Study Title

Evaluation of Insect-Protected, Insect-Protected Roundup Ready™, and Roundup Ready™ Maize Lines in the 1995 European Field Trial 95-BTRR-01

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Study Completed On

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Laboratory Project ID

Study 95-10-50-03
CHW Project 6103-185

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 maize lines until after their use in this study [160.105(a)].

2. The data contained in Appendices 1 and 3 was generated in other studies conducted under GLP, not as part of this study.

3. The data contained in Attachment 2 was not generated as part of this study and was not generated under protocol. The experimental data, documentation, experimental procedures and standard operating procedures used by trained personnel were consistent with the practices required by GLP standards and good scientific principles to provide reliable and accurate results.

Roy Fischer 12/19/96
Sponsor Date

Patricia Sanders Dec 19, 1996
Study Director Date

Quality Assurance Statement

This signed statement indicates that the Quality Assurance Unit has monitored this study and reviewed the study data and final report. These reviews indicate that the report accurately describes the methods and standard operating procedures, and that the reported results accurately reflect the raw data of the study.

The following is a list of reviews on the study reported herein.

Date of Inspection	Type of Inspection	Date Reported to Study Director	Date Reported to Management
Jul 21, 1995	Protocol Review	Jul 21, 1995	Jul 21, 1995
Aug 3, 1995	Sample Grinding	Aug 4, 1995	Aug 4, 1995
Aug 15, 1995	ELISA Analysis	Aug 15, 1995	Aug 15, 1995
May 16-22, 1996	Raw Data and Report	May 22, 1996	May 22, 1996
Nov 20, 1996	Final Review	Nov 20, 1996	Nov 20, 1996

Clyde L. Livingston

Clyde L. Livingston
Quality Assurance Representative
Monsanto Company

20 Dec 1996
Date

Signatures of Approval

Study Number: 95-10-50-03

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Study Initiation Date: July 28, 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 Sanders — Dec. 19, 1996
Study Director — Date

[signature] — 12/19/96
Sponsor — Date

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Abbreviations

ADF	Acid detergent fibre
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
IPM	Insect Protected Maize
IPM/RR	Insect Protected Maize Roundup Ready™
KCl	potassium chloride
kg	Kilogram
LOD	Limit of Detection
lb	pound
M	Molar
mg	Milligram
MgCl2	magnesium chloride
mL	Milliliter
mM	Millimolar
N.A.	Not Applicable
NaCl	sodium chloride
N.D.	Not detected

Abbreviations (cont'd.)

NDF	Neutral detergent fibre
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

Maize lines have been modified to express a protein from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 [Cry IA(b)] (Höfte and Whitely, 1989). This protein, CryIA(b), has insecticidal activity against the European Corn Borer (ECB, *Ostrinia nubilalis*) insect pest and the pink borer (*Sesamia cretica*). These maize lines, MON 801, 809 and 810, will be referred to as insect-protected maize (IPM) lines throughout this report. In addition to the *cryIA(b)* gene, genes encoding CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) (Padgette *et al.*, 1996) and glyphosate oxidoreductase (GOX) (Padgette *et al.*, 1994) may also be present. The CP4 EPSPS and *gox* genes were present to enable selection of cells in tissue culture that contained the *cryIA(b)* gene. The Roundup Ready™ maize lines, MON 830, 831 and 832, contain only the CP4 EPSPS and *gox* genes, whose proteins confer tolerance to glyphosate (the active ingredient in the herbicide Roundup®) at the whole plant level. The insect-protected Roundup Ready (IPM/RR) maize lines, MON 802 and 805, contain all three genes, *cryIA(b),* CP4 EPSPS and *gox*. The maize transformation vectors used to produce these maize lines include 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 vector in media containing kanamycin. The *nptII* gene was under the control of a bacterial-specific promoter and therefore, does not produce the NPTII protein in plant cells. The control lines have background genetics representative of their respective test lines, but have not been genetically modified and therefore, do not express the CryIA(b), CP4 EPSPS or GOX proteins.

The purpose of this study was to evaluate insect-protected (IPM), insect-protected Roundup Ready (IPM/RR), and Roundup Ready (RR) maize lines grown under field conditions. This study was designed to estimate the levels of CryIA(b), CP4 EPSPS and/or GOX proteins in leaf, forage and grain samples from several maize lines. In addition, compositional analyses were performed on forage and grain samples.

Plant samples were collected from insect-protected, insect-protected Roundup Ready, Roundup Ready and control maize plants grown in the 1995 European field trials as representative of commercially grown maize. Therefore, data collected on protein expression levels and compositional components were representative of the levels expected in the commercial crop of these maize lines. The forage and grain samples produced in this study are appropriate for the compositional analyses.

Expression levels of CryIA(b), CP4 EPSPS and GOX proteins varied for each maize line analyzed yet were sufficient to confer the observed phenotypes, insect-protection and/or glyphosate tolerance.

The levels of the major components of maize grain (protein, fat, ash, neutral detergent fibre, acid detergent fibre, carbohydrates, moisture, amino acids and fatty acids) were similar between the test and control samples, and typical of the values published (Watson, 1982) and observed (Sanders and Patzer, 1995; and Sanders *et al.,* 1996). The major components of forage (protein, fat, ash, neutral detergent fibre, acid detergent fibre, carbohydrate and dry matter content) were similar between the maize test lines and the control line, MON 820 and within the published literature ranges (Watson, 1982). It was concluded that each of these maize lines are substantially equivalent in composition and representative of maize grain currently in commerce.

II. INTRODUCTION

A. Background

Maize lines have been modified to express a protein from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 [Cry IA(b)] (Höfte and Whitely, 1989). This protein, CryIA(b), has insecticidal activity against the European Corn Borer (ECB, *Ostrinia nubilalis*) insect pest and the pink borer (*Sesamia cretica*). These maize lines, MON 801, 809 and 810, will be referred to as insect-protected maize (IPM) lines throughout this report. In addition to the *cryIA(b)* gene, genes encoding CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) (Padgette *et al.*, 1996) and glyphosate oxidoreductase (GOX) (Padgette *et al.*, 1994) may also be present. The CP4 EPSPS and *gox* genes were present to enable selection of cells in tissue culture that contained the *cryIA(b)* gene. The Roundup Ready™ maize lines, MON 830, 831 and 832, contain only the CP4 EPSPS and *gox* genes, whose proteins confer tolerance to glyphosate (the active ingredient in the herbicide Roundup®) at the whole plant level. The insect-protected Roundup Ready (IPM/RR) maize lines, MON 802 and 805, contain all three genes, *cryIA(b)*, CP4 EPSPS and *gox*. The maize transformation vectors used to produce these maize lines include 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 vector in media containing kanamycin. The *nptII* gene was under the control of a bacterial-specific promoter and therefore, does not produce the NPTII protein in plant cells. The control lines have background genetics representative of their respective test lines, but have not been genetically modified and therefore, do not express the CryIA(b), CP4 EPSPS or GOX proteins.

B. Purpose

The purpose of this study was to evaluate insect-protected (IPM), insect-protected Roundup Ready (IPM/RR), and Roundup Ready (RR) maize lines grown under field conditions. This study was designed to estimate the levels of CryIA(b), CP4 EPSPS and/or GOX proteins in leaf, forage and grain samples from several maize lines. In addition, compositional analyses were performed on forage and grain samples.

III. MATERIALS

A. Test substances

The test substances for this study were: the IPM maize lines MON 801, MON 809 and MON 810; the IPM/RR maize lines MON 802 and MON 805; and the RR maize lines MON 830, MON 831 and MON 832. Two additional IPM maize lines, MON 813 and MON 814, were planted and sampled but were not pursued as commercial candidates, so only limited analyses were performed.

B. Control substances

The control substances for this study, MON 820 and MON 821, have not been genetically modified, but have background genetics representative of the test substances. MON 820 was the control for test lines MON 801, 802, 805, 810, 830, 831 and 832. MON 821 was the control for test lines MON 809, 813 and 814.

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 seed. Full characterization of the test and control substances was the purpose of this study.

Southern blot analysis of maize lines planted in this study was performed concurrent with this study to confirm maize line identity.

D. Reference substance

There was no reference substance for this study.

Appropriate standards were used in each assay as reference substances 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.

CryIA(b) protein standard for ELISA. The trypsin-resistant core of CryIA(b) protein (lot #I92017) used in the ELISA was prepared by trypsinization of full length CryIA(b) protein purified from *E. coli* containing plasmid pMAP40 (Heeren *et al.,* 1992). The purified protein was stored as a 1.8 mg/mL tryptic fragment of CryIA(b) protein solution in 100 mM sodium carbonate, pH 10 at approximately -80°C. Characterization of the standard has been described previously (EPA MRID #43468001).

CP4 EPSPS protein standard for ELISA. CP4 EPSPS protein standard (lot #5192245, prepared 12-12-92) was purified from *E. coli* expressing an *Agrobacterium* species strain CP4 EPSPS gene with a 90%+ purity (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 GOX protein was purified from *E. coli*, lot #LAH4/13/92 #8 characterized previously (Padgette *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 CryIA(b), 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 maize.

IV. METHODS

A. Summary of experimental design

Insect-protected, insect-protected Roundup Ready, Roundup Ready and control maize plants were grown in France and Italy (Study 95-BTRR-01). The field trials were conducted at five locations: Segoufielle, FR; Mogliano Veneto TV, IT; Beaumont sur Lève, FR; Le Castera, FR; and Montadet, FR.

These sites provided a variety of environmental conditions which were representative of regions where insect-protected and/or glyphosate tolerant maize lines would be grown as a commercial product.

Young leaf, forage and grain samples were collected from these plants as described in the Study Protocol (Attachment 1). These tissues were evaluated for CryIA(b), CP4 EPSPS and GOX protein levels using sensitive and specific ELISA assays developed and validated for each protein. The Italy site was destroyed before the forage and grain samples could be collected. Forage and grain harvested from the four remaining sites was used for the compositional analyses.

B. Field trial

Test and control maize plants were grown at five European sites under conditions typical for maize in each region. The locations encompass a range of environmental conditions and insect pressure from agronomically important pests. Up to twenty-five seed of each maize 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. Leaf, forage and grain samples from the maize plants were shipped promptly to Monsanto facilities, St. Louis, Missouri and stored according to the protocol (Attachment 1).

C. ELISA analytical methods

Extraction of protein from maize tissues. Maize tissues were processed and extracts prepared according to SOPs (Appendix 2). 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.

CryIA(b) ELISA. A direct double antibody sandwich enzyme-linked immunosorbent assay, ELISA, has been developed and validated to quantitate the levels of CryIA(b) protein in genetically modified maize plants (Ledesma *et al.*, 1995a, 1995b). The ELISA validation summary is contained in Appendix 3. CryIA(b) protein levels in tissue extracts were measured by ELISA according to SOP BtM-PRO-068-01. The leaf extraction buffer was PBST (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, and 0.07% Tween-20; SOP BtM-PRO-068) for the CryIA(b) ELISA. The forage and grain samples were extracted in TBA buffer (100mM Trizma base, 10mM sodium borate, 0.05% (v/v) Tween-20, 5mM $MgCl_2$ and 0.2% (w/v) L-ascorbic acid, pH 7.5). Tissue extracts were treated with trypsin to produce the trypsin resistant fragment of CryIA(b) protein for detection by ELISA. Trypsinolysis was stopped by addition of a serine protein inhibitor, phenylmethylsulfonyl fluoride (PMSF). Tryptic fragment of CryIA(b) protein was measured using a direct double antibody sandwich ELISA using rabbit anti-CryIA(b) and a polyclonal antibody conjugated to alkaline phosphatase (AP). Para-nitrophenyl phosphate (pNPP) was the AP substrate used for color development. Quantitation of sample CryIA(b) protein concentration was accomplished by extrapolation (based on sample absorbance value) from a tryptic fragment of CryIA(b) protein standard curve. The CryIA(b) ELISA measures the levels, in ng/mL, of tryptic fragment of CryIA(b) protein in maize tissue protein extracts. The ng/mL value obtained in the ELISA was multiplied by 2 to convert these data to levels of full-length CryIA(b) protein. The molecular weight of the tryptic fragment is approximately one-half the molecular weight of the plant-expressed full-length CryIA(b) protein.

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 maize plants (Elswick, 1995a, 1995b). The ELISA validation summary is contained in Appendix 3. CP4 EPSPS protein levels in maize tissue protein extracts were measured by a direct double antibody sandwich ELISA according to SOP BtM-PRO-076-01. 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 absorbance 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 maize plants (Davies, 1994; Davies and Sanders, 1995a). The ELISA validation summary is contained in Appendix 3. The ELISA procedure is described in detail in SOP BtM-PRO-037-00. 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) (SOP BtM-PRO-037-00). GOX protein concentration in samples was quantitated by extrapolation from the standard curve of GOX protein.

