.05% Tween 20). This assay used goat anti-CP4 EPSPS antibody to capture and rabbit anti-CP4 EPSPS conjugated to horseradish peroxidase to quantitate CP4 EPSPS protein levels. A horseradish peroxidase substrate, TMB, (3,3',5,5' Tetramethylbenzidene) was added for color development. Quantitation of sample CP4 EPSPS concentration was accomplished by extrapolation (based on sample absorbency value) from a CP4 EPSPS protein standard curve.

GOX ELISA. A direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) has been developed and validated to quantitate the levels of GOX protein in genetically modified corn plants (Davies, 1994; Davies and Sanders, 1995a). This ELISA uses goat anti-GOX antibody and alkaline phosphatase conjugated to that antibody as the two major assay reagents. Para-nitrophenyl phosphate (pNPP) was added for color development. The extraction buffer for the GOX ELISA was TBA+CHAPS (100 mM Tris, 100 mM sodium borate, 5 mM $MgCl_2$, 0.05% (v/v) Tween 20, 6.5 mM CHAPS, 0.2% (w/v) L-ascorbic acid, pH 7.8). GOX protein concentration in samples was quantitated by extrapolation from the standard curve of GOX protein.

Total soluble protein. Total soluble protein in corn tissue extracts was measured by the method of Bradford (1976) using the microtiter plate application of the Bio-Rad Protein Assay according to SOP. Bovine serum albumin (Sigma, St. Louis, MO) was used as the protein standard.

D. Compositional analytical methods

Preparation of samples for compositional analyses. Approximately 100 g of test (MON 830, MON 831 and MON 832) and control (MON 822) forage and grain samples were ground to a fine powder according to SOP and shipped to Corning Hazleton, Inc. for compositional analyses. Line identification and sample integrity were preserved by careful labeling and storage under conditions to preserve sample stability.

Grain was analyzed for proximates (protein, fat, ash, neutral detergent fiber, acid detergent fiber, and moisture), amino acid composition, fatty acid profile, alpha tocopherols, calcium and phosphorus. Forage samples were analyzed for proximates, calcium and phosphorus.

Acid Detergent Fiber (ADF). The sample was placed in a fritted vessel and washed with a boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. An acetone wash removed the fats and pigments. The lignocellulose fraction was collected on the frit and determined gravimetrically (USDA Agricultural Handbook No. 379, 8, 1970 modified). There is no analytical reference substance for this method.

Alpha Tocopherol by HPLC (EFD2). The grain samples were saponified to break down any fat and release the tocopherols. A portion of the saponified mixture is then extracted with organic solvent. The alpha tocopherol is quantitated on an HPLC silica column using fluorescence

detection. The limit of detection for this study was 0.0020 mg/g. Reference Standards: USP Alpha Tocopherol, lot number L (Cort *et al.*, 1983).

Amino Acid Composition (TAAP)
Grain samples were hydrolyzed with hydrochloric acid, and adjusted to pH 2.2. The individual amino acids were quantitated using an automated amino acid analyzer. This assay was based on previously published references (AOAC method 982.30, 1990). The reference substances used for these analyses were: K18 (Beckman, lot #A304008), L-Tryptophan (Sigma Chemical, lot #52H0717), Cysteic Acid Monohydrate (Sigma Chemical, lot #83H2607), Methionine Sulfone (Sigma Chemical, lot #12H3349). The limit of detection was 0.1 mg/g.

Ash (ASHM). Volatile organic matter was driven off when the sample was ignited at 550°C in an electric furnace. The residue was quantitated gravimetrically and calculated to determine percent ash (AOAC method 923.03, 1990). There was no analytical reference substance for this analysis. The limit of detection was 0.1%.

Calcium (CAA). The samples were dried, precharred, and ashed overnight at 500°to 550° C. The ashed samples were treated with nitric acid and then taken to dryness, reashed, and put into a solution of 4% hydrochloric acid. The amount of calcium was determined at a wavelength of 422.7 nm by comparing the signal of the unknown sample, measured by the atomic absorption spectrophotometer, with the signal of the standard solutions. All solutions contain 1% lanthanum and 5% hydrochloric acid. The limit of detection for this study was 0.001% (AOAC methods 965.09, 968.08, 985.35, 1990 modified). Reference Standards: Fisher 1000 ppm Calcium Solution, lot number 940982-24.

Carbohydrates (CHO). Carbohydrates were calculated by difference using the fresh weight-derived data and the following equation (USDA Agricultural Handbook No. 8, 1975):

% carbohydrates = 100% - (% protein + % fat + % ash + % moisture)

There was no analytical reference substance for these analyses.

Fat (FAAH). The forage sample was hydrolyzed with hydrochloric acid at elevated temperature. The fat was extracted using ether and hexane. The extracts were washed with a dilute alkali solution and filtered through a sodium sulfate column. The extract was then evaporated, dried, and weighed. The limit of detection for this study was 0.1% (AOAC methods

922.06, 954.02, 1990 modified). There was no analytical reference substance for this method.

Fat (FSOX). The grain sample was weighed into a cellulose thimble containing sand or sodium sulfate and dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was evaporated, dried and weighed (AOAC method 960.39). The limit of detection for this study was 0.1%. There was no analytical reference substance for these analyses.

Fatty Acid Profile (FAC)
The lipid in the grain samples was extracted, saponified with 0.5N sodium hydroxide in methanol, and methylated with 14% boron trifluoride:methanol. The resulting methyl esters were extracted with heptane containing an internal standard. The methyl esters of the fatty acids were analyzed by gas chromatography using external standards for quantitation (AOCS method Ce 1-62, 1981). The limit of detection varied between 0.11% and 0.14%. The reference substances are listed in the study data files.

Moisture (M100). The sample was dried in a vacuum oven at 100°C to a constant weight (approximately 5 hours) (AOAC methods 926.08 and 925.09, 1990). The moisture loss was determined gravimetrically (AOAC methods 926.08 and 925.09, 1990 modified). There was no analytical reference substance for these analyses. The limit of detection was 0.1 %.

Neutral Detergent Fiber Enzyme Method (NDFE). The sample was placed in a fritted vessel and washed with a boiling detergent solution that dissolved the protein, carbohydrate, enzyme, and ash. An acetone wash removed the fats and pigments. The hemicellulose, cellulose and lignin fractions were collected on the frit and determined gravimetrically (AACC method 32.20, 1983 modified). There is no analytical reference substance for this method.

Phosphorus (PTA). The samples were dried, precharred, and ashed overnight at 500°to 550° C. The ashed samples were treated with nitric acid, reashed, and dissolved in hydrochloric acid to yield a solution of 4% hydrochloric acid. The amount of phosphorus is determined colormetrically at a wavelength of 420 nm by comparing aliquots of the samples, each reacted with molybdovanadate solution, to standards prepared in the same manner (AOAC methods 965.17, 962.11, 1990 modified). The limit of

detection for this study was 0.001%. Reference Standards: SPEX 10,000 ppm Phosphorus Solution, lot number E-87P.

Protein (PGEN). Protein and other organic nitrogen in the sample was converted to ammonia by digesting the sample with sulfuric acid containing a mercury catalyst mixture. The acid digest was made alkaline, and the ammonia was distilled and titrated with a standard acid. The percent nitrogen was determined and converted to protein using the factor 6.25 (AOAC methods 955.04C and 979.09, 1990; Bradstreet, R.B. 1965; Kalthoff and Sandell, 1948). There was no analytical reference substance for these analyses. The limit of detection was 0.1 %.

E. Control of bias

The test and control lines in the 1995 U.S. field trial were planted in a non-systematic manner at each of five field sites. Samples were collected from all plants within the plots. Corn tissues were ground thoroughly and mixed before extraction to minimize tissue bias. In addition, where appropriate, plant tissue matrix was added to analytical reference standards to control for matrix effects.

During the validation of each ELISA method used in this study, the accuracy of the system was evaluated and each method optimized to minimize assay bias. The reported expression levels were not corrected for assay bias.