Total soluble protein. Total soluble protein in maize tissue extracts was measured by the method of Bradford (1976) using the microtiter plate application of the Bio-Rad Protein Assay according to SOP (Appendix 2). Bovine serum albumin (Sigma, St. Louis, MO) was used as the protein standard.

D. Compositional analytical methods

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

Preparation of samples for compositional analyses. Approximately 100g of several test and control forage and grain samples (MON 801, 802, 805, 810, 830, 831, 832, 820 and 821) were ground to a fine powder according to SOP and shipped to Corning Hazleton, Inc. (Madison, WI) for compositional analyses. Line identification and sample integrity were preserved by careful labelling and storage under conditions to preserve sample stability.

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. There was no analytical reference substance for these analyses.

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.

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 and 954.02, 1990). 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. The thimble was dried to remove excess moisture. Pentane was dripped through the sample to remove the fat. The extract was evaporated, dried and weighed (AOAC methods 960.39). This method was used for the grain sample analysis. There was no analytical reference substance for these analyses.

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.

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.

Crude Fibre (CFIB). Crude fibre is the loss on ignition of dried residue remaining after digestion of the samples with 1.25% sulfuric acid and 1.25% sodium hydroxide solutions under specfic conditions (AOAC method 962.09, 1990). There was no reference substance for this method.

Neutral Detergent Fibre Enzyme Method (NDFE). The sample was placed in a fitted 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 3220, 1977; USDA Agricultural Handbook No. 379, 8, 1970). There is no analytical reference substance for this method.

Acid Detergent Fibre (ADF). The sample was placed in a fitted 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). There is no analytical reference substance for this method.

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).

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 reference substances are listed in the study data files.

E. Control of bias

The test and control lines in the 1995 European field trial were planted in a non-systematic manner at each of five field sites. Maize 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. Accuracy is defined by two components: extraction efficiency and recovery of spike protein. These values for each protein are in Appendix 3. The reported expression levels were not corrected for assay bias.

F. Data reduction and statistical analyses

CryIA(b), CP4 EPSPS and GOX protein concentrations from ELISA data were calculated using validated computer systems and software. Absorbance 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, Berberich *et al.*, 1995).

The concentration of CryIA(b), CP4 EPSPS and GOX protein in the maize 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 amendments

1. Protocol Amendment #1 deleted the statistical analysis of the composition data from the study. The crude fibre assay for forage and grain samples was replaced by the neutral detergent fibre and acid detergent fibre assays. The Laboratory Information Management Systems reports would not be included in the analytical subreport.

2. Amendment #2 outlined the deletion of test lines MON 813 and MON 814 from the study. These deletions were made based on the business decision not to continue these lines as commercial candidates.

3. Amendment #3 added the crude fibre assay for forage to the compositional analysis.

V. RESULTS AND DISCUSSION

A. Field trials

The IPM, IPM/RR and RR maize lines were grown under conditions representative of the major maize-growing region of the European Union. Approximately twenty-five seeds were planted of each line at each of five sites. Leaf, forage and grain samples from insect-protected, insect-protected Roundup Ready, Roundup Ready and control plants were collected, labelled, shipped, and stored in a manner to preserve line identity and sample integrity. Table 1 lists the test and control substance identifiers assigned to each line and grain samples.

1. Test and control substance characterization

Sample analysis. Characterization of the test substance included analysis of the test and control plant samples for CryIA(b), CP4 EPSPS and GOX protein levels as part of the study. The test lines MON 813 and MON 814 were dropped from the study and are not discussed further in this report but the expression data was included in the archived study files.

Southern blot analysis. The identity of the IPM (MON 801, 809, 810, 813 and 814) and IPM/RR (MON 802 and 805) test substances was confirmed by Southern blot analysis (Appendix 1). The same test and control seed batches were planted in field trials in the US (Study #95-01-50-01/02) and EU (Study #95-BTRR-01/02). Southern blot analysis was performed on leaf material collected from one US site as representative of the line at all US and EU field sites. For the IPM and IPM/RR maize lines, the DNA pattern was compared to the pattern for the grain batch planted in the 1994 U.S. field trials. Southern blot analysis gave a unique DNA pattern for each maize line. The unique DNA pattern for each line was identical between seed planted in the 1994 U.S. trials and seed planted in these trials, verifying line identity. The control lines, MON 820 and 821 did not contain a CryIA(b) fragment, confirming their identities as controls. These results are summarized in Appendix 1. The raw data has been archived as part of Study 95-01-50-01.

The RR maize lines (MON 830, 831 and 832) were planted in trials conducted under GLP for the first time in 1995. A unique "fingerprint" DNA pattern was determined for each RR maize line as test substance characterization (Appendix 1).

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 labelled bag, frozen on dry ice and shipped frozen to Monsanto, St. Louis facility. All samples arrived frozen and were transferred to approximately -80°C storage. The leaf samples of maize line MON 801 from the Beaumont sur Lève, FR site were not received.

Forage. Two forage plants (leaves, ears, tassel and stalk) were collected at soft dough stage from each site in France. The two plants of each line were pooled and treated as a single sample. Forage plants were frozen and delivered to Monsanto Louvain-la-Neuve (LLN) on dry ice. The plants were ground to a fine powder on dry ice then shipped on dry ice to Monsanto, St. Louis facility. The samples were stored at approximately -80°C.

Grain. All grain was harvested at physiological maturity and dried to approximately 13% moisture prior to shelling. The ears were harvested from all plants at each site in France. Ears were shelled, and the grain placed into bag(s) labeled with unique batch MON numbers consisting of 3-digit maize line MON number and 2-digit numbers (Table 1). The grain was shipped to and stored at Monsanto, St. Louis facility at ambient temperature.

B. Protein expression in maize plant samples

Tables 2, 3 and 4 summarize the CryIA(b), CP4 EPSPS and GOX protein levels, respectively, in the plant samples. The RR lines do not contain the *cryIA(b)* gene and therefore, samples from these lines were not analyzed for the CryIA(b) protein. The mean CryIA(b), CP4 EPSPS and GOX protein levels for each 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 each site. All samples and extracts were analyzed within the timeframe of demonstrated protein stability for CryIA(b) (Ledesma and

Sanders, 1995a,b,c), CP4 EPSPS (Elswick and Sanders, 1995a,b,c) and GOX (Davies and Sanders, 1995b,c,d).

1. CryIA(b) protein levels in maize tissues

Table 2 summarizes the levels of CryIA(b) protein in young leaf, forage and grain samples of all IPM and IPM/RR maize lines. For the IPM lines, MON 801, 809 and 810, the level of CryIA(b) protein ranged from <0.14 to 9.39 µg/g fwt in young leaf tissue, <0.04 to 5.56 µg/g fwt in forage, and <0.07 to 0.79 µg/g fwt in grain.

For the IPM/RR lines, MON 802 and 805, the level of CryIA(b) protein ranged from 1.15 to 7.23 µg/g fwt in young leaf tissue, <0.04 to 3.79 µg/g fwt in forage, and 0.63 to 5.02 µg/g fwt in grain.

The RR lines do not contain the *cryIA(b)* gene and therefore, samples from these lines were not analyzed for the CryIA(b) protein.

2. CP4 EPSPS protein levels in maize tissues

Table 3 summarizes the levels of CP4 EPSPS protein levels in the young leaf, forage and grain samples from all maize lines. For the IPM maize lines (MON 801, 809 and 810) the level of CP4 EPSPS protein ranged from <0.49 to 16.99 µg/g fwt in young leaf tissue, <0.35 to 9.66 µg/g fwt in forage, and <0.012 to 4.11 µg/g fwt in grain.

For the IPM/RR maize lines, MON 802 and 805, the level of CP4 EPSPS protein ranged from 1.23 to 38.74 µg/g fwt in young leaf tissue, <0.35 to 13.33 µg/g fwt in forage, and 0.24 to 7.55 µg/g fwt in grain.

For the RR maize lines, MON 830, 831 and 832, the level of CP4 EPSPS protein ranged from 25.92 to 64.63 µg/g fwt in young leaf tissue, 7.53 to 46.16 µg/g fwt in forage, and 3.93 to 7.74 µg/g fwt in grain.

3. GOX protein levels in maize tissues

Table 4 summarizes the levels of GOX protein in the young leaf, forage and grain samples. For the IPM maize lines (MON 801, 809 and 810) the level of GOX protein ranged from <0.44 to 2.59 µg/g fwt in young leaf tissue, and was below the limit of detection in forage tissue and grain.

For the IPM/RR maize lines (MON 802 and 805), the level of GOX protein ranged from 1.54 to 28.71 µg/g fwt in young leaf tissue, <2.78 to 9.67 µg/g fwt in forage tissue, and <1.26 to 4.55 µg/g fwt in harvested grain.

For the RR maize lines, MON 830, 831 and 832, the level of GOX protein ranged from 3.45 to 48.3 µg/g fwt in young leaf tissue, 2.02 to 16.73 µg/g fwt in forage, and 1.63 to 7.16 µg/g fwt in grain.

C. Compositional analyses of grain and forage samples

The compositional parameters included proximate analyses (protein, fat, ash, neutral detergent fibre, acid detergent fibre and moisture), amino acid composition and fatty acid profile. 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 6103-185) which has been archived. The mean values for each component for each test sample across all sites was calculated. These values were calculated from the values measured for each sample, one from each of four sites. The range represents the minimum and maximum values from the analyses of samples across all sites.

1. Proximate analysis of maize grain

The levels of the major components of maize grain (protein, fat, ash, neutral detergent fibre, acid detergent fibre, carbohydrates, and moisture) were determined for grain of seven test lines and one control line harvested from four field trials conducted under GLP in France in 1995. Table 5a summarizes the results of these analyses. Proximate analysis was not performed for maize line MON 809 since the data was not needed for this line. The levels of each of these components were similar for the test lines and the control line, MON 820. The values for both the test and control lines were also comparable to the published literature (Watson, 1987; Jugenheimer, 1976) and observed ranges (Sanders and Patzer, 1995; and Sanders *et al.*, 1996) (Table 5b).

2. Amino acid composition of maize grain

Amino acid composition was completed on maize grain samples and the results are presented in Table 6. The reported values for each amino acid (mg/g) were converted to percent of total protein. Amino acid composition was not generated on maize line MON 809 since the data was not needed for this line. 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 and within the range previously observed for two control lines with similar genetic background (Sanders and Patzer,

1995; and Sanders *et al.,* 1996). These differences are due to the genetic background and not to the insertion of these genes.

3. Fatty acid profile of maize grain

The fatty acid composition was determined for the grain of the seven test lines and the results are summarized in Table 7. Ten fatty acids, for which the measured values were below the limit of detection of the assay (caprylic, capric, lauric, myristic, myristoleic, eicosadienoic, eicosatrienoic, arachidonic, pentadecanoic, and heptadecenoic) were excluded from the table. A fatty acid profile was not generated for maize line MON 809 since the data was not needed for this line. The fatty acid values were similar between the test and control samples, and typical of the values published (Watson, 1982) and observed (Sanders and Patzer, 1995; and Sanders *et al.,* 1996).

4. Proximate analyses of forage

The major components of forage of the maize test and control lines were measured and the results presented in Table 8. The values for protein, fat, ash, neutral detergent fibre, acid detergent fibre, carbohydrate and dry matter content were similar between the maize test lines and the control line, MON 820 and within the published literature ranges (Watson, 1982).

VI. CONCLUSIONS

Plant samples collected from insect-protected, insect-protected Roundup Ready, Roundup Ready and control maize plants grown in the 1995 European field trials were representative of commercially grown maize. Therefore, data collected on protein expression levels and compositional components were representative of the levels expected in the commercial crop of these maize lines. The forage and grain samples produced in this study are appropriate for the compositional analyses.

Expression levels of CryIA(b), CP4 EPSPS and GOX proteins varied for each maize line analyzed yet were sufficient to confer the observed phenotpyes, insect-protection and/or glyphosate tolerance.

The levels of the major components of maize grain (protein, fat, ash, neutral detergent fibre, acid detergent fibre, carbohydrates, moisture, amino acids and fatty acids) were similar between the test and control samples, and typical of the values published (Watson, 1982; 1987; Jugenheimer, 1976) and observed (Sanders and Patzer, 1995; and Sanders *et al.,* 1996).

It was concluded that each of these maize lines are substantially equivalent in composition and representative of maize grain currently in commerce.