F. Data reduction and statistical analyses

CP4 EPSPS and GOX protein concentrations from ELISA data were calculated using validated computer systems and software. Absorbency readings from the ELISA and total soluble protein determinations were recorded using a Bio-Rad Model 3550 plate reader and were collected directly onto a formatted Microsoft® Excel (version 3.0) file using proprietary software developed by Monsanto ("ELISAread" program, King *et al.*, 1993). The raw data for each microtiter plate were transformed into concentration values using a validated Microsoft® Excel (version 3.0) Macro program and validated templates designed specifically for each method (Donovan *et al.*, 1993; Elswick, 1995c).

The concentration of CP4 EPSPS and GOX protein in the corn tissue extracts (via ELISA methods) was transformed to µg protein/g fresh wt of tissue using the tissue:volume ratios for each extraction. These calculations were executed using verified Microsoft® Excel (version 4.0) worksheets. The mean

expression and standard deviation across all sites for each test line was calculated by Microsoft® Excel (version 4.0) spreadsheet. No additional statistical analyses were performed on the expression or composition data.

G. Protocol amendment

1. Amendment #1 included the following changes: deletion of the statistical analysis of compositional data; sample identifier for forage samples; forage was analyzed for acid detergent fiber and neutral detergent fiber rather than crude fiber; grain was analyzed for acid detergent fiber and neutral detergent fiber rather than crude fiber; grain was analyzed for alpha tocopherol rather than total tocopherols; grain was analyzed for calcium and phosphorus; and LIMS data sheets were deleted from the analytical subreport.

V. Results and Discussion

A. Field trial

The Roundup Ready corn lines were grown under conditions representative of the major corn-growing region of the United States (Peters *et al.*, 1996). Approximately twenty-five to thirty-five seeds were planted of each line at each of six sites. The plant stand at the West Lafayette, IN site was insufficient to produce the necessary plant samples, so this field site was deleted from the field study. Emergence ranged between 72-96% across all lines at all five remaining sites. Young leaf, forage and grain samples were collected from each line, labeled, shipped, and stored in a manner to preserve line identity and sample integrity.

1. Test and control substance characterization

Sample analysis. Characterization of the test substances included analysis of the test and control plant samples for CP4 EPSPS and GOX protein levels as part of this study.

2. Plant samples

Young leaf sampling. One young leaf from each of the plants of each line was collected at all sites, when plants were approximately V4-6 stage. The leaves of each line were pooled and placed into a labeled bag, frozen on dry ice and shipped frozen to Monsanto, St. Louis. The leaf pools contained between 18-32 leaves, depending upon the line and site.

Forage. Two forage plants (leaves, ears, tassel and stalk) at early dent stage were collected and pooled from each test and control line at one site

(Jerseyville, Il). Forage plants were frozen and delivered to Monsanto, St. Louis on dry ice. The samples were stored at approximately -80°C.

Grain. The ears were harvested from all plants in the trial. The grain pools contained between 12-29 ears, depending upon the line and site. All grain was harvested at physiological maturity and dried to approximately 13% moisture prior to shelling. The ears were shelled, and the grain placed into bags labeled with unique batch MON numbers. The grain was shipped to and stored at Monsanto, St. Louis facility at ambient temperature.

B. Protein expression in corn plant samples

Tables 1 and 2 summarize the CP4 EPSPS and GOX protein levels in the plant samples. The mean of CP4 EPSPS and GOX protein levels for the test line across all sites was calculated. These values were calculated from the protein levels measured for each site. The range represents the minimum and maximum values from the analyses of samples across all sites. The forage protein levels were measured from a pool of two plants collected at one site. All samples and extracts were analyzed within the timeframe of demonstrated protein stability for CP4 EPSPS (Elswick and Sanders, 1995a,b,c) and GOX (Davies and Sanders, 1994; 1995b,c).

1. CP4 EPSPS protein levels in corn tissues. Table 1 summarizes the levels of CP4 EPSPS protein levels in the young leaf, forage and grain samples from all corn lines. The level of CP4 EPSPS protein ranged from 32.32 to 59.84 µg/g fwt in young leaf tissue, 18.85 to 28.79 µg/g fwt in forage, and 1.90 to 16.02 µg/g fwt in grain. The CP4 EPSPS protein levels measured in samples collected from Roundup treated plants were similar to levels in samples from unsprayed plants of the same lines (Table 1, Sanders *et al.*, 1997a).

Table 1. Levels of CP4 EPSPS Protein in Leaf, Forage and Grain Samples

		CP4 EPSPS protein (µg / g fwt)			
		Glyphosate Treated			Untreated
Maize Line		Mean[1]	Std Dev[2]	Range[3]	Range[4]
A. Leaf					
MON 830	RR	41.34	2.68	37.67 - 44.09	43.33 - 57.47
MON 831	RR	40.73	5.33	32.32 - 45.87	38.82 - 51.12
MON 832	RR	48.98	6.72	42.30 - 59.84	45.11 - 57.31
B. Forage[5]					
MON 830	RR	27.61	NA[6]	NA	NA
MON 831	RR	18.85	NA[6]	NA	NA
MON 832	RR	28.79	NA[6]	NA	NA
E. Grain					
MON 830	RR	4.63	1.77	1.90 - 6.85	1.72 - 5.31
MON 831	RR	7.51	2.91	4.46 - 11.12	4.48 - 7.28
MON 832	RR	8.21	5.01	2.23 - 16.02	4.55 - 8.44

1: The mean and standard deviation were calculated from the analyses of plant samples, one from each of five field sites unless noted otherwise.
2: Standard Deviation.
3: Minimum and maximum values from the analyses of samples across five sites unless noted otherwise.
4: Minimum and maximum values from the analyses of samples across five sites (Study 95-01-46-01, Sanders *et al.*, 1997a).
5: Value was determined from the analyses of one sample from one site.
6: Not applicable; only one sample was analyzed.

2. GOX protein levels in corn tissues. Table 2 summarizes the levels of GOX protein in the young leaf, forage and grain samples. The level of GOX protein ranged from 3.35 to 21.18 µg/g fwt in young leaf tissue, 2.27 to 6.67 µg/g fwt in forage, and 1.27 to 5.98 µg/g fwt in grain. The GOX protein levels measured in samples collected from Roundup treated plants were similar to levels in samples from unsprayed plants of the same lines (Table 2, Sanders *et al.*, 1997a).

Table 2. Levels of GOX Protein in Leaf, Forage and Grain Samples

		GOX protein (µg / g fwt)			
		Glyphosate Treated			Untreated
Maize Line		Mean[1]	Std Dev[2]	Range[3]	Range[4]
A. Leaf					
MON 830	RR	10.13	1.49	9.05 - 12.72	12.98 - 26.17
MON 831	RR	13.37	5.90	5.98 - 21.18	11.73 - 21.66
MON 832	RR	4.10	0.67	3.35 - 4.78	3.03 - 6.71
B. Forage[5]					
MON 830	RR	6.67	NA[6]	NA	NA
MON 831	RR	6.22	NA[6]	NA	NA
MON 832	RR	2.27	NA[6]	NA	NA
E. Grain					
MON 830	RR	2.88	0.49	2.41 - 3.47	1.60 - 4.50
MON 831	RR	4.16	1.98	1.27 - 5.98	1.94 - 4.46
MON 832	RR	2.53	0.56	1.89 - 3.05	1.25 - 3.13

1: The mean and standard deviation were calculated from the analyses of plant samples, one from each of five field sites unless noted otherwise.
2: Standard Deviation.
3: Minimum and maximum values from the analyses of samples across five sites unless noted otherwise.
4: Minimum and maximum values from the analyses of samples across five sites (Study 95-01-46-01, Sanders *et al.*, 1997a).
5: Value was determined from the analyses of one sample from one site.
6: Not applicable; only one sample was analyzed.