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Table 1. Test and Control Substance Identification

Line Number[1]	Maize Line MON Number	Seed Batch MON Number[2]	Grain MON Numbers[3]
Insect-protected lines:			
576-01-1	801	80110	80121,23-25
572-16-1	809	80910	80921,23-25
658-06-1	810	81010	81021,23-25
600-14-2	813[4]	81310	81321,23-25
654-04-1	814[4]	81410	81421,23-25
Insect-protected Roundup Ready lines:			
599-04-2	802	80210	80221,23-25
631-03-1	805	80510	80521,23-25
Roundup Ready lines:			
481-10-1	830	83010	83082-86
574-04-2	831	83110	83182-86
591-03-2	832	83210	83282-86
Control lines:			
BC2F1xMo17	820	82010	82021,23-25
PI-T3/PI-3	821	82110	82121,23-25

1: Line number used in USDA planting and shipping permits.
2: Unique seed batch identifier for the batch of seed planted.
3: Unique grain batch identifier for each batch of grain harvested from each of 4 sites.
4: Line was dropped from the study by Amendment #2.

Table 2. Levels of CryIA(b) Protein in Leaf, Forage and Grain Samples

		CryIA(b) protein (µg / g fwt)		
Maize Line		**Mean[1]**	**Std Dev[2]**	**Range[3]**
A. Leaf				
MON 801	IPM	0.15	0.01	<0.14[4] - 0.16
MON 809	IPM	0.45	0.13	0.22 - 0.55
MON 810	IPM	8.60	0.74	7.59 - 9.39
MON 802	IPM/RR	6.34	0.87	5.05 - 7.23
MON 805	IPM/RR	3.41	1.80	1.15 - 5.49
B. Forage[5]				
MON 801	IPM	<0.04[4]	N.A.[6]	N.A.
MON 809	IPM	1.23	0.27	0.88 - 1.54
MON 810	IPM	4.80	0.75	4.11 - 5.56
MON 802	IPM/RR	3.34	0.33	3.03 - 3.79
MON 805	IPM/RR	1.02	0.13	<0.04 - 1.15
E. Grain[7]				
MON 801	IPM	0.08	0.01	<0.07 - 0.09
MON 809	IPM	0.51	0.23	0.25 - 0.79
MON 810	IPM	0.53	0.12	0.42 - 0.69
MON 802	IPM/RR	3.96	0.93	2.85 - 5.02
MON 805	IPM/RR	0.90	0.33	0.63 - 1.39

1: The mean and standard deviation were calculated from the analyses of plant samples, one from each of four field sites unless noted otherwise. MON 801 leaf values are from four samples.
2: Standard Deviation.
3: Minimum and maximum values from the analyses of samples across sites.
4: The threshold of detection of the assay. Some samples were below this value.
5: The mean, standard deviation and range were calculated from the analyses of plant sample(s) from four sites. A sample was a pool of two plants from each site.
6: Not applicable; the mean values were below the threshold of detection.
7: The mean, standard deviation and range were calculated from the analyses of pooled grain samples from each of four sites.

Table 3. Levels of CP4 EPSPS Protein in Leaf, Forage and Grain Samples

		CP4 EPSPS protein (µg / g fwt)		
Maize Line		**Mean[1]**	**Std Dev[2]**	**Range[3]**
A. Leaf				
MON 801	IPM	<0.49[4]	N.A.[5]	N.A.
MON 809	IPM	8.91	4.91	4.26 - 16.99
MON 810[6]	IPM	N.A.	N.A.	N.A.
MON 802	IPM/RR	31.78	6.87	21.32 - 38.74
MON 805	IPM/RR	1.84	0.79	1.23 - 3.21
MON 830	RR	49.08	9.23	38.09 - 60.40
MON 831	RR	41.63	13.30	25.92 - 58.50
MON 832	RR	49.60	9.91	38.02 - 64.63
B. Forage[7]				
MON 801	IPM	<0.35[4]	N.A.[5]	N.A.
MON 809	IPM	8.87	0.88	7.67 - 9.66
MON 810	IPM	<0.35[4]	N.A.[5]	N.A.
MON 802	IPM/RR	10.14	2.16	8.78 - 13.33
MON 805	IPM/RR	1.99	0.62	<0.35 - 2.61
MON 830	RR	22.62	7.74	15.99 - 32.76
MON 831	RR	17.48	1.85	15.59 - 19.98
MON 832	RR	28.15	15.92	7.53 - 46.16
E. Grain[8]				
MON 801	IPM	<0.12[4]	N.A.[5]	N.A.
MON 809	IPM	3.06	1.41	1.00 - 4.11
MON 810[6]	IPM	N.A.	-	-
MON 802	IPM/RR	6.51	0.92	5.52 - 7.55
MON 805	IPM/RR	0.39	0.19	0.24 - 0.64
MON 830	RR	5.33	0.19	5.12 - 5.55
MON 831	RR	6.07	1.53	3.93 - 7.39
MON 832	RR	6.97	1.22	5.15 - 7.74

1: The mean and standard deviation were calculated from the analyses of five 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 all sites.
4: The threshold of detection of the assay. All samples were below this value.
5: Not applicable. The mean values were below the threshold of detection.
6: Not applicable. MON 810 line does not contain the CP4 EPSPS gene (Kania *et al.*, 1995).
7: The mean, standard deviation and range were calculated from the analyses of plant samples from four sites. A sample was a pool of two plants from each site.
8: The mean, standard deviation and range were calculated from the analyses of pooled grain samples from each of four sites.

Table 4. Levels of GOX Protein in Leaf, Forage and Grain Samples

		GOX protein (µg / g fwt)		
Maize Line		**Mean[1]**	**Std Dev[2]**	**Range[3]**
A. Leaf				
MON 801	IPM	<0.44[4]	N.A.[5]	N.A.
MON 809	IPM	2.47	0.17	<0.73 - 2.59
MON 810[6]	IPM	---	---	---
MON 802	IPM/RR	13.29	8.77	7.74 - 28.71
MON 805	IPM/RR	2.65	1.19	1.54 - 4.13
MON 830	RR	24.70	14.88	9.37 - 48.3
MON 831	RR	23.03	9.06	8.82 - 32.56
MON 832	RR	6.50	2.35	3.45 - 10.03
B. Forage[7]				
MON 801	IPM	<0.57[4]	N.A.[5]	N.A.
MON 810[6]	IPM	---	---	---
MON 802	IPM/RR	5.00	3.22	2.41 - 9.67
MON 805	IPM/RR	5.14	3.39	<2.78 - 9.06
MON 830	RR	12.88	3.84	8.73 - 16.73
MON 831	RR	12.70	2.62	9.83 - 16.12
MON 832	RR	7.74	4.17	2.02 - 11.78
E. Grain[8]				
MON 801	IPM	<1.19[4]	N.A.[5]	N.A.
MON 809	IPM	<0.82[4]	N.A.[5]	N.A.
MON 810[6]	IPM	---	---	---
MON 802	IPM/RR	3.01	1.56	<1.26 - 4.11
MON 805	IPM/RR	3.04	1.05	2.24 - 4.55
MON 830	RR	5.09	1.30	3.74 - 6.87
MON 831	RR	5.86	1.17	4.33 - 7.16
MON 832	RR	1.93	0.27	1.63 - 2.27

1: The mean and standard deviation were calculated from the analyses of five 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 from sites.
4: The threshold of detection of the assay. All samples were below this value.
5: Not applicable. The mean values were below the threshold of detection.
6: Not applicable. MON 810 line does not contain the gox gene (Kania et al., 1995).
7: The mean, standard deviation and range were calculated from the analyses of plant samples from four sites. A sample was a pool of two plants from each site.
8: The mean, standard deviation and range were calculated from the analyses of pooled grain samples from each of four sites.

Table 5. Summary of Proximate Analysis of Maize Grain

Characteristic	MON 801	MON 802	MON 805	MON 810	MON 830	MON 831	MON 832	MON 820[a]
	Mean[b] (Range)[c]	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)
Protein[d]	12.3	12.0	12.2	11.8	11.3	11.6	11.8	10.8
	(11.4-13.0)	(11.4-12.7)	(11.4-13.3)	(10.8-12.2)	(10.2-12.8)	(10.4-12.9)	(11.4-12.4)	(9.0-11.8)
Fat[d]	2.6	2.8	2.8	3.0	2.9	3.4	3.1	3.0
	(2.4-3.0)	(2.7-2.9)	(2.6-3.1)	(2.8-3.3)	(2.6-3.1)	(3.1-3.6)	(2.9-3.3)	(2.4-3.5)
Ash[d]	1.5	1.4	1.5	1.4	1.4	1.4	1.4	1.4
	(1.3-1.6)	(1.3-1.5)	(1.4-1.6)	(1.3-1.5)	(1.3-1.5)	(1.2-1.6)	(1.2-1.6)	(1.2-1.6)
NDF[d,e]	12.6	12.9	12.1	12.1	11.2	11.6	12.5	12.4
	(11.6-13.7)	(11.2-14.3)	(11.2-14.2)	(10.7-13.6)	(10.2-12.1)	(9.8-12.5)	(11.8-12.8)	(9.3-15.3)
ADF[d,f]	3.9	4.1	4.3	3.4	3.8	3.8	4.0	3.9
	(3.3-4.3)	(3.8-4.5)	(3.2-5.2)	(2.7-4.1)	(3.5-3.9)	(3.1-4.3)	(3.5-5.2)	(3.1-5.3)
Carbohydrate[d]	83.5	83.7	83.5	84.1	84.4	83.8	83.7	84.9
	(82.3-84.6)	(83.0-84.1)	(82.0-84.3)	(83.1-84.8)	(82.7-86.3)	(82.3-84.9)	(83.3-84.0)	(83.7-86.3)
Moisture %	12.2	12.4	12.1	13.3	12.2	12.5	12.5	12.1
	(11.3-12.6)	(11.7-13.3)	(11.6-12.7)	(12.1-15.2)	(11.6-12.5)	(11.9-12.9)	(12.2-12.6)	(11.3-12.3)

[a] Values are average of two sets of measurements. MON 820 is the control maize line.

[b] Value reported is mean of four samples, one from each field site.

[c] Range denotes the lowest and highest individual values across sites for each line.

[d] Percent dry weight of sample.

[e] Neutral detergent fibre

[f] Acid detergent fibre

Table 5a. Summary of Proximate Analysis of Maize Grain: Literature References

Component	Literature Mean	Literature Range	Literature Reference	MON 800/818 Range[a]
Protein %	9.5	6.0-12.0	Watson, 1987	11.2-13.5
	12.3	9.7-16.1	Jugenheimer, 1976	
Fat (oil) %	4.3	3.1-6.7	Watson, 1987	2.6-4.2
	4.6	2.5-6.1	Jugenheimer, 1976	
Ash %	1.4	1.1-3.9	Watson, 1987	1.5-1.8
Carbohydrate		not reported		81.7-83.8
Moisture %	16.0	7-28	Watson, 1987	10.6-15.8

[a] Sanders and Petzer (1995) and Sanders et al., (1996; range for two control lines with similar genetic backgrounds; five samples of MON 800, one from each of five US field sites in 1993 and six samples of MON 818, one from each of six US field sites in 1994

Table 6. Amino Acid Composition of Maize Grain[a]

Amino Acids	MON 801	MON 802	MON 805	MON 810	MON 830	MON 831	MON 832	MON 820[b]	Literature[c]	MON 800/818[d]
	Mean[e] (Range)[f]	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Range	Mean Range
Nutritionally Essential										
Methionine	1.5 (1.4-1.5)	1.6 (1.6-1.6)	1.5 (1.5-1.6)	1.4 (1.4-1.6)	1.7 (1.6-2.1)	1.6 (1.4-1.6)	1.6 (1.5-1.8)	1.5 (1.4-1.7)	1.0-2.1	1.5-2.6
Cystine	1.9 (1.5-1.9)	2.1 (2.0-2.1)	2.0 (1.8-2.2)	1.9 (1.9-2.1)	2.1 (1.9-2.4)	2.0 (1.8-2.2)	2.1 (1.9-2.2)	2.1 (1.9-2.4)	1.2-1.6	1.8-2.3
Lysine	3.0 (2.7-3.5)	2.8 (2.5-3.1)	3.0 (2.9-3.1)	2.9 (2.7-3.1)	3.0 (2.9-3.2)	3.1 (2.9-3.4)	2.8 (2.9-3.5)	3.1 (3.0-3.5)	2.0-3.8	2.9-3.4
Tryptophan	0.6 (0.6-0.7)	0.7 (0.6-0.7)	0.6 (0.6-0.7)	0.6 (0.4-0.6)	0.6 (0.6-0.9)	0.6 (0.5-0.6)	0.6 (0.5-0.6)	0.6 (0.6-0.7)	0.5-1.2	0.4-0.8
Threonine	3.4 (3.4-3.6)	3.5 (3.5-3.7)	3.5 (3.3-3.7)	3.7 (3.6-3.7)	3.6 (3.4-3.9)	3.7 (3.4-3.9)	3.8 (3.7-3.8)	3.7 (3.3-3.8)	2.9-3.9	3.7-4.2
Isoleucine	4.2 (3.8-4.2)	4.0 (3.9-4.1)	3.8 (3.4-4.2)	3.8 (3.4-4.3)	4.1 (3.8-4.2)	3.7 (3.6-3.8)	3.9 (3.8-4.1)	3.9 (3.7-4.3)	2.6-4.0	3.6-4.0
Histidine	3.2 (3.0-3.1)	3.0 (2.8-3.1)	2.9 (2.8-3.0)	3.0 (2.9-3.0)	3.0 (2.9-3.1)	3.1 (2.9-3.4)	3.0 (3.0-3.2)	3.1 (2.9-3.2)	2.3-2.8	2.8-3.2

[a] Values are expressed as percent of total protein.

[b] Values are the average of two measurements; MON 820 is the control maize line.

[c] Watson, 1982. Values are percent of total protein (10.1% total protein [N×6.25]).

[d] Sanders and Parker (1995) and Sanders et al., (1996) range for two control lines with similar genetic backgrounds.

[e] Value reported is mean of four samples, one from each field site.

[f] Range denotes the lowest and highest individual values across sites for each line.

Table 6. Amino Acid Composition of Maize Grain[a] (cont'd.)

Amino Acids	MON 801	MON 802	MON 806	MON 810	MON 830	MON 831	MON 832	MON 820[b]	Literature[c]	MON 800/818[d]
	Mean[e] (Range[f])	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Range	Mean Range
Nutritionally Essential										
Valine	4.3	4.8	4.8	4.7	4.7	4.5	4.9	4.8		
	(4.3-4.7)	(4.6-5.3)	(4.1-5.3)	(4.4-4.9)	(4.5-4.9)	(4.3-4.7)	(4.7-5.0)	(4.4-4.9)	2.1-6.2	4.3-4.9
Leucine	14.2	14.4	14.1	14.5	14.2	14.3	14.4	14.2		
	(13.9-14.5)	(14.0-14.8)	(13.9-14.2)	(13.9-15.3)	(13.5-14.9)	(13.9-14.5)	(14.3-14.6)	(13.3-15.3)	7.8-15.2	13.6-16.0
Arginine	3.5	3.3	3.4	3.9	3.3	3.9	4.3	4.1		
	(3.5-3.5)	(3.0-3.6)	(3.3-4.1)	(3.6-4.1)	(2.9-4.2)	(3.5-4.3)	(3.9-4.6)	(3.8-4.3)	2.9-6.9	4.2-5.0
Phenylalanine	5.6	5.7	5.5	5.6	5.6	5.7	5.8	5.6		
	(5.5-5.5)	(5.5-5.5)	(5.4-5.7)	(5.4-5.9)	(5.4-5.8)	(5.4-6.0)	(5.3-6.3)	(5.3-6.0)	2.9-6.7	6.2-5.6
Glycine	3.4	3.2	3.4	3.5	3.5	3.4	3.6	3.6		
	(3.3-3.7)	(3.1-3.3)	(3.3-3.4)	(3.4-3.7)	(3.3-3.6)	(3.3-3.7)	(3.3-3.6)	(3.2-3.9)	2.6-4.7	3.6-4.2

[a] Values are expressed as percent of total protein.

[b] Values are the average of two measurements; MON 820 is the control maize line.

[c] Watson, 1982. Values are percent of total protein [10.1% total protein (Nx6.25)].

[d] Sanders and Pater (1993) and Sanders *et al.*, (1990) range for two control lines with similar genetic backgrounds.

[e] Value reported is mean of four samples one from each field site.

[f] Range denotes the lowest and highest individual values across sites for each line.

Table 6. Amino Acid Composition of Maize Grain[a] (cont'd.)

Amino Acids	MON 801	MON 802	MON 805	MON 810	MON 830	MON 831	MON 832	MON 820[b]	Literature[c]	MON 800/818[d]
	Mean[e] (Range)[f]	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Range	(Range)
Nonessential										
Alanine	8.1	8.0	8.0	8.2	7.9	8.1	8.2	8.1		
	(7.9-8.3)	(7.7-8.2)	(7.9-8.3)	(7.9-8.4)	(7.6-8.3)	(8.0-8.2)	(8.0-8.5)	(7.5-8.6)	6.4-9.9	7.8-8.1
Aspartic Acid	7.1	6.7	6.9	7.1	6.7	7.0	7.1	6.9		
	(6.6-7.6)	(6.3-7.0)	(6.9-7.1)	(6.9-7.3)	(6.3-7.2)	(6.8-7.2)	(6.9-8.2)	(6.6-7.3)	6.8-7.2	6.3-7.3
Glutamic Acid	20.6	21.5	21.0	21.3	20.8	20.9	21.4	20.9		
	(19.9-21.3)	(20.6-21.6)	(20.7-21.3)	(20.8-21.8)	(19.8-21.7)	(20.7-21.4)	(21.1-22.3)	(19.5-22.1)	12.4-19.6	19.9-21.6
Proline	10.2	10.0	9.1	9.7	9.2	10.3	10.0	9.7		
	(10.0-10.6)	(9.3-10.8)	(8.8-9.5)	(9.5-9.8)	(8.9-10.0)	(9.5-10.8)	(9.6-10.5)	(9.2-10.1)	6.6-10.3	9.0-9.8
Serine	6.6	5.2	5.2	5.5	6.3	5.5	5.5	5.3		
	(6.2-6.7)	(4.9-5.4)	(4.9-5.3)	(5.4-5.5)	(5.1-6.4)	(5.4-5.6)	(5.4-5.5)	(4.9-5.5)	4.2-5.5	5.1-6.0
Tyrosine	4.1	4.1	4.0	4.0	4.1	4.1	4.1	4.0		
	(4.0-4.2)	(3.9-4.3)	(3.9-4.1)	(3.9-4.2)	(3.9-4.4)	(4.0-4.3)	(4.1-4.2)	(3.5-4.3)	2.9-4.7	3.8-4.3

[a] Values are expressed as percent of total protein.

[b] Values are the average of two measurements; MON 820 is the control maize line.

[c] Watson, 1982. Values are percent of total protein (10.1% total protein [N=6.26]).

[d] Sanders and Porter (1995) and Sanders et al., (1996) range for two control lines with similar genetic backgrounds

[e] Value reported is mean of four samples, one from each field site.

[f] Range denotes the lowest and highest individual values across sites for each line

Table 7. Fatty Acid Composition of Maize Grain[a]

Fatty Acids	MON 801	MON 802	MON 805	MON 810	MON 830	MON 831	MON 832	MON 820[b]	Literature[c]	MON 800/818[c]
	Mean[d] (Range)[e]	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Range	(Range)
Palmitic (16:0)	9.6	9.9	10.0	10.5	10.1	10.1	10.2	10.2		
	(9.3-9.7)	(9.8-10.0)	(9.9-10.2)	(10.3-10.8)	(10.1-10.2)	(9.9-10.2)	(10.0-10.4)	(9.9-10.7)	7-19	(10.2-10.8)
Stearic (18:0)	1.5	1.6	1.4	1.5	1.5	1.6	1.6	1.5		
	(1.4-1.6)	(1.4-1.6)	(1.4-1.6)	(1.4-1.7)	(1.4-1.5)	(1.6-1.7)	(1.4-1.5)	(1.4-1.6)	1-3	(1.6-2.1)
Oleic (18:1)	21.6	21.5	21.3	22.0	22.0	23.0	22.0	22.4		
	(20.6-22.6)	(20.6-22.0)	(21.0-22.0)	(21.0-23.9)	(20.8-23.1)	(21.9-23.7)	(21.6-23.1)	(21.8-23.5)	20-46	21.3-23.9
Linoleic (18:2)	66.8	65.4	65.9	64.0	64.7	63.6	64.7	64.0		
	(64.7-66.7)	(64.0-65.9)	(64.0-66.2)	(63.3-64.6)	(63.7-66.3)	(62.9-64.5)	(63.6-65.4)	(62.7-65.1)	35-70	61.7-65.0
Linolenic (18:3)	0.9	1.1	1.1	1.1	1.0	1.0	0.9	1.0		
	(0.8-0.9)	(1.0-1.2)	(1.0-1.2)	(1.0-1.1)	(1.0-1.1)	(1.0-1.1)	(0.8-1.0)	(1.0-1.1)	0.8-2	0.9-1.1
Arachidic (20:0)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3		
	(0.3-0.3)	(0.3-0.3)	(0.3-0.3)	(0.3-0.3)	(0.3-0.3)	(0.3-0.4)	(0.3-0.3)	(0.3-0.4)	0.1-2	0.4-0.4
Eicosenoic (20:1)	0.3	0.3	0.3	0.3	0.2	0.2	0.3	0.3		
	(0.2-0.3)	(0.3-0.3)	(0.3-0.3)	(0.2-0.3)	(0.2-0.3)	(0.2-0.2)	(0.2-0.3)	(0.2-0.3)	not reported	0.3-0.3
Palmitoleic (16:1)	N.D.[f]	N.D.	N.D.	0.2	N.D.	0.1	0.1	0.2		
				(0.2-0.2)		(0.1-0.1)	(0.1-0.1)	(0.1-0.2)	not reported	not reported
Behenic (22:0)	0.2	0.1	0.2	0.2	0.1	0.1	0.2	0.2		
	(0.1-0.2)	(0.1-0.2)	(0.1-0.2)	(0.2-0.2)	(0.1-0.2)	(0.1-0.2)	(0.1-0.2)	(0.1-0.2)	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] Values are the average of two measurements; MON 820 is the control maize line.

[c] Watson, 1988.

[d] Sanders and Patzer (1995) Sanders et al. (1995) range for two control lines with similar genetic backgrounds.

[e] Value reported is mean of four samples, one from each field site.

[f] Range denotes the lowest and highest individual values across sites for each line.

[g] N.D.: Value was below the limit of detection of the assay (LOD < 0.1%).

Table 8. Summary of Proximate Analysis of Forage

Characteristic	MON 801	MON 802	MON 803	MON 810	MON 830	MON 831	MON 832	MON 820[a]	
	Mean[b] (Range)[c]	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Mean (Range)	Literature[d] (Range)
Protein[e]	7.1 (6.6-7.3)	6.9 (6.7-7.2)	7.3 (6.6-8.6)	7.3 (6.6-8.4)	6.7 (6.3-7.2)	7.4 (6.6-7.9)	6.8 (6.7-8.0)	5.8 (4.5-7.4)	3.5-16.0
Fat[e]	1.6 (1.6-1.9)	1.8 (0.5-2.6)	1.5 (1.1-1.8)	1.4 (1.3-1.7)	1.8 (1.2-2.3)	1.1 (0.3-2.1)	1.6 (0.7-2.4)	1.2 (1.1-1.4)	0.7-6.7
Ash[e]	3.1 (2.5-3.4)	3.4 (2.5-3.9)	3.8 (3.0-5.0)	3.2 (3.1-3.6)	3.3 (3.0-3.7)	3.5 (3.0-4.2)	3.4 (2.8-3.7)	3.4 (2.9-4.4)	1.3-10.5
NDF[e,f]	41.0 (38.9-44.8)	42.4 (28.4-44.1)	40.9 (38.5-42.5)	25.4 (20.9-41.4)	40.1 (37.8-42.0)	39.9 (37.0-44.4)	41.4 (35.6-45.2)	42.6 (29.3-48.3)	not reported
ADF[e,g]	32.6 (29.4-39.8)	33.4 (28.3-30.5)	26.9 (26.5-36.7)	24.7 (22.6-27.2)	27.8 (26.1-29.5)	29.0 (27.1-30.7)	28.1 (24.1-34.4)	27.3 (25.6-29.2)	not reported
Carbohydrate[e]	85.3 (87.5-88.6)	87.3 (86.6-88.4)	88.1 (87.1-90.4)	83.0 (83.0-89.6)	88.2 (87.7-89.7)	88.0 (87.1-90.2)	88.4 (87.7-90.2)	85.8 (85.6-89.1)	not reported
Dry Matter %[h]	30.0 (28.0-32.5)	30.1 (28.3-31.5)	28.2 (26.5-31.3)	30.0 (25.7-32.4)	29.8 (27.5-31.0)	28.5 (24.8-31.5)	27.6 (25.6-29.7)	28.7 (25.6-31.3)	15.5-46.7

[a] MON 820 is the control maize line.

[b] Value reported is mean of four samples, one from each field site.

[c] Range denotes the lowest and highest individual values across sites for each line.

[d] Watson, 1988.

[e] Percent dry weight of sample.

[f] Neutral detergent fiber.