C. Compositional analyses of grain and forage samples

The compositional parameters included proximate analyses (protein, fat, ash, neutral detergent fiber, acid detergent fiber, carbohydrates and moisture), amino acid composition, fatty acid profile, calcium, phosphorus and alpha tocopherol. The values reported for the compositional analyses at Corning Hazleton Inc. were expressed as percent dry weight of the sample using the measured moisture content. The analytical data was summarized in an Analytical Subreport (CHW 6103-187) which has been archived. The mean values for each component for each test sample across all sites were calculated. These values were calculated from the values measured for each sample, one from each of five sites. The range represents the minimum and maximum values from the analyses of samples across all sites.

1. Proximate analysis of corn grain. The levels of the major components of corn grain (protein, fat, ash, neutral detergent fiber, acid detergent fiber, carbohydrates, and moisture) were determined for grain of three Roundup Ready lines and one control line harvested from five field trials conducted under GLP in the United States in 1995. Table 3a summarizes the results of these analyses. The levels of each of these components were similar for the Roundup Ready lines and the control line, MON 822. The values for both the test and control lines were also comparable to the published literature (Watson, 1987; Jugenheimer, 1976) and previously observed ranges for controls with similar genetic background (Sanders and Patzer, 1995; and Sanders *et al.,* 1996a,b; 1997a,b) (Table 3b).

Table 3a. Summary of Proximate Analysis of Corn Grain[a]

Analyte	MON 822[b] Mean[c] (Range)[d]	MON 830 Mean (Range)	MON 831 Mean (Range)	MON 832 Mean (Range)
Protein	10.9 (10.0 - 11.9)	12.0 (11.2 - 13.2)	11.5 (10.5 - 12.5)	11.9 (10.9 - 13.6)
Fat	3.1 (2.9 - 3.4)	3.0 (2.6 - 3.2)	3.2 (3.0 - 3.5)	3.2 (3.0 - 3.3)
Ash	1.5 (1.3 - 1.6)	1.6 (1.5 - 1.7)	1.5 (1.4 - 1.6)	1.6 (1.3 - 1.8)
Neutral Detergent Fiber	13.5 (12.0 - 14.6)	12.8 (11.2 - 15.7)	12.9 (12.4 - 14.0)	12.8 (12.2 - 14.2)
Acid Detergent Fiber	4.6 (4.0 - 5.3)	4.8 (4.5 - 5.3)	4.4 (3.8 - 4.8)	5.0 (4.4 - 6.4)
Carbohydrate	84.5 (83.3 - 85.1)	83.5 (82.4 - 84.2)	83.7 (83.0 - 84.5)	83.3 (81.6 - 84.5)
Moisture	10.6 (9.4 - 11.9)	10.7 (9.1 - 13.6)	10.9 (9.7 - 14.2)	10.5 (9.7 - 12.2)

a: Percent dry weight of sample, except for moisture.
b: MON 822 is the non-transgenic control corn line.
c: Value reported is mean of five samples, one from each field site.
d: Range denotes the lowest and highest individual values across five sites for each line.

Table 3b. Literature References

Component	Literature Mean	Literature Range	Reported Range
Protein %	9.5	6.0-12.0[a]	9.0-13.6[c]
	12.3	9.7-16.1[b]	
Fat (oil) %	4.3	3.1-5.7[a]	2.4-4.2[c]
	4.6	2.9-6.1[b]	
Ash %	1.4	1.1-3.9[a]	1.2-1.8[c]
Neutral Detergent Fiber %	9.5	8.3-11.9[a]	9.6-15.3[d]
Acid Detergent Fiber %	3.3	3.0-4.3[a]	3.1-5.3[d]
Moisture %	16.0	7-23[a]	9.7-15.8[c]

a: Watson, 1987
b: Jugenheimer, 1976
c: Range for five control lines with similar genetic background (Sanders and Patzer, 1995; Sanders *et al.*, 1996a,b; 1997a,b).
d: Range for two control lines with similar genetic background (Sanders *et al.*, 1996b; 1997b).

2. Amino acid composition of corn grain. Amino acid composition was completed on corn grain samples and the results are presented in Table 4. The reported values for each amino acid (mg/g) were converted to percent of total protein. The values for all amino acids were similar between the test and control samples. The values for cystine, histidine and glutamic acid were slightly higher than the published literature range (Watson, 1982) but similar to the non-modified control, MON 822, and within the range previously observed for other control lines with similar genetic background (Sanders and Patzer, 1995; and Sanders *et al.,* 1996a,b).

Table 4. Amino Acid Composition of Corn Grain[a]

Amino Acid	MON 822[b] Mean[c] (Range)[d]	MON 830 Mean (Range)	MON 831 Mean (Range)	MON 832 Mean (Range)	Literature[e] Range	Reported[f] Range
Nutritionally essential						
Methionine	2.0 (1.8 - 2.3)	1.9 (1.8 - 2.1)	1.8 (1.6 - 2.1)	1.9 (1.7 - 2.1)	1.0-2.1	1.3-2.6
Cystine	2.4 (2.1 - 2.7)	2.3 (2.1 - 2.4)	2.3 (2.0 - 2.6)	2.3 (2.1 - 2.5)	1.2-1.6	1.8-2.5
Lysine	3.1 (2.9 - 3.3)	2.8 (2.8 - 2.9)	3.1 (2.4 - 3.7)	3.1 (2.8 - 3.5)	2.0-3.8	2.6-3.5
Tryptophan	0.8 (0.7 - 0.9)	0.7 (0.6 - 0.9)	0.7 (0.7 - 0.7)	0.7 (0.7 - 0.8)	0.5-1.2	0.4-1.0
Threonine	3.8 (3.5 - 3.9)	3.7 (3.6 - 3.8)	3.6 (3.3 - 3.9)	3.7 (3.6 - 3.8)	2.9-3.9	3.3-4.2
Isoleucine	3.6 (3.2 - 3.9)	3.6 (3.5 - 3.8)	3.6 (3.4 - 3.7)	3.7 (3.6 - 3.8)	2.6-4.0	3.4-4.3
Histidine	3.1 (2.9 - 3.3)	3.1 (3.0 - 3.1)	3.1 (2.9 - 3.4)	3.2 (3.0 - 3.5)	2.0-2.8	2.8-3.4
Valine	4.7 (4.2 - 5.0)	4.7 (4.5 - 4.8)	4.9 (4.5 - 5.3)	4.9 (4.6 - 5.3)	2.1-5.2	4.3-5.3
Leucine	13.5 (12.6 - 14.5)	13.8 (13.4 - 14.4)	13.4 (12.8 - 14.5)	13.5 (13.2 - 14.4)	7.8-15.2	12.0-15.8
Arginine	4.0 (3.6 - 4.4)	3.8 (3.6 - 3.9)	3.9 (3.2 - 4.9)	4.1 (3.6 - 4.4)	2.9-5.9	3.5-5.0
Phenylalanine	5.5 (5.0 - 5.8)	5.5 (5.4 - 5.6)	5.6 (5.4 - 5.7)	5.5 (5.3 - 5.8)	2.9-5.7	4.9-6.1
Glycine	3.9 (3.7 - 4.1)	3.7 (3.6 - 3.7)	3.8 (3.2 - 4.4)	3.9 (3.5 - 4.2)	2.6-4.7	3.2-4.2
Nonessential						
Alanine	7.8 (7.3 - 8.4)	7.9 (7.7 - 8.2)	7.8 (7.3 - 8.0)	7.9 (7.6 - 8.1)	6.4-9.9	7.2-8.8
Aspartic acid	6.8 (6.4 - 7.1)	6.7 (6.7 - 6.8)	6.7 (6.1 - 7.6)	6.9 (6.8 - 7.2)	5.8-7.2	6.3-7.5
Glutamic acid	20.5 (19.5 - 21.5)	20.7 (20.4 - 21.5)	20.2 (19.2 - 20.9)	20.6 (20.2 - 21.4)	12.4-19.6	18.6-22.8
Proline	9.1 (8.7 - 9.4)	9.2 (8.9 - 9.4)	8.8 (8.4 - 9.2)	9.1 (8.7 - 9.4)	6.6-10.3	8.9-10.1
Serine	5.4 (5.2 - 5.5)	5.3 (5.1 - 5.6)	5.2 (5.0 - 5.7)	5.3 (5.1 - 5.5)	4.2-5.5	4.9-6.0
Tyrosine	3.9 (3.7 - 4.1)	3.8 (3.6 - 4.1)	3.9 (3.7 - 4.1)	3.9 (3.3 - 4.2)	2.9-4.7	3.7-4.3

[a] Values are expressed as percent of total protein.
[b] MON 822 is the non-transgenic control line.
[c] Value reported is mean of five samples, one from each field site (Groth and Sanders, 1997).
[d] Range denotes the lowest and highest individual values across sites for each line.
[e] Watson, 1982. Values are per cent of total protein [10.1% total protein (Nx6.25)].
[f] Range for three control lines with similar genetic background (Sanders and Patzer, 1995; Sanders *et al.*, 1996a,b).