[g] Acid detergent fiber.

Table 8. Summary of Proximate Analysis of Forage (cont'd.)

Characteristic	MON 809	MON 813	MON 814	MON 821[a]	
	Mean[b] (Range)[c]	Mean (Range)	Mean (Range)	Mean (Range)	Literature[d] (Range)
Protein[e]	6.8 (6.0-7.5)	6.5 (5.8-6.9)	6.5 (5.7-7.1)	6.6 (5.9-7.3)	3.5-15.9
Fat[e]	1.6 (1.2-2.1)	1.7 (1.1-2.2)	2.3 (2.0-2.7)	2.0 (1.5-2.8)	0.7-6.7
Ash[e]	3.3 (3.2-3.7)	3.1 (2.9-3.3)	3.1 (2.7-3.4)	3.3 (2.7-3.5)	1.3-10.5
NDF[e,f]	43.3 (42.5-44.6)	39.1 (34.8-45.6)	37.8 (36.2-40.1)	38.9 (36.5-43.5)	not reported
ADF[e,g]	30.1 (27.4-32.8)	25.8 (21.3-29.4)	24.7 (23.9-25.3)	26.7 (24.3-30.6)	not reported
Carbohydrate[e]	88.2 (87.4-89.5)	88.7 (88.0-89.6)	88.1 (87.3-89.3)	88.1 (87.4-88.8)	not reported
Dry Matter %	29.1 (27.8-31.5)	32.3 (29.4-33.6)	32.0 (30.8-33.7)	32.3 (30.1-35.3)	12.5-46.7

[a]: MON 821 is the control maize line.

[b]: Value reported is mean of four samples, one from each field site.

[c]: Range denotes the lowest and highest individual values across sites for each line.

[d]: Watson, 1982.

[e]: Percent dry weight of sample.

[f]: Neutral detergent fibre.

[g]: Acid detergent fibre.

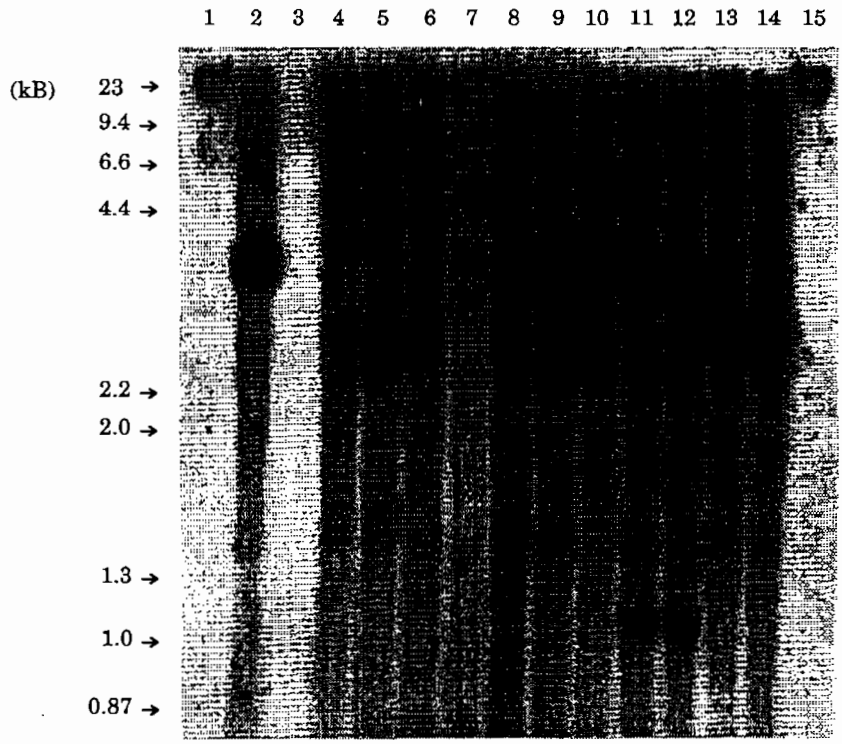
Appendix 1

Test and Control Substance Characterization

Southern blot analysis. The identity of the IPM (MON 801, 809, 810, 813 and 814) and IPM/RR (MON 802 and 805) test substances was confirmed by Southern blot analysis. The same test and control seed batches were planted in field trials in the US (Study #95-01-50-01/02) and EU (Study #95-BTRR-01/02). Southern blot analysis was performed on leaf material collected from one US site as representative of the line at all US and EU field sites. The DNAs were digested with NcoI/EcoRI and the blot probed with *cryIA(b)* DNA. For the IPM and IPM/RR maize lines, the DNA pattern was compared to the pattern for the grain batch planted in the 1994 U.S. field trials. Southern blot analysis gave a unique DNA pattern for each maize line. The unique DNA pattern for each line was identical between seed planted in the 1994 U.S. trials and seed panted in these trials, verifying line identity (Figure A1 and A2). The control lines, MON 820 and 821 did not contain a CryIA(b) fragment, confirming their identities as controls. The raw data has been archived as part of Study 95-01-50-01.

The RR maize lines (MON 830, 831 and 832) were planted in trials conducted under GLP for the first time in 1995. The DNAs were digested with NotI/KpnI and NdeI and the blot probed with *gox* DNA. A unique "fingerprint" DNA pattern was determined for each RR maize line as test substance characterization (Figure A3).

Figure A1. Test and Control Substance Characterization: Southern Blot Analysis of Insect-Protected Maize Lines MON 809 and 810, Insect-Protected Roundup Ready Maize Lines MON 802 and 805, and control lines MON 820 and 821[1]

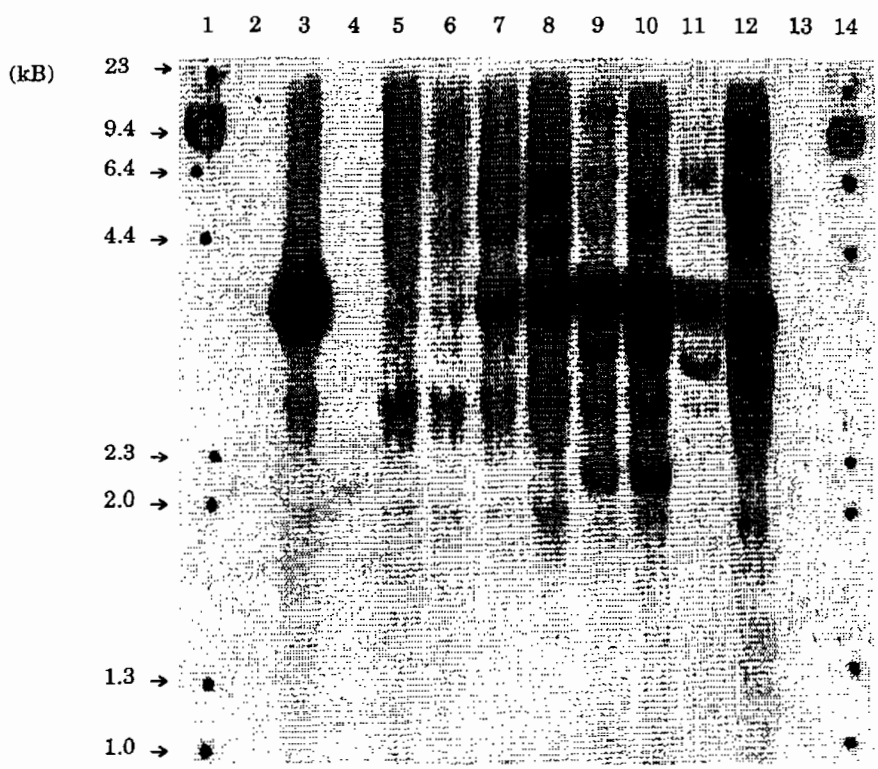


Lane

1 Boehringer Manheim Molecular Weight Markers
2 20 pg of plasmid PV-ZMBK07 and PV-ZMGT10 with 10 µg of MON 820 control DNA
3 Empty
4 MON 81800: 10 µg of DNA from control line seed batch planted in 1994 U.S. field trials
5 MON 82010: 10 µg of DNA from control line seed batch planted in 1995 U.S. and E.U. field trials
6 MON 82110: 10 µg of DNA from control line seed batch planted in 1995 U.S. and E.U. field trials
7 MON 80200: 10 µg of DNA from test line seed batch planted in 1994 U.S. field trials
8 MON 80210: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials
9 MON 80500: 10 µg of DNA from test line seed batch planted in 1994 U.S. field trials
10 MON 80510: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials
11 MON 80900: 10 µg of DNA from test line seed batch planted in 1994 U.S. field trials
12 MON 80910: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials
13 MON 81000: 10 µg of DNA from test line seed batch planted in 1994 U.S. field trials
14 MON 81010: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials
15 Boehringer Manheim Molecular Weight Markers

1: All DNAs were digested with NcoI/EcoRI. The blot was probed with ^{32}P-labeled full length *cryIA(b)* DNA.

Figure A2. Test and Control Substance Characterization: Southern Blot Analysis of Insect-Protected Maize Lines MON 801, 813 and 814[1]

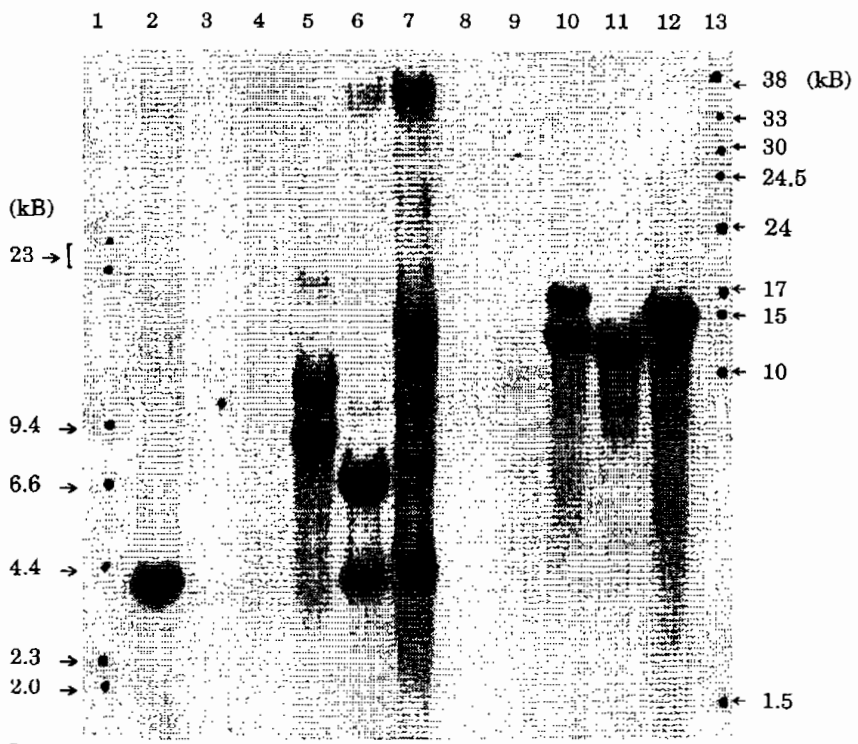


Lane

1 Boehringer Manheim Molecular Weight Markers
2 Empty
3 20pg of plasmids PV-ZMBK07 and PV-ZMGT10 with 10µg of MON 821 control DNA.
4 Empty
5 MON 82110: 10 µg of DNA from control line seed batch planted in 1995 U.S. and E.U. field trials.
6 MON 81900: 10 µg of DNA from control line seed batch planted in 1994 U.S. field trials.
7 MON 81300: 10 µg of DNA from test line seed batch planted in 1994 U.S. field trials.
8 MON 81310: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
9 MON 81400: 10 µg of DNA from test line seed batch planted in 1994 U.S. field trials.
10 MON 81410: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
11 MON 80100: 10 µg of DNA from test line seed batch planted in 1994 U.S. field trials.
12 MON 80110: 10 µg of DNA from test line seed batch planted in 1995 E.U. field trials.
13 Empty
14 Boehringer Manheim Molecular Weight Markers

1: All DNAs were digested with NcoI/EcoRI. The blot was probed with ^{32}P-labeled full length *cryIA(b)* DNA.

Figure A3. Test and Control Substance Characterization: Southern Blot Analysis of Roundup Ready Maize Lines MON 830, 831 and 832[1]



Lane

1 Boehringer Manheim Molecular Weight Markers
2 20pg of plasmid PV-ZMGT10 with 10µg of MON 820 control DNA.
3 Empty
4 MON 82010: 10 µg of DNA from control line seed batch planted in 1995 U.S. and E.U. field trials.
5 MON 83010: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
6 MON 83110: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
7 MON 83210: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
8 Empty
9 MON 82010: 10 µg of DNA from control line seed batch planted in 1995 U.S. and E.U. field trials.
10 MON 83010: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
11 MON 83110: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
12 MON 83210: 10 µg of DNA from test line seed batch planted in 1995 U.S. and E.U. field trials.
13 New England BioLabs Mono Cut Mix Molecular Weight Markers

1: DNAs in lanes 2, 4-7 were digested with NotI/KpnI; DNAs in lanes 9-12 were digested with NdeI. The blot was probed with ^{32}P-labeled full length *gox* DNA.

Appendix 2

Standard Operating Procedures

BtM-PRO-067-01	Preparation of Protein Extracts of Corn Tissues
BtM-PRO-068-01	Procedure for Quantitative HD-1 ELISA for Corn Tissues
BtM-PRO-076-01	Procedure for the Direct ELISA for the Extraction & Quantitative Analysis of CP4 5-Enol-Pyruvyl-Shikimate-3-Phosphate Synthase in Corn Leaf, Seed, and Whole Plant Tissues.
BtM-PRO-037-01	Procedures for Extraction and Quantitative ELISA for Glyphosate Oxidoreductase (GOX) in Corn Leaf, Seed and Whole Plant Tissue
BtC-PRO-015-00	Bio-Rad Protein Assay (96-well plate application)
GG-PRO-015-01	Bio-Rad Protein Assay (96-well plate application)
GEN-EQP-019-01	Operation and Use of a Brinkman Polytron
GEN-PRO-012-02	Procedure for Conjugation of Alkaline Phosphatase to Purified Antibody
GEN-COM-002-00	Procedure for the NPD Regulatory Sciences Computer Data Handling System

Appendix 3: ELISA Validation Summaries

CryIA(b) Protein ELISA Validation Summary[1]

I. Precision

QC Sample[2] Variability:	≈ 13.9 % CV
Variability in Tissue:	≈ 11.8 % CV for corn leaf ≈ 21.1 % CV for corn whole plant ≈ 32.4 % CV for corn grain

II. Accuracy

Extraction Efficiency[3]:	≈ 88% for corn leaf (1:50 tissue to buffer ratio, t:b) ≈ 83% for corn forage (1:50 t:b ratio) ≈ 88% for corn grain (1:100 t:b ratio)
Spike and Recovery[4]:	≈ 78% from corn leaf ≈ 65% from corn forage ≈ 77% from corn grain

III. Range

Limit of Detection[5]:	≈ 0.17 µg/g fwt for corn leaf ≈ 0.06 µg/g fwt for corn forage ≈ 0.06 µg/g fwt for corn grain
Range of Quantitation:	0.32 - 12.8 ng/mL tryptic CryIA(b)

IV. Assay Evaluation Criteria

Quality control (QC) sample[2]:	± 3 standard deviations from the mean (46.44 - 127.94 ng/mL)
Value of the buffer blank:	< 0.229 OD at 405 nm/655 nm ref.
OD of highest standard:	0.8 - 1.2 OD

CryIA(b) Protein ELISA Validation Summary (cont'd.)

R2 value from std. curve: > 0.98 (approximately)

Mean % Error for curve fit: < 10 % (approximately)

Variability in sample replicates: < 10 % CV (approximately)

Range for quadratic curve fit parameters a, b, c:
± 3 standard deviations from the mean

a: -2.383 to -1.697 b: 0.686 to 1.121 c: -0.115 to 0.021

V. Summary of Spike and Recovery of CryIA(b) Protein from Corn Forage and Senescence Tissues

A. Spike and Recovery (Tryptic Fragment of CryIA(b) Protein)

Spike Levels (ng/ml)	Recovered (ng/ml)	Recovery (%)	Mean % Recovery
Forage Matrix, MON 820			
2.5	1.64[6]	66	
30.06	19.23[6]	64	65
Senescence Matrix, MON 820			
2.5	1.64	65	
10.0	3.79	38	52
Buffer Control, PBSTO			
2.5	2.23	89	
10.0	8.19	82	84
30.0	23.97	80	

CryIA(b) Protein ELISA Validation Summary (cont'd.)

VI. Summary of Extraction Efficiency of CryIA(b) Protein from Corn Forage and Senescence Tissues

B. Extraction Efficiency

	Tissue:buffer ratio	Ext. Efficiency Range %	Mean
Forage, MON 810	1:50	76 - 86	83
Senescence, MON 810	1:50	65 - 73	69

1: Study #93-01-39-07, (Ledesma, *et al.*, 1995b).
2: Quality control sample is a cotton seed extract which expresses a very stable, truncated form of CryIA(b) protein.
3: Extraction efficiency was evaluated during either assay development or during the course of the study (Ledesma, *et al.*, 1995a).
4: Spike and recovery values are the mean of two spike levels.
5: Limit of detection values are calculated from the average mean µg/g fwt of two control lines, MON 820 and MON 821.
6: Value is an average of 2 non-consecutive results.

CP4 EPSPS Protein ELISA Validation Summary[1]

I. Precision

QC1 (low range) Variability:	≈ 15.9 % CV
QC2 (mid range) Variability:	≈ 6.6 % CV
Variability in Tissue:	≈ 10.9 % CV Leaf tissue ≈ 15.0 % CV Whole Plant tissue ≈ 25.4 % CV Grain tissue

II. Accuracy

Extraction Efficiency[2]:	≈ 84% from leaf (1:20 tissue:buffer ratio) ≈ 94% from whole plant (1:50 tissue:buffer ratio) ≈ 93% from grain (1:100 tissue:buffer ratio)
Spike and Recovery[3]:	≈ 98% (≈20%CV) from leaf ≈ 99% (≈11%CV) from whole plant ≈ 96% (≈12%CV) from grain

III. Range

Limit of Detection[4]:	≈ 0.49µg/g fwt for corn leaf ≈ 0.36µg/g fwt for corn forage ≈ 0.16µg/g fwt for corn grain
Range of Quantitation[2]:	0.10-2.0 ng CP4 EPSPS/250 µl well ±2 Standard Deviations (SD)

CP4 EPSPS Protein ELISA Validation Summary (cont'd.)

IV. Assay Evaluation Criteria[2]

Quality Controls:	± 2 SD of the mean of the historical QC data. (QC1: 0.213-0.553 ng/well) (QC2: 0.594-1.347 ng/well)
Value of the buffer blank:	< 0.101 OD
Standard #1:	OD ≥ 0.030
Standard #7:	OD ≥ 0.810
R^2 of the standard curve:	≥ 0.985
Variability of triplicate wells:	≤ 10% CV

1: Study #94-01-39-06, Elswick, E. 1995b
2: Elswick, E. 1995a
3: % Recovery of spiked CP4 EPSPS protein. Mean of nine data points at low (0.4 ng) and mid (1 ng) spike concentrations.
4: Limit of detection values are calculated from the average mean µg/g fwt of two control lines, MON 820 and MON 821.

GOX Protein ELISA Validation Summary

I. Precision[1]

QC Sample[2] Variability:	≈ 20% CV for leaf tissue ≈ 17% CV for grain tissue
Variability in Tissue:	≈ 47% CV for leaf tissue ≈ 31% CV for whole plant tissue ≈ 32% CV for grain tissue

II. Accuracy

Extraction Efficiency[3]:	≈ 79% from leaf tissue (1:100 tissue to buffer ratio) ≈ 88% from whole plant tissue (1:60 tissue to buffer ratio) ≈ 81% from grain tissue (1:100 tissue to buffer ratio)
Spike and Recovery[4]:	≈ 51% from leaf tissue ≈ 73% from whole plant tissue ≈ 80% from grain tissue

III. Range

Limit of Detection[5]:	≈ 1.6 µg/g fwt for corn leaf ≈ 2.0 µg/g fwt for forage ≈ 1.1 µg/g fwt for corn grain
Range of Quantitation:	0.375 ng to 6.0 ng/well leaf 0.75 ng to 6.0 ng/well whole plant 0.75 ng to 6.0 ng/well grain

IV. Assay Evaluation Criteria[1]

Absorbance of the Buffer Blank:	< 0.4833

GOX Protein ELISA Validation Summary (cont'd.)

Quality Control Sample:	mean of leaf QC= 2.96 ng/well std dev=0.57 range=1.25 to 4.67 ng/well mean of seed QC =1.85 ng/well std dev of 0.31 range=0.92 to 2.78 ng/well
Coefficient of Variance of Replicated Wells:	< 10% CV
Coefficient of Determination (R^2 value):	> 0.985

1: Study #93-01-39-09, Davies and Sanders 1995a.
2: Quality Control sample is control extract, spiked with GOX protein standard.
3: Davies, 1994.
4: Study #93-01-39-09, Davies and Sanders 1995a. Means of ≥7 data points at three spike levels.
5: Limit of detection values are calculated by averaging the values generated from individual ELISA plates for control lines MON 820 and 821.

Attachment 1:

Protocol

Study #: 95-10-50-03

Corning Hazleton Project Identification: 6103-185

Study Title: Evaluation of Insect Protected, Insect Protected Roundup Ready™, and Roundup Ready™ Corn Produced in the 1995 European Field Trial 95-BTRR-01

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

Primary Testing Facility:
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Principal Investigator:
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Phone (314) 537-7460
FAX (314) 537-6880

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:

Ray L. Fuchs Date: 7/28/95

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

Study Director:

Patricia Sanders Date: 7/28/95

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

Reviewed by:

Principal Investigator:

Mark E. Groth Date: 7/28/95

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

Principal Investigator:

Diane Henning Date: 8/2/95

Diane Henning
Corning Hazleton, Inc.

Monsanto Quality Assurance:

DeAnn Holden
Quality Assurance Auditor
Monsanto Company

Date: 7/31/95

Corning Hazleton Quality Assurance:

Jonathan C Kelly
QA Representative
Corning Hazleton, Inc.

Date: 8/3/95

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GLP/QC Coordinator
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Date: 7/31/95

Co-investigators acknowledging study protocol:

Bibi E. Ledesma
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Glen Rogan
Glen Rogan
Monsanto Company
CEREGEN

Date: 7/28/95

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Frances Brostrom
Olsten Temps, contracted to
CEREGEN

Date: 7/28/95

1.0 Purpose:

The purpose of this study is to evaluate insect protected (IPC), insect protected Roundup Ready™ and glyphosate tolerant (Roundup Ready™) corn lines grown under field conditions. Some of these corn lines have been modified to express a protein from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 [Cry IA(b) Höfte and Whiteley, 1989] (abbreviated as *B.t.k.* HD-1) which has insecticidal activity against the European Corn Borer (ECB) insect pest (*Ostrinia nubilalis* Hubner). Genes encoding CP4 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) and glyphosate oxidoreductase (GOX) may also be present. In addition to the *B.t.k.* HD-1 gene, the CP4 EPSPS and/or *gox* genes are present to enable selection of cells in tissue culture that contain the *B.t.k.* HD-1 gene and to confer glyphosate tolerance to the corn plant for some lines. The control lines have background genetics representative of the test lines, but have not been genetically modified and therefore, do not express the *B.t.k.* HD-1, CP4 EPSPS or GOX proteins. The control lines provide a background matrix for the analytical evaluation of *B.t.k.* HD-1, CP4 EPSPS and GOX protein expression levels in the corn tissues collected from field-grown corn plants. The test lines will be compared to the control line for each analyte measured in the compositional analyses.

This study is designed to estimate the levels of *B.t.k.* HD-1, CP4 EPSPS and/or GOX proteins in leaf, forage and grain samples of insect protected (IPC), insect protected Roundup Ready™ (IPC/RR) and glyphosate tolerant (Roundup Ready™, RR) corn plants grown under field conditions. 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-BTRR-01 in Europe.

2.0 Timelines:

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

3.0 Experimental design:

3.1 Test Substances:

The test substances are defined as the following corn lines:

MON Number	Seed Batch Number	Seed Pedigree	Line Phenotype
801	80110	BC1F5xMo17	IPC
802	80210	BC3F3xMo17	IPC/RR
805	80510	BC2F3xMo17	IPC/RR

809	80910	PI-2/PI-1	IPC
810	81010	BC1F4xMo17	IPC
813	81310	PI-T3/PI-3	IPC
814	81410	PI-T3/PI-3	IPC
830	83010	BC2F3xMo17	RR
831	83110	BC2F3xMo17	RR
832	83210	BC2F3xMo17	RR

Any of the test and control 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 Control Substances:

The control substances are defined as corn lines MON 820 and MON 821, each of which have a genetic background similar to their respective test lines, which are defined below.

MON Number	Seed Batch Number	Line Pedigree	Corresponding Test Lines
820	82010	BC2F1xMo17	MON 801, 802, 805, 810, 830, 831, 832
821	82110	PI-T3/PI-3	MON 809, 813, 814

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 95-01-50-01 or during this study.