3. Fatty acid profile of corn grain. The fatty acid composition was determined for the grain of the three test lines and the control line and the results are summarized in Table 5. Nine fatty acids, for which the measured values were below the limit of detection of the assay (arachidonic, capric, caprylic, eicosadienoic, lauric, myristic, myristoleic, pentadecanoic, and heptadecenoic) were excluded from the table. The fatty acid values were similar between the test and control samples, and typical of the values published (Watson, 1982) and values previously observed for controls with similar genetic background (Sanders and Patzer, 1995; and Sanders *et al.*, 1996a,b; 1997a,b).

Table 5. Fatty Acid Composition of Corn Grain[a]

Component	MON 822[b] Mean[c] Range[d]	MON 830 Mean Range	MON 831 Mean Range	MON 832 Mean Range	Literature[e] Range	Reported[f] Range
Linoleic (18:2)	61.4 (55.9 - 63.6)	62.4 (60.2 - 63.3)	60.9 (58.8 - 62.2)	63.2 (61.1 - 64.4)	35-70	60.2-66.1
Oleic (18:1)	24.1 (22.6 - 27.5)	23.7 (23.1 - 25.0)	25.0 (24.0 - 26.2)	23.1 (22.1 - 24.2)	20-46	20.6-25.4
Palmitic (16:0)	10.5 (10.0 - 12.0)	10.3 (10.0 - 10.6)	10.2 (9.9 - 10.7)	10.2 (10.0 - 10.7)	7-19	9.9-10.8
Palmitoleic (16:1)	0.2 (0.2 - 0.2)	0.2 (0.2 - 0.2)	0.2 (0.2 - 0.2)	0.2 (0.2 - 0.2)	not reported	0.1-0.2
Stearic (18:0)	1.8 (1.6 - 2.2)	1.7 (1.6 - 1.9)	1.9 (1.7 - 2.0)	1.6 (1.5 - 1.8)	1-3	1.4-2.1
Linolenic (18:3)	1.0 (0.9 - 1.0)	0.9 (0.8 - 1.0)	0.9 (0.9 - 1.0)	0.8 (0.7 - 0.8)	0.8-2	0.8-1.1
Arachidic (20:0)	0.4 (0.4 - 0.5)	0.4 (0.4 - 0.5)	0.4 (0.4 - 0.5)	0.4 (0.4 - 0.4)	0.1-2	0.3-0.4
Eicosenoic (20:1)	0.3 (0.3 - 0.3)	0.3 (0.3 - 0.3)	0.3 (0.3 - 0.3)	0.3 (0.3 - 0.4)	not reported	0.2-0.3
Behenic (22:0)	0.2 (0.2 - 0.3)	0.2 (0.2 - 0.2)	0.2 (0.2 - 0.2)	0.2 (0.2 - 0.2)	not reported	0.1-0.2

[a]: Value of fatty acid is % of total lipid. Other fatty acids were below the limit of detection of the assay.
[b]: MON 822 was the control corn line.
[c]: Values presented are means (five samples for each line).
[d]: Range denotes the lowest and highest individual value across sites for each line.
[e]: Watson, 1982.
[f]: Range for five control lines with similar genetic background (Sanders and Patzer, 1995; Sanders *et al.*, 1996a,b; 1997a,b).

4. Tocopherol, calcium and phosphorus analysis of corn grain. Tocopherols are naturally present in corn oil and have vitamin E potency (Watson, 1982). The alpha tocopherol was measured and the results summarized in Table 6. The tocopherol values were similar for the test lines and the control line and comparable to the previously observed ranges for controls with similar genetic background (Sanders and Patzer, 1995; Sanders *et al.,* 1996a; 1997a). The values are slightly lower than published literature (Watson, 1982; Watson 1987) which could be attributed to differences in analytical methodology and/or variance among corn genotypes.

The calcium and phosphorus levels were determined and the results presented in Table 6. The calcium values were similar for the test lines and the control line and comparable to the previously observed ranges for controls with similar genetic background (Sanders and Patzer, 1995; Sanders *et al.,* 1996a; 1997a). The values are lower than published literature (Watson, 1982) which could be attributed to differences in analytical methodology or variance among corn genotypes. The phosphorus values were similar for the test lines and the control line and comparable to the published ranges (Watson, 1982) and to previously observed ranges for controls with similar genetic background (Sanders and Patzer, 1995; Sanders *et al.,* 1996a; 1997a).

Table 6. Analysis of Tocopherol and Inorganic Components of Corn Grain[a]

Component	MON 822[b] Mean[c] Range[d]	MON 830 Mean Range	MON 831 Mean Range	MON 832 Mean Range	Literature Range	Reported[g] Range
A. Tocopherol						
alpha (mg/g)	0.012 (0.010 - 0.015)	0.008 (0.007 - 0.010)	0.012 (0.010 - 0.015)	0.012 (0.011 - 0.015)	0.04-0.09[e] 0.14-0.23[f]	.008-.013
B. Inorganic Components						
Calcium %	0.005 (0.004 - 0.006)	0.006 (0.005 - 0.006)	0.006 (0.005 - 0.006)	0.005 (0.005 - 0.006)	0.01-0.1[e]	.003-.005
Phosphorus %	0.320 (0.303-0.339)	0.332 (0.313 - 0.354)	0.339 (0.318 - 0.380)	0.337 (0.321 - 0.356)	0.26-0.75[e]	0.288-0.363

[a]: Values on a dry weight basis.
[b]: MON 822 was the control line.
[c]: Value reported is mean of five samples, one from each field site.
[d]: Range denotes the lowest and highest individual values across sites for each line.
[e]: Watson, 1982.
[f]: Watson, 1987
[g]: Range for three control lines with similar genetic background (Sanders and Patzer, 1995; Sanders *et al.,* 1996a; 1997a).

5. Proximate analyses of forage. The major components of forage of the corn test and control lines were measured and the results presented in Table 7. The values for protein, fat, ash, carbohydrate, neutral detergent fiber, acid detergent fiber, calcium, phosphorus and dry matter content were similar between the test lines and the control line, MON 822. The values for both the test and control lines were also comparable to the published literature (Watson, 1982) and previously observed ranges for controls with similar genetic background (Sanders *et al.*, 1996b; 1997b).

Table 7. Summary of Analysis of Forage[a]

Component	MON 822[b]	MON 830	MON 831	MON 832	Literature[c] Range	Reported[d] Range
Protein	7.3	6.1	6.9	6.8	3.5 - 15.9	4.8-8.4
Moisture	71.1	71.4	72.4	73.4	53.3 - 87.5	68.7-73.5
Fat	1.5	1.5	2.4	1.5	0.7 - 6.7	1.4-2.1
Ash	4.2	4.2	4.6	4.3	1.3 - 10.5	2.9-5.1
Carbohydrate	86.9	88.1	86.2	87.6	not reported	84.6-89.1
Neutral Detergent Fiber	40.1	36.4	37.7	41.7	not reported	39.9-46.6
Acid Detergent Fiber	25.4	23.3	25.1	23.6	not reported	21.4-29.2
Calcium	0.241	0.185	0.218	0.164	0.2 - 0.6	not reported
Phosphorus	0.282	0.216	0.228	0.216	0.15 - 0.55	not reported

[a]: Values reported are single analysis on one sample from one site. Therefore, ranges are not available. Values reported are percentages on a dry weight basis, except moisture.
[b]: MON 822 was the control line.
[c]: Watson, 1982.
[d]: Range for two control lines with similar genetic background (Sanders *et al.*, 1996b; 1997b).

VI. Conclusions

Plant samples collected from Roundup Ready and control corn plants grown in the 1995 United States field trials were representative of commercially grown corn. Therefore, data collected on protein expression levels and compositional components were representative of the levels expected in the commercial crop of these corn lines following treatment with Roundup.

Expression levels of CP4 EPSPS and GOX proteins varied for each corn line analyzed yet are sufficient to confer glyphosate tolerance to the plants. The CP4 EPSPS and GOX protein levels measured in samples collected from Roundup treated plants were similar to levels in samples from unsprayed plants of the same lines.

The levels of the major components of corn grain (protein, fat, ash, neutral detergent fiber, acid detergent fiber, carbohydrates, moisture, amino acids and fatty acids) were similar between the test and control samples, and typical of the values published. Forage components (protein, ash, acid detergent fiber, neutral detergent fiber, fat, carbohydrates, calcium, phosphorus and dry matter) were similar between the test and control samples, and typical of the values published.

It was concluded that these corn lines are substantially equivalent in composition to the control corn line and representative of corn grain currently in commerce.

VII. Acknowledgements

The authors would like to acknowledge Roy Fuchs for his critical review of this report. We thank Diana Kester for the quality control review of data presented in this report and Heather Seitzsinger for tissue processing and extract preparations.

VIII. References

AACC Method 32.20, 1983. *In* American Association of Cereal Chemists, 8th Edition, modified.

AOAC 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 982.30. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 922.06, 954.02. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 965.17, 962.11. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 965.09, 968.08, 985.35. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 960.39. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 955.04C, 979.09. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 926.08, 925.09. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOAC Method 923.03. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

AOCS Method Ce 1-62. 1981. *In* Official and Tentative Methods of the American Oil Chemists Society. American Oil Chemists Society, Champaign, Illinois, modified.

Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein Dye-Binding. Anal. Biochem. 72: 248-254.

Bradstreet, R.B. 1965. The Kjelhahl Method for Organic Nitrogen, Academic Press, New York, New York, modified.

Cort, W.M., Vincente, T.S., Waysek, E.H., and Williams, B.D. 1983. Journal of Agricultural Food Chemistry, 31:1330-1333, modified.

Davies, S.R. 1994. Development of an ELISA for the Detection and Quantitation of Glyphosate Oxidoreductase (GOX). Monsanto Technical Report St. Louis, MSL-13528.

Davies, S.R. and Sanders, P. 1994. Stability of the GOX Protein in Corn Whole Plant Tissue and Frozen Corn Whole Plant Extracts. Monsanto Technical Report St. Louis, MSL-13759. Study 94-01-39-04.

Davies, S.R. and Sanders, P. 1995a. Validation of a Direct ELISA to Detect and Quantitate Glyphosate Oxidoreductase, GOX, Protein in Corn Plant Tissues. Monsanto Technical Report St. Louis, MSL-13527. Study 93-01-39-09.

Davies, S.R. and Sanders, P. 1995b. Stability of the GOX Protein in Corn Leaf Tissue and Frozen Corn Leaf Extracts. Monsanto Technical Report St. Louis, MSL-13791. Study 93-01-39-12.

Davies, S.R. and Sanders, P. 1995c. Stability of the GOX Protein in Corn Seed and Extracts of Corn Seed. Monsanto Technical Report St. Louis, MSL-13760. Study 93-01-39-10.

Donovan, D.E., Nida, D.N., Biest, N.A., Nickson, T.E., Holden, L.R., and Fuchs, R.L. 1993. Validation of ELISARead CP4-V3.0.SLV Excel Macro and Template, Monsanto Technical Report St. Louis, MSL-12597.

Elswick, E. 1995a. Development of a Direct ELISA Method to Quantitate CP4 EPSPS in Genetically Modified Corn Plants Using a Horseradish Peroxidase Conjugate. Monsant Technical Report St. Louis, MSL-13564.

Elswick, E. 1995b. Validation of a Direct ELISA to Detect and Quantitate CP4 5-Enol-Pyruvyl Shikimate 3-Phosphate Protein in Corn Plant Tissues. Monsanto Technical Report St. Louis, MSL-13576. Study 94-01-39-06.

Elswick, E. 1995c. Validation of the ELISA CP4_HRP_V3.0.SLV Excel Macro and Template. Monsanto Technical Report St. Louis, MSL-13563.

Elswick, E. and Sanders, P. 1995a. Stability of CP4 EPSPS Protein in Corn Seed and Extracts of Corn Seed. Monsanto Technical Report St. Louis, MSL-13763. Study 94-01-39-05.

Elswick, E. and Sanders, P. 1995b. Stability of CP4 Protein in Whole Corn Plants and Extracts of Whole Corn Plant Tissue. Monsanto Technical Report St. Louis, MSL-13764. Study 94-01-39-03.

Elswick, E. and Sanders, P. 1995c. Stability of CP4 Protein in Corn Leaf Tissue and Frozen Corn Leaf Extracts. Monsanto Technical Report St. Louis, MSL-13762. Study 94-01-39-11.

Groth, M.E., and P.R. Sanders. 1997. Evaluation of Insect-Protected, Insect-Protected Roundup Ready, and Roundup Ready Corn Lines in the 1995 U.S. Field Trials. Monsanto Technical Report, St. Louis, MSL-14573. Study 95-01-50-01.

Harrison, L.A., M.R. Bailey, R.M. Leimgruber, C.E. Smith, D.L. Nida, M.L. Taylor, M. Gustafson, B. Heeren, and S.R. Padgette. 1993. Characterization of Microbially Expressed Protein: CP4 EPSPS. Monsanto Technical Report, St. Louis, MSL-12901.

Harrison, L.A., Bailey, M.R., Beist, N.A., Basse, J., Leimgruber, R.M., Nickson, T.E., and Smith, C. 1994. Characterization of Microbially Expressed Proteins: GOX(M4-C1) and GOXv247(M4-C1). Monsanto Technical Report, St. Louis, MSL-12965.

Höfte, H. and Whiteley, H.R. 1989. Insecticidal Crystal Proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53: 242-255.

Jugenheimer, R.W. 1976. Corn Improvement, Seed Production, and Uses. John Wiley & Sons, Inc. New York, New York, USA.

Kalthoff, I.M. and Sandell, E.B. 1948. Quantitative Inorganic Analysis, MacMillan, New York, modified.

King, R.S., Witt, A.F., Fuchs, R.L. 1993. Elisaread Data Transfer Validation. Elisaread v5.911 MAC, Monsanto Technical Report, St. Louis, MSL-12303.

Padgette, S.R., D.B. Re, G.F. Barry, D.A. Eichholtz, X. Delannay, R.L. Fuchs, G.M. Kishore and R.T. Fraley. 1996. New Weed Control Opportunities: Development of Soybeans with a Roundup Ready™ Gene. *In* Herbicide-Resistant Crops: Agricultural, Environmental, Economic, Regulatory and Technical Aspects. S.O. Duke, Ed. CRC Press, Boca Raton, pp 53-84.

Peters, T.J., Dodson, H. and Sanders, P.R. 1996. Evaluation of Insect-Protected Roundup Ready™ and Roundup Ready™ Corn Lines in 1995 U.S. Field Trials Following Roundup® Application. Study Number 95-01-50-02, MSL-14954, an unpublished study conducted by Monsanto Company.

Sanders, P.R. and Patzer, S.S. 1995. Compositional Analyses of MON 801 Grain and Silage from the 1993 and 1994 Corn Field Trials. Monsanto Technical Report, St. Louis, MSL-14180.