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 *B.t.k.* HD-1, CP4 EPSPS and/or 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 *B.t.k.* HD-1, CP4 EPSPS and GOX protein levels in leaf, forage and grain samples.

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-BTRR-01), site number, 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-BTRR-01.

Field sites	Site Number	Site Code
F-32600 Segoufielle, France	1	SF
I-31021 Mogliano Veneto TV, Italy	2	MV
F-31870 Beaumont sur Lève, France	3	BL
F-31530 Le Castera, France	4	LC
F-32220 Montadet, France	5	MD

All samples will be ground to a fine powder as needed according to SOP. Monsanto will perform the *B.t.k.* HD-1, 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. Samples will be collected from multiple field 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 ten test lines and two control lines 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-BTRR-01. Leaf and forage samples will be shipped on dry ice and stored at approximately -80°C. 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 be collected and pooled. There will be one leaf sample per test and control line for each site (12 lines/site X 5 sites = 60 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 all sites at soft dough stage. The two forage samples for each line will be pooled and ground to a fine powder as per Study Plan 95-BTRR-01 (12 lines/site X 5 sites = 60 samples). An aliquot will be shipped to the Study Director. Additional grinding, according to SOP BtM-PRO-067 may be necessary before protein extracts are prepared.

4.1.3 Grain Samples

The ears of all plants will be harvested, shelled and shipped as part of Study 95-BTRR-01. 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 (12 lines/site X 4 sites = 48 samples). Grain samples will not be collected from Site 2 due to the late planting of the trial.

4.2 Analytical Methods:

Samples of test and control corn lines will be assayed for *B.t.k.* HD-1, CP4 EPSPS and/or 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 labelled with a unique number which includes the study number, tissue type, line MON number, and site code. 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 *B.t.k.* HD-1, CP4 EPSPS and/or 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-068, BtM-PRO-076 and BtM-PRO-037 respectively.

ELISA and total protein assay data will be collected and the *B.t.k.* HD-1, 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 ten test lines and two control lines in this study. Samples will be labelled with the Study #, a unique sample identifier and date. See section 4.1 for additional details. 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 forage and grain samples will be shipped to:

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

5.1.1 Forage Samples
Two plants from each line will be collected at all sites at soft dough stage. The two plants of each line will be combined during grinding to a powder. Additional grinding may be necessary before shipment to CHW. Approximately 200 gm of each ground forage sample will be shipped to CHW.

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. Approximately one kilogram of grain from each line from each site will be ground to a fine powder and an aliquot 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) 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), and 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 processing/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 [redacted]

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:

Höfte, H. & Whiteley, H. R. 1989. Insecticidal Crystal Proteins of *Bacillus thuringiensis*. Microbiological Reviews 53: 242-255.

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
BtM-PRO-068:	Procedure for Quantitative HD-1 ELISA for Corn Tissues
BtC-PRO-015	BioRad Protein Assay (96-well [illegible] plication)
GEN-COM-002	Procedure for the NPD Regulatory Sciences Computer Data Handling System

Attachment 1

Table 1. Summary of plant samples of corn lines for protein expression level determinations

	Site Numbers and Site Codes				
	1 SF	2 MV	3 BL	4 LC	5 MD
Young leaf	X	X	X	X	X
Forage	X	X	X	X	X
Grain	X	-*	X	X	X

Table 2. Summary of plant samples of corn lines for compositional analyses

	Site Numbers and Site Codes				
	1 SF	2 MV	3 BL	4 LC	5 MD
Forage	X	X	X	X	X
Grain	X	-*	X	X	X

*Site number 2 will be planted in mid-July, representative of forage maize growing conditions. Grain will not be harvested.

Attachment 2

GRAIN SAMPLE MON NUMBERS

	SITE NUMBER / CODE*			
	1	3	4	5
Corn	SF	BL	LC	MD
Line #	F-32600	F-31870	F-31530	F-32220
Test lines:				
802	80221	80223	80224	80225
805	80521	80523	80524	80225
801	80121	80123	80124	80125
809	80921	80923	80924	80925
810	81021	81023	81024	81025
813	81321	81323	81324	81025
814	81421	81423	81424	81425
830	83021	83023	83024	83025
831	83121	83123	83124	83125
832	83221	83223	83224	83225
Control lines:				
820	82021	82023	82024	82025
821	82121	82123	82124	82125

*Site number 2 will be planted in mid-July, representative of forage maize growing conditions. Grain will not be harvested.

Study Number: 95-10-50-03 **Amendment #:** 1
CHW Number: 6103-185

Study Title: Evaluation of Insect Protected, Insect Protected Roundup Ready™, and Roundup Ready™ Corn Produced in the 1995 European Field Trial 95-BTRR-01

Date Change Implemented: October 30, 1995

Project: Corn

Page No/s. &/or Section/s: Pg 7, Sec 3.8; Pg 10, Sec 5.2; Pg 11, Sec 7.0

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.

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

Amended as Follows:

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

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); 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); acid detergent fiber (ADF), neutral detergent fiber (NDFE), amino acid profile (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. 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.

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 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 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 **Date:** 10/30/95

Patricia R. Sanders

Sponsor/Testing Facilities Management Representative:

Roy L. Fuchs — **Date:** 10/30/95

Signatures of Acknowledgement

Mark Groth — **Date:** 10/30/95

Diane Henning — **Date:** 11/6/95

Signature of Review by QA

Clyde Livingston - Ceregen — **Date:** 29 Nov 1995

Corning Hazleton, Inc. — **Date:** 11/6/95

cc:

Study Number: 95-10-50-03 **Amendment #:** 2
CHW Number: 6103-185

Study Title: Evaluation of Insect Protected, Insect Protected Roundup Ready™, and Roundup Ready™ Corn Produced in the 1995 European Field Trial 95-BTRR-01

Date Change Implemented: December 22, 1995

Project: Corn

Page No/s. &/or Section/s: Pg 5 and 6, Sec 3.0

Protocol originally stated:

3.1 Test Substances:

The test substances are defined as the following corn lines:

MON Number	Seed Batch Number	Seed Pedigree	Line Phenotype
801	80110	BC1F5xMo17	IPC
802	80210	BC3F3xMo17	IPC/RR
805	80510	BC2F3xMo17	IPC/RR
809	80910	PI-2/PI-1	IPC
810	81010	BC1F4xMo17	IPC
813	81310	PI-T3/PI-3	IPC
814	81410	PI-T3/PI-3	IPC
830	83010	BC2F3xMo17	RR
831	83110	BC2F3xMo17	RR
832	83210	BC2F3xMo17	RR

3.2 Control Substances:

The control substances are defined as corn lines MON 820 and MON 821, each of which have a genetic background similar to their respective test lines, which are defined below.

MON Number	Seed Batch Number	Line Pedigree	Corresponding Test Lines
820	82010	BC2F1xMo17	MON 801, 802, 805, 810, 830, 831, 832
821	82110	PI-T3/PI-3	MON 809, 813, 814

Amended as Follows:

3.1 Test Substances:

The test substances are defined as the following corn lines:

MON Number	Seed Batch Number	Seed Pedigree	Line Phenotype
801	80110	BC1F5xMo17	IPC
802	80210	BC3F3xMo17	IPC/RR
805	80510	BC2F3xMo17	IPC/RR
809	80910	PI-2/PI-1	IPC
810	81010	BC1F4xMo17	IPC
830	83010	BC2F3xMo17	RR
831	83110	BC2F3xMo17	RR
832	83210	BC2F3xMo17	RR

3.2 Control Substances:

The control substances are defined as corn lines MON 820 and MON 821, each of which have a genetic background similar to their respective test lines, which are defined below.

MON Number	Seed Batch Number	Line Pedigree	Corresponding Test Lines
820	82010	BC2F1xMo17	MON 801, 802, 805, 810, 830, 831, 832
821	82110	PI-T3/PI-3	MON 809

Reason for Amendment and how this change will impact the Study:
The corn lines MON 813 and MON 814 have been dropped as commercial candidates and therefore, no additional analyses will be performed on these lines.

Signatures of Approval

Study Director:

Patricia R. Sanders **Date:** 12/22/95

Patricia R. Sanders

Sponsor/Testing Facilities Management Representative:

Roy L Fuchs — **Date:** 1/10/96

Signatures of Acknowledgement

Mark Groth — **Date:** 12/22/95

Diane Henning — **Date:** 1/4/96

Signatures of Review by QA

Clyde Livingston - Ceregen — **Date:** 22 Dec 1995

Corning Hazleton, Inc. — **Date:** 1-4-96

cc: Bibi Ledesma

Monsanto
CEREGEN
Regulatory Sciences

Protocol Amendment Form
SOP Reference: GEN-POL-005 Page 1 of 1

[redacted]

Study Number: 95-10-50-03 **Amendment #: 3**

Study Title: Evaluation of Insect Protected, Insect Protected Roundup Ready and Roundup Ready Corn Produced in the 1995 European Field Trial 95-BTRR-01

Date Change Implemented: 3/7/96

Project: Corn

Page No/s. &/or Section/s: Amendment #1

Protocol (Amendment #1) originally stated: The following analyses will be performed on the forage samples: proximates: moisture (M100), protein (PGEN), fat (FAAH), ash (ASHM), carbohydrates (CHO); acid detergent fiber (ADF), neutral detergent fiber (NDFE).

Amended as Follows: Some forage samples may also be analysed for total crude fiber (CFIB).

Reason for Amendment and how this change will impact the Study: After reviewing the NDF and ADF values, it was decided to also perform total crude fiber analysis on some lines.

Signatures of Approval

Study Director:

Patricia Sanders **Date:** 3/7/96

Sponsor/Testing Facilities Management Representative:

Roy Huel **Date:** 3/8/96

Signatures of Acknowledgement

Diane Henning **Date:** 3/20/96

Signature of Review by QA

QA Rep.: Scott C Rumsey **Date:** 3/21/96

Attachment 2:

Protein Expression and Composition Data on Maize Progeny

This attachment contains data on corn hybrids of MON 810, MON 802 and MON 805 grown in Italy or France. The text was taken directly from the following 90/220 dossiers submitted to the French Commission du Génie Biomoléculaire:

MON 810: Application to Place on the Market Genetically Modified Higher Plants: Insect-Protected Maize.

MON 802 Volume I-A: Application to Place on the Market Genetically Modified Higher Plants: Insect-Protected Maize.

MON 805 Volume I-B: Application to Place on the Market Genetically Modified Higher Plants: Insect-Protected Maize.

The tables are numbered as in these original documents. See the original 90/220 dossiers for the references.

(c) Expression levels in the tissues of progeny from MON 810 from the 1995 European field trials

Field trials were conducted by both Italy and France to produce leaf, forage and grain samples for expression analysis of Insect-Protected maize hybrids. The five Insect-Protected maize hybrids were developed through crossing of the MON 810 event into commercial inbreds. Non-modified versions of the same hybrids were used as the controls. Leaf samples were collected at the Italy site only, while forage and grain samples were collected at both sites. The CryIA(b) protein levels were assessed in the maize samples using a validated ELISA (Table B.9). The ELISAs for CP4 EPSPS and GOX proteins were not performed since the genes are not present in maize line MON 810 (the absence of these proteins was confirmed in the previous field trials). Field trials were approved under permit numbers B/IT/95-23 and 95.03.06 for Italy and France, respectively.

Table B.9 Summary of CryIA(b) Protein Levels in Tissues of Progeny from maize line MON 810 grown in the 1995 E.U. field trials[1]

	CryIA(b) Protein (µg/g fwt)	
Leaf	mean[2]	9.26
	range[3]	8.20-10.51
Forage[4]	mean	4.52
	range	4.00-5.11
Grain[5]	mean	0.46
	range	0.35-0.60

1: There were five hybrids planted at two field sites. All values are expressed as µg/g fresh weight of tissue.
2: The means were calculated from the analysis of an aliquot of pooled sample from Italy site.
3: The range is the minimum and maximum values from the analysis of samples from Italy site.
4: The mean and range were calculated from the analysis of one or two plants collected from both sites.
5: The mean and range were calculated from the analysis of pooled grain samples collected from both sites.

The level of CryIA(b) protein in progeny of MON 810 ranged from 8.20 - 10.51 mg/g fwt in young leaf tissue, 4.00 - 5.11 mg/g fwt in forage tissue, and 0.35 - 0.60 mg/g fwt in harvested grain. The CryIA(b) protein levels are similar for MON 810 plants derived from backcrosses to B73/Mo17 and commercial hybrids(Table B.9).