Sanders, P.R., Henning, D.M., and Groth, M.E. 1996a. Compositional Analysis of Insect-Protected and Insect-Protected Roundup Ready™ Corn Lines from the 1994 U.S. Field Trials. Study Number 94-01-50-18, MSL-14326, an unpublished study conducted by Monsanto Company.

Sanders, P.R., M.E. Groth, B.E. Ledesma and J.R. Kania. 1996b. Evaluation of Insect Protected, Insect Protected Roundup Ready™, and Roundup Ready™ Maize Lines in the 1995 European Field Trial 95-BTRR-01. Study Number 95-10-50-03, MSL-14615, an unpublished study conducted by Monsanto Company.

Sanders, P.R., M.E. Groth, B.E. Ledesma and J.R. Kania. 1997a. Expression and Compositional Analyses of Roundup Ready™ Corn Lines MON 830, MON 831 and MON 832 in the 1995 U.S. Field Trial. Study Number 95-01-46-01, MSL-14724, an unpublished study conducted by Monsanto Company.

Sanders, P.R., M.E. Groth and B.E. Ledesma. 1997b. Evaluation of Insect Protected Roundup Ready™ and Roundup Ready™ Maize Lines in the 1995 European Field Trial 95-BTRR-02. Study Number 95-10-50-04, MSL-14383, an unpublished study conducted by Monsanto Company.

USDA Agriculture Handbook No. 379,8. 1970. Forage and Fiber Analysis. *In* Agricultural Handbook No. 379, 8. United States Department of Agriculture, Washington, D.C., modified.

Watson, S.A. Corn: Amazing Maize. General Properties. 1982. pg 3-29. *In* CRC Handbook of Processing and Utilization in Agriculture, Volume II: Part 1 Plant Products. I.A. Wolff (ed). CRC Press, Inc., Florida.

Watson, S.A. 1987. Structure and composition. pp. 53-82. *In* Corn Chemistry and Technology. Watson, S.A. and R.E. Ramstad, Eds. American Association of Cereal Chemists, Inc., St. Paul, Minnesota.

Attachment

Protocol

Study #: 95-01-46-02

Corning Hazleton Project Identification: 6103-187

Study Title: Evaluation of Roundup Ready™ Corn Produced in the 1995 U.S. Field Tests Following Roundup® Application

Sponsor:
Monsanto Company
CEREGEN
700 Chesterfield Parkway North
St. Louis, MO 63198

Primary Testing Facility:
Monsanto Company
CEREGEN
700 Chesterfield Parkway North
St. Louis, MO 63198

Study Director:
Patricia Sanders, Molecular Biologist
Monsanto Company
CEREGEN
700 Chesterfield Parkway North GG4K
St. Louis, MO 63198
Phone (314) 537-6412
FAX (314) 537-6567

Principal Investigator:
Mark Groth, Biologist
Monsanto Company
CEREGEN
700 Chesterfield Parkway North
St. Louis, MO 63198
Phone (314) 537-7460

CHW Testing Facility:
Corning Hazleton, Inc. (CHW)
Wisconsin Facility
3301 Kinsman Blvd.
Madison, WI 53704

CHW Principal Investigator:
Diane Henning
Corning Hazleton, Inc.
Wisconsin Facility
P.O. Box 7545
Madison, WI 53707
Phone (608) 242-2712

Approved By:

Sponsor/ Testing Facility Management Rep:

Date: 7/28/95

R.L. Fuchs, Ph.D., Associate Fellow
Regulatory Science
GG4G, (314) 537-6438
Monsanto Company

Study Director:

Patricia Sanders Date: 7/27/95

Patricia Sanders, Molecular Biologist
GG4K, Lab GG4210, (314) 537-6412
Monsanto Company

Reviewed by:

Principal Investigator:

Mark E. Groth Date: 7/27/95

Mark Groth
Today's Temporary, contracted to
Monsanto Agricultural Group
CEREGEN

Principal Investigator:

Date: 7/27/95

Diane Henning
Corning Hazleton, Inc.

Monsanto Quality Assurance:

Date: 7/31/95

Quality Assurance Auditor
Monsanto Company

Corning Hazleton Quality Assurance:

Date: 8/3/95

QA Representative
Corning Hazleton, Inc.

Quality Control:

Date: 7/27/95

R. S. King
GG4F, (314) 537-7362
GLP/QC Coordinator
Monsanto Company

Co-investigators acknowledging study protocol:

Bibi Ledesma

Date: 7-27-95

Bibi Ledesma
Today's Temporary, contracted to
CEREGEN

Date: 7-27-95

Glen Rogan
Monsanto Company
CEREGEN

Date: 7/27/95

Frances Brostrum
Olsten Temps, contracted to
CEREGEN

1.0 Purpose:

The purpose of this study is to evaluate glyphosate tolerant (Roundup Ready™) corn lines grown under field conditions. The corn lines contain the genes encoding CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) (Padgette et al., 1993) and glyphosate oxidoreductase (GOX) (Padgette et al., 1994). The CP4 EPSPS and *gox* genes are present to enable selection of genetically modified cells in tissue culture and to confer glyphosate tolerance to the corn plant. The control line has background genetics representative of the test lines, but has not been genetically modified and therefore, does not express the CP4 EPSPS or GOX proteins. The control line provides a background matrix for the analytical evaluation of the CP4 EPSPS and GOX protein expression levels in the corn tissues collected from field-grown corn plants. The test line will be compared to the control line for each analyte measured in the compositional analyses.

This study is designed to estimate the levels of CP4 EPSPS and GOX proteins in leaf, forage and grain samples of Roundup Ready™ corn plants following Roundup® application. In addition, compositional analyses will be performed on forage and grain samples. Samples for this study will be collected from the GLP field Study #95-01-50-02.

2.0 Timelines:

2.1 Proposed experimental start date: July 28, 1995
2.2 Proposed experimental termination date: April 30, 1996

3.0 Experimental design:

3.1 Test Substances:

The test substances are the corn lines MON 830, 831 and 832 (BC2F3xMo17) which contain the genes encoding CP4 EPSPS and GOX proteins. Any of these lines may be deleted at any time during this study. The deletion and reason(s) for the deletion of a test substance will be documented by amendment to the study protocol.

3.2 The Control Substance:

The control substance is the non-transformed corn line MON 822 (BC2F1xMo17) which has a genetic background similar to the test lines.

3.3 Reference Substance:
There will be no reference substance for this study. Appropriate standards will be used in each assay as reference substances for the analytical procedures.

3.4 Test and Control Substance Characterization:
The identity of the test and control substances will be determined by the Study Director prior to their use in the study by verifying the chain-of-custody documentation supplied with the samples collected from the corn lines. The corn lines will be characterized as part of Study #95-01-50-02.

3.5 Test System:
The test system is the panel of analytical biochemical methods. Validated Enzyme Linked Immunosorbent Assay (ELISA) will be performed to quantitate the CP4 EPSPS and GOX protein levels in the leaf, forage and grain samples.

Compositional analyses will be performed by published methods which are currently used to evaluate nutritional quality in corn products for commercial purposes.

3.6 Justification of Test System:
The ELISAs have been validated for each protein and designed to measure the CP4 EPSPS and GOX protein levels in leaf, forage and grain samples from corn.

Compositional analyses methods are validated assays which are currently used to evaluate nutritional quality in corn products for commercial purposes. All methods have been validated according to CHW Standard Operating Procedures (SOPs).

3.7 Description of Experimental Design:
Young leaf, forage and grain samples will be collected from the field sites for analysis. All plant samples will be labelled with the field Study number (95-01-50-02), site code, line MON number, sample type, and date of collection. The samples and a Sample Handling Form will be transferred to Monsanto as outlined in Study 95-01-50-02.

Field sites	Site Code
Jerseyville, IL	JV
Van Horne, IA	VH
Ames, IA	IA
Mead, NE	NE
Monmouth, IL	MN

All samples will be ground to a fine powder according to SOP. Monsanto will perform the CP4 EPSPS and GOX protein expression level determinations and Corning Hazleton, Inc. will perform the compositional analyses.

3.8 Proposed Statistical Methods:

The mean expression level (µg / g fresh tissue) will be reported for each protein by line for each tissue across sites with a standard deviation for that mean.

Compositional analyses will be reported on a dry weight basis where appropriate. The mean across sites will be reported for each analyte. Statistical analyses will be performed, comparing test and control means, using the SAS statistical program (SAS Institute, 1990) and the details described in the statistical analysis subreport as part of study final report.

3.9 Control of Bias:

The leaf and grain samples will be collected from all corn plants of each line at multiple sites. The tissues will be ground thoroughly and mixed well before extraction to minimize tissue bias. In addition, where appropriate, the plant tissue matrix will be included in the reference standard curve to control for matrix effects.

4.0 Protein Expression Level Determinations at Monsanto

4.1 Samples

There are three test lines and one control line in this study. All samples for analyses will be obtained from each site and sent to the appropriate destination as described in the protocol for Study #95-01-50-02. At a minimum, all samples will be labelled with the field Study 95-01-50-02, site code, sample type, MON number, and date of collection. Leaf and forage samples will be shipped on dry ice and stored at approximately -80°C. Ears or kernels will be shipped at ambient temperature and stored at ambient temperature or approximately 4°C. A summary of expected samples is contained in Attachment 1, Table 1.

4.1.1 Leaf Samples

The youngest immature whorl leaf from each plant of a line will collected and pooled. There is one leaf sample per test and control line for each site (4 lines/site X 5 sites = 20 samples). Young leaf samples will be collected from 5 field sites.

4.1.2 Forage Samples

Two forage plants from each line will be collected at soft dough stage. The sample identifier will include the field study # and line MON number, followed by an "A" or "B".

4.1.3 Grain Samples

The ears of all plants will be harvested, dried and shelled as part of Study #95-01-50-02. The grain samples will be assigned MON numbers as designated in Attachment 2. The MON number is the unique sample identifier. Approximately one kilogram of grain from each line from each site will be ground to a fine powder. An aliquot will be removed for ELISA analyses.

4.2 Analytical Methods:

Samples of test and control corn lines will be assayed for CP4 EPSPS and GOX protein levels by ELISA. Appropriate worksheets will be used during data collection which will delineate the sample location within the microtitre plates.

4.2.1 Sample Processing

Processing and extraction of corn tissues will be completed according to SOP BtM-PRO-067, BtM-PRO-037 and BtM-PRO-076. Each extract will be assigned a unique extract identifier. Extracts will be stored at approximately -80°C until analyzed. All extracts will be evaluated for total protein according to SOP BtC-PRO-015 as a quality check on the consistency of extraction among samples.

4.2.2 ELISA analyses

The levels of CP4 EPSPS and GOX proteins in leaf, forage and grain samples will be measured by ELISA according to the appropriate SOP for that protein in corn tissues, BtM-PRO-076 and BtM-PRO-037 respectively.

ELISA and total protein assay data will be collected and the CP4 EPSPS and GOX protein concentrations calculated using validated data handling systems developed at Monsanto.

4.2.3 Any additional analyses or re-analyses will be documented and justified in the raw data file. Not all analyses will necessarily be performed on all samples from all lines.

5.0 Compositional Analyses at Corning Hazleton, Inc. (CHW)

5.1 Samples

There are three test lines and one control line in this study. Samples will be labelled with the Study #, a unique sample identifier and date. A summary of expected samples is contained in Attachment 1, Table 2. Samples will be stored in a freezer set to maintain approximately -20°C ± 10°C. Any remaining test or control material, including original sample receipt containers will be returned to the Sponsor after completion of analyses. The grain and forage samples will be shipped on dry ice to:

Diane Henning
Corning Hazleton, Inc.
Wisconsin Facility
3301 Kinsman Blvd.
Madison, WI 53704

5.1.1 Forage Samples

Two forage plants from each line will be collected at soft dough stage. The sample identifier will include the Study # and line MON number, followed by an "A" or "B". Approximately 100 gm of each ground forage sample (A and B for each line) will be pooled and shipped to CHW. There will be 1 forage sample for each line.

5.1.2 Grain Samples

The grain samples will be assigned MON numbers as designated in Attachment 2. The MON number is the unique sample identifier. An aliquot of the ground corn grain of each line from each site will be shipped to CHW.

5.2 Analytical Methods

Grain and forage samples will be assayed by the following CHW approved methods:

5.2.1 Forage Samples

The following analyses will be performed on the forage samples: proximates: moisture (M100), protein (PGEN), fat (FAAH), ash (ASHM), calories (CALC) carbohydrates (CHO); and crude fiber (CFIB).

5.2.2 Grain Samples

The following analyses will be performed on the grain samples:

proximates: moisture (M100), protein (PGEN), fat (FSOX), ash (ASHM), calories (CALC) carbohydrates (CHO); crude fiber (CFIB), amino acid (TAAP), and fatty acid profile (FAC).

5.2.3 Any additional analyses or re-analyses will be documented and justified in the raw data file. Not all analyses will necessarily be performed on all samples from all lines.

6.0 Records to be Maintained:

6.1 Monsanto Facility.

All raw data including ELISA worksheets, computer printouts, and extraction worksheets shall be archived upon completion of the study. Excess samples will be retained until notified of final disposition by the Sponsor.

Records will be retained of all sampling and observational raw data, the protocol and all deviations and amendments thereto, and copies of all letters, memoranda, and other correspondence related to this study. Upon completion of the study, raw data will be transferred to the archives of the Sponsor.

6.2 Corning Hazleton, Inc.

Original data or copies will be available at CHW to facilitate auditing the study during its progress and before acceptance of the final subreport. When the final subreport is completed, original paper data, computer printouts, chromatographs, worksheets, data sheets, original notes by investigators, forms specified by SOP and magnetically encoded records, will be retained in the archives of CHW in accordance with 21 CFR 58.

The following supporting records will be retained at CHW but will not be archived with the study data: refrigerator and freezer temperature records, instrument calibration and maintenance records.

7.0 CHW Final Subreport:

A quality control checked and Quality Assurance accepted analytical subreport generated by the CHW Principal Investigator will be submitted to the Monsanto Study Director to be used in preparation of the final report. This will include a Quality Assurance accepted summary spreadsheet which includes the results reported in LIMS reports and results calculated on dry weight basis. A final subreport including a data summary spreadsheet, a copy of the Laboratory

Information Management Systems (LIMS) reports, reference standards (where applicable) for each assay and Method Summaries will be submitted to the Study Director. The raw data and final subreport will be audited by the Quality Assurance Unit of CHW in accordance with CHW Standard Operating Procedures (SOPs). One copy of the draft report and two copies of the final subreport will be provided.

8.0 Study Conduct Statement:

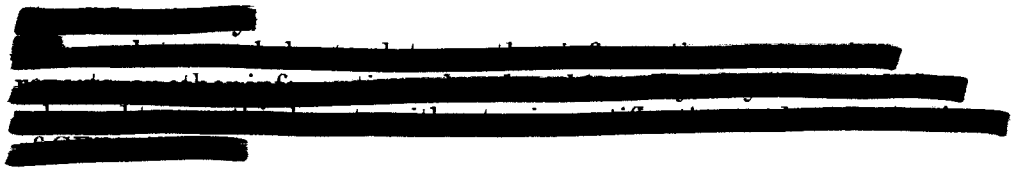
8.1 Monsanto Facility.

This experiment shall be conducted in accordance with the protocol. Any change, revision, or deviation from this protocol should be documented promptly according to SOP #GEN-POL-005 and communicated to the Study Director immediately. (If the Study Director is unavailable, deviations should be communicated to the Principal Investigator or GLP/QC Coordinator who will inform the Study Director as soon as possible.) All specimens will be identified clearly with the Study # and date collected. All data and information will be recorded directly and promptly in indelible ink. The exceptions are electronically captured data, for which a printout will be generated and included with other study data. All entries will be dated on the day of entry and signed or initialed by the person entering the information. Computer printouts will have dates and initials of the person responsible for their generation. All data sheets must contain the Study number. Any change in entries will be made so as not to obscure the original entry, must indicate the reason for the change and must be dated and signed (or initialed) at the time of the change.