In summary, the level of CryIA(b) protein in MON 810 plants is similar when plants are grown in different geographics and when the gene is present in different genetic backgrounds. The level of expression remains consistently high to provide season long control of the targeted insect pests.

iv. Compositional analyses of progeny of MON 810

Field trials were conducted in both Italy and France in 1995 to produce forage and grain samples for the compositional analysis of Insect-Protected maize hybrids of line MON 810. Five Insect-Protected maize hybrids were developed through crossing of the MON 810 event. Nonmodified versions of the same hybrids were used as controls. Field trials were approved under permit number B/IT/95-23 in Italy and permit number 95.03.06 in France.

Grain from four or five plants of each insect protected maize hybrid and control hybrid was pooled by site, ground to a fine powder and analyzed by Corning Hazleton, Inc. The samples were analyzed by AOAC methodology (Association of Official Analytical Chemists) for protein, ash, acid detergent fiber (ADF), neutral detergent fiber (NDF), fat, moisture content, amino acid composition and fatty acid profile according to published methods (AOAC, 1990).

Forage plant samples were collected from the field trials conducted in Italy and France. One or two plants of each Insect-Protected maize hybrid or control hybrid were pooled and processed by standard procedures. Processed, dried samples were analyzed by NIR for neutral detergent fiber, acid detergent fiber, crude fiber, crude protein, ash, in vitro digestibility (cellulase method), soluble sugars, dry matter, and in situ dry matter disappearance. All analyses were conducted at Pioneer Hi-Bred Intl. in Johnston, Iowa. Results are reported as percentages of the dry weight of the sample. The forage samples were analyzed on a NIR Systems 6500 scanning near infrared spectrometer. The spectra were recorded from 1100 to 2500 nanometers. All spectra were measured in the reflectance mode. Calibrations used for prediction of constituent values for both grain and forage samples were internal calibrations developed by Pioneer Hi-Bred Intl. In addition, the forage samples were analyzed by AOAC methods at Corning Hazleton, Inc.

The compositional analysis data presented in Tables C.10 through C.13 are expressed as ranges. The range represents the minimum and maximum levels measured across the Insect-Protected or control hybrids at a given location. Many of the nutritional characteristics measured in these studies are known to vary widely across hybrids and environments (Perry, 1988). Therefore, it is most informative to analyze a range of values for nutritional characteristics when reporting results of such studies.

A. Proximate analysis on grain from progeny of maize line MON 810

The results of proximate analyses as performed by AOAC methodology on grain of maize line MON 810 are summarized in Table C.10. The values for all parameters are similar for the control hybrid and MON 810 hybrids, within and between the field sites. The measured ranges are similar to the published literature ranges (Watson, 1987).

Table C.10. Proximate Analyses on Grain from Progeny of Maize Line MON 810

	Italy Site Ranges		France Site Ranges	
	Control	MON 810	Control	MON 810
Protein %	9.1-10.4	8.4-11.0	10.1-11.2	10.7-13.7
Ash %	1.3-1.6	1.4-1.6	1.3-1.5	1.3-1.7
ADF %	2.4-4.1	2.2-3.2	2.3-2.9	3.1-3.6
NDF %	8.0-9.7	7.7-9.5	7.2-9.4	8.5-9.4
Total fat %	3.1-3.8	3.6-4.8	3.3-4.3	3.2-4.9
Carbohydrates, %	84.2-86.4	82.9-86.4	83.4-85.3	79.8-84.7
Calories C/100g	410-412	412-418	412-416	411-418
Dry Matter %	87.7-89.7	87.1-89.4	78.0-80.5	63.5-78.9

B. Amino acid composition of grain from progeny of maize line MON 810

The amino acid composition of grain from progeny of maize line MON 810 and the control is summarized in Table C.11. The range of values for each amino acid are similar for the control and MON 810 hybrids. The values are also similar to those reported in the literature (Watson, 1982).

Table C.11. Amino Acid Composition of Grain from Progeny of Maize Line MON 810

	Italy site		France site	
	Range[a]		Range[a]	
	% of Total Protein		% of Total Protein	
Amino Acid	Control	MON 810	Control	MON 810
Nutritionally essential				
Methionine	1.8-2.1	1.7-2.2	1.8-2.0	1.7-2.3
Cystine	2.1-2.3	2.1-2.3	1.9-2.2	1.9-2.3
Lysine	3.1-3.4	2.9-3.6	2.9-3.3	2.8-3.2
Tryptophan	0.6-0.7	0.6-0.7	0.5-0.6	0.6-0.6
Threonine	3.8-3.9	3.5-3.9	3.7-3.9	3.6-4.0
Isoleucine	3.6-4.4	3.7-4.5	3.9-4.3	3.9-4.7
Histidine	2.7-3.1	2.9-3.1	2.7-3.0	2.8-3.1
Valine	4.4-5.0	4.6-5.0	4.3-5.1	4.8-5.3
Leucine	13.3-13.6	12.8-13.6	13.4-14.2	14.0-14.8
Arginine	4.4-4.8	4.3-5.0	3.9-4.3	3.9-4.4
Phenylalanine	5.3-5.8	5.3-5.8	5.5-5.7	5.6-6.0
Glycine	3.8-4.0	3.7-4.2	3.6-3.8	3.5-4.0
Non-essential				
Alanine	7.7-8.2	7.6-8.0	7.7-8.2	7.9-8.6
Aspartic acid	6.7-7.2	6.7-7.2	6.5-7.1	6.4-7.3
Glutamic acid	20.0-20.4	19.2-20.1	19.9-21.0	20.7-21.6
Proline	9.1-9.7	9.3-10.0	9.0-9.7	9.5-9.9
Serine	5.2-5.7	5.0-5.3	5.1-5.7	5.2-5.6
Tyrosine	4.2-4.3	4.1-4.5	4.2-4.6	4.3-4.8

[a] Range denotes the lowest and highest individual values across 5 hybrids at each site.

C. Fatty acid profile of grain from progeny of maize line MON 810

The fatty acid profile of grain from progeny of line MON 810 is summarized in Table C.12. The range of values for each fatty acid are similar for the control hybrids and the MON 810 hybrids and were within the reported literature ranges (Watson, 1982).

Table C.12 Fatty Acid Profile of Grain from Progeny of Maize Line MON 810[a]

Component	Italy site Range[b]		France site Range[b]	
	Control	MON 810	Control	MON 810
Linoleic (18:2)	53.2-60.6	62.2-65.8	55.3-60.7	61.8-65.2
Oleic (18:1)	23.8-32.3	20.1-24.2	23.9-30.0	20.6-24.2
Palmitic (16:0)	10.6-12.2	10.1-11.6	10.7-12.3	10.4-11.8
Stearic (18:0)	1.3-1.5	1.4-1.6	1.4-1.6	1.5-1.6
Linolenic (18:3)	1.2-1.5	1.1-1.3	1.2-1.4	1.0-1.2
Arachidic (20:0)	0.3-0.4	0.3-0.4	0.3-0.4	0.3-0.4
Eicosenoic (20:1)	0.3-0.4	0.3-0.4	0.3-0.4	0.3-0.4
Behenic (22:0)	0.1-0.2	0.1-0.2	0.1-0.2	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. There were 5 control hybrids and 5 MON 810 hybrids.

[b]: Range denotes the lowest and highest individual value across all hybrids.

D. Compositional analyses on forage from progeny of maize line MON 810

Tables C.13 (a and b) summarize the results of the compositional analysis of forage samples of the control and MON 810 hybrids. Table C.13a summarizes the data from NIR analysis, Table C.13b contains the AOAC data. All data are expressed on a dry weight basis.

Table C.13a. Near-Infrared Reflectance (NIR) Spectroscopy Results on Forage from Progeny of Maize Line MON 810

	Italy Site Range[a]		France Site Range[a]	
	Control	MON 810	Control	MON 810
Crude Protein	7.4-8.6	8.0-9.4	7.4-8.4	7.3-8.3
Ash	4.8-5.7	4.5-5.4	3.5-4.6	3.8-5.3
Crude Fibre	21.4-25.1	19.0-22.6	19.3-24.6	20.4-23.7
ADF[b]	25.2-30.7	24.1-28.0	22.0-28.6	23.3-27.7
NDF[c]	47.8-54.4	46.3-51.2	43.2-54.0	45.3-51.8
Starch	15.4-28.6	22.2-30.6	12.9-32.2	8.2-24.4
Soluble Sugars	7.4-18.6	6.5-15.7	14.9-23.7	21.0-28.1
Dry Matter	94.8-95.1	92.6-95.5	93.2-95.1	94.2-95.2
IVDC[d]	65.6-71.5	69.2-73.0	69.1-76.5	70.9-76.1
ISDMD[e]	36.8-40.9	38.1-41.9	38.7-42.9	40.2-44.3

a: Range denotes the lowest and highest individual values of 5 hybrids tested.
b: Acid detergent fiber.
c: Neutral detergent fiber.
d: *in vitro* digestibility-cellulose method.
e: *in situ* dry matter disappearance.

Table C13b. AOAC Results on Forage from Progeny of Maize Line MON 810

	Italy site Range[a]		France site Range[a]	
	Control	MON 810	Control	MON 810
Protein %	6.3-7.3	6.5-7.7	6.4-8.8	5.9-7.6
Ash %	3.8-4.8	4.0-4.7	2.7-3.4	2.7-3.9
ADF[b] %	19.7-29.0	17.3-22.4	19.6-25.2	18.0-20.5
NDF[c] %	31.5-35.5	29.7-33.7	30.0-35.3	29.5-32.8
Total fat %	1.7-2.4	1.8-2.5	1.2-2.1	1.0-2.1
Carbohydrates, %	86.1-87.8	85.4-87.1	88.2-89.0	87.1-89.8
Calories C/100g	390-394	390-395	395-398	393-398
Dry Matter %	29.0-34.5	29.6-36.2	33.7-38.3	34.2-40.0

[a] Range denotes the lowest and highest individual values of 5 hybrids tested.
[b] Acid detergent fiber.
[c] Neutral detergent fiber.

In summary, within a given field trial location, either France or Italy, the compositional data was comparable across all hybrids. This is evidenced by the overlap in the range of values for each characteristic. The NIR and AOAC results are consistent, validating the utility of either method. These data demonstrate that under similar growing conditions, the composition of the grain and forage of the Insect-Protected maize hybrids are equivalent to the control hybrids grown commercially.

(c) Expression Levels in the Tissues of Progeny of Line MON 802 from the 1995 European Field Trials

Forage and grain samples were collected from progeny of MON 802 planted in a field trial in Italy. The five Insect-Protected Roundup Ready maize hybrids were developed through crossing of the MON 802 event into commercial hybrids. Non-modified versions of the same hybrids were used as the controls. The CryIA(b), CP4 EPSPS and GOX protein levels were assessed in the maize samples using validated ELISAs (Table B.8).

Table B.8. Summary of CryIA(b), CP4 EPSPS and GOX Protein Levels in Tissues of Progeny from Maize Line MON 802 Grown in the 1995 European Field Trials[1]

		Protein (µg / g fwt)		
		CryIA(b)	CP4 EPSPS	GOX
Forage[2]	range[3]	1.35-2.03	3.95-7.60	0.79-3.05
Grain[4]	range	0.67-4.64	2.97-9.33	<1.1[5]

[1]: There were five hybrids planted at one field site. All values are expressed as µg / g fresh weight of tissue.
[2]: Two plants of each hybrid were pooled and analyzed.
[3]: The range is the minimum and maximum values from the analysis of five samples, one of each hybrid.
[4]: The range was determined from the analysis of pooled grain samples, one sample from each of five hybrids.
[5]: Values were below the Limit of Detection (1.1 µg/g) for the assay.

The level of CryIA(b) protein in progeny of MON 802 ranged from 1.35 - 2.03 µg/g fwt in forage tissue and 0.67 - 4.64 µg/g fwt in harvested grain. The CryIA(b) protein levels are similar for MON 802 plants derived from backcrosses to B73/Mo17 and commercial hybrids (Table B.8).

In summary, the level of CryIA(b), CP4 EPSPS and GOX proteins in MON 802 plants is similar when plants are grown in different geographies and when the genes are present in different genetic backgrounds. The CryIA(b) protein level of expression remains consistently high to provide season long control of the targeted insect pests.

iv. Compositional analyses of progeny from maize line MON 802

Grain samples were collected from a field trial conducted in Italy in 1995 for the compositional analysis of hybrids of maize line MON 802. Six maize hybrids were developed through crossing of the MON 802 event. Nonmodified versions of the same hybrids were used as controls.

Grain from three to five plants of each insect-protected Roundup Ready maize hybrid and control hybrid was pooled by site, ground to a fine powder and analyzed by Corning Hazleton, Inc. The samples were analyzed by AOAC methodology (Association of Official Analytical Chemists) for protein, ash, acid detergent fiber (ADF), neutral detergent fiber (NDF), fat, moisture content, and amino acid composition according to published methods (AOAC, 1990).