8.2 Corning Hazleton, Inc.

This experiment shall be conducted in accordance with the protocol and CHW SOPs. Any change, revision, or deviation from this protocol should be documented promptly and communicated to the Study Director immediately. CHW Quality Assurance Unit will monitor the study conduct and the final subreport.

9.0



10.0 GLP Compliance:

This experiment will be conducted in compliance with the United States FDA Good Laboratory Practice Regulations (21 CFR Part 58).

11.0 References:

Padgette, S.R., Barry, G.F., Re, D.B., Weldon, M., Eichholtz, D.A., Kolacz, K.H., and Kishore, G.M. 1993. Purification, Cloning, and Characterization of a Highly Glyphosate-tolerant EPSP Synthase from *Agrobacterium* sp. Strain CP4. Monsanto Technical Report St. Louis, MSL-12738.

Padgette, S. R., Taylor, M.L., Barry, G.F., Huber, T., Harrison, L.A. and Kishore, G.M. 1994. Characterization of Glyphosate Oxidoreductase. Monsanto Technical Report St. Louis, MSL-13234.

SAS Institute, Inc. 1990. SAS/STAT® User's Guide, Version 6, Fourth Edition, Volumes 1 and 2; SAS Procedures Guide®, Version 6, Third Edition; Cary, NC.

12.0 Monsanto Study Specific SOPs:

BtM-PRO-037 :	Procedures for Extraction and Quantitative ELISA for Glyphosate Oxidoreductase (GOX) in Corn Leaf, Seed and Whole Plant Tissue
BtM-PRO-076 :	Procedure for the Direct ELISA for the Quantitative Analysis of CP4 5-Enol Pyruvyl Shikimate 3-Phosphate Synthase in Corn Leaf, Seed and Whole Plant Tissues
BtM-PRO-067 :	Preparation of Protein Extracts of Corn Tissues
BtC-PRO-015	BioRad Protein Assay (96-well plate application)
GEN-COM-002	Procedure for the NPD Regulatory Sciences Computer Data Handling System

Attachment 1

Table 1. Summary of plant samples of corn lines MON 822, 830, 831, and 832 for protein expression level determinations

	Site codes				
	JV	VH	IA	NE	MN
Young leaf	X	X	X	X	X
Forage	X				
Grain	X	X	X	X	X

Table 2. Summary of plant samples of corn lines MON 822, 830, 831, and 832 for compositional analyses

	Site codes				
	JV	VH	IA	NE	MN
Forage	X				
Grain	X	X	X	X	X

Attachment 2

GRAIN SAMPLE MON NUMBERS

Corn Line MON #	Site Codes JV	VH	IA	NE	MN
Control Line					
822	82262	82263	82264	82265	82266
Test Lines					
830	83062	83063	83064	83065	83066
831	83162	83163	83164	83165	83166
832	83262	83263	83264	83265	83266

Study Number: 95-01-46-02 **Amendment #:** 1
CHW Number: 6103-187

Study Title: Evaluation of Roundup Ready™ Corn Produced in the 1995 U.S. Field Tests Following Roundup® Application

Date Change Implemented: November 6, 1995

Project: Corn

Page No/s. &/or Section/s: Pg 6, Sec 3.8; Pg 8, Sec 5.1 and 5.2; Pg 9, Sec 7.0; Pg 11, Sec 11.0

Protocol originally stated:

3.8 Proposed Statistical Methods:
Statistical analyses will be performed, comparing test and control means, using the SAS statistical program (SAS Institute, 1990) and the details described in the statistical analysis subreport as part of study final report.

5.1.1 Forage Samples
Two forage plants from each line will be collected at soft dough stage. The sample identifier will include the Study # and line MON number, followed by an "A" or "B". Approximately 100 gm of each ground forage sample (A and B for each line) will be pooled and shipped to CHW. There will be 1 forage sample for each line.

5.2.1 Forage Samples
The following analyses will be performed on the forage samples: proximates: moisture (M100), protein (PGEN), fat (FAAH), ash (ASHM), calories (CALC) and carbohydrates (CHO); and crude fiber (CFIB).

5.2.2 Grain Samples
The following analyses will be performed on the grain samples: proximates: moisture (M100), protein (PGEN), fat (FSOX), ash (ASHM), calories (CALC) carbohydrates (CHO); crude fiber (CFIB), amino acid (TAAP), and fatty acid profile (FAC).

7.0 CHW Final Subreport:
A quality control checked and Quality Assurance accepted analytical subreport generated by the CHW Principal Investigator will be submitted to the Monsanto Study Director to be used in preparation of the final report. This will include a Quality Assurance accepted

summary spreadsheet which includes the results reported in LIMS reports and results calculated on dry weight basis. A final subreport including a data summary spreadsheet, a copy of the Laboratory Information Management Systems (LIMS) reports, reference standards (where applicable) for each assay and Method Summaries will be submitted to the Study Director.

11.0 References

SAS Institute, Inc. 1990. SAS/STAT® User's Guide, Version 6, Fourth Edition, Volumes 1 and 2; SAS Procedures Guide®, Version 6, Third Edition; Cary, NC.

Amended as Follows:

3.8 Proposed Statistical Methods: No statistical analysis of the data will be performed.

5.1.1 Forage Samples

Two forage plants from each line will be collected at soft dough stage. The plants will be pooled during processing and the sample identifier will include the Study #, "S" for silage, 3-digit line number and 2-letter site code. Approximately 100 gm of each ground silage sample will be shipped to CHW.

5.2.1 Forage Samples

The following analyses will be performed on the forage samples: proximates: moisture (M100), protein (PGEN), fat (FAAH), ash (ASHM), carbohydrates (CHO); calcium (CAA), phosphorus (PTA), acid detergent fiber (ADF) and neutral detergent fiber (NDFE).

5.2.2 Grain Samples

The following analyses will be performed on the grain samples: proximates: moisture (M100), protein (PGEN), fat (FSOX), ash (ASHM), and carbohydrates (CHO); calcium (CAA), phosphorus (PTA), acid detergent fiber (ADF), neutral detergent fiber (NDFE), amino acid profile (TAAP), fatty acid profile (FAC) and alpha tocopherol (EFD2).

7.0 CHW Final Subreport:

A quality control checked and Quality Assurance accepted analytical subreport generated by the CHW Principal Investigator will be submitted to the Monsanto Study Director to be used in preparation of the final report. A final subreport including a data summary spreadsheet, reference standards (where applicable) for each assay and Method Summaries will be submitted to the Study Director.

11.0 References
(Reference for statistical analysis, SAS, deleted)

Reason for Amendment and how this change will impact the Study:
Statistical analysis of the data will not be performed. The statistical analysis has been of marginal utility in previous studies and deemed unnecessary for this study.

The forage samples were pooled during harvest and therefore, there will not be an "A" and "B" sample for analysis.

The crude fiber assay for forage and grain samples will be replaced by the acid detergent fiber assay and the neutral detergent fiber assay. This change will improve the utility of the fiber data generated. The alpha tocopherol, calcium and phosphorus analyses have been added to the protocol.

The Laboratory Information Management Systems (LIMS) reports (if generated) will not be included in the analytical subreport. This change will eliminate unnecessary paperwork and reduce the chance of transcription errors.

Signatures of Approval

Study Director:

Patricia R Sanders
Patricia R. Sanders

Date: 11/13/95

Sponsor/Testing Facilities Management Representative:

Roy L Fuchs
Roy L. Fuchs

Date: 11/13/95

Signatures of Acknowledgement

Mark E. Groth
Mark Groth — **Date:** 11/10/95

Diane Henning
Diane Henning — **Date:** 11/16/95

Signature of Review by QA

Clyde L. Livingston
Clyde Livingston - Ceregen — **Date:** 10 Nov 1995

Jonathan C Kreuger
Corning Hazleton, Inc. — **Date:** 11/16/95

cc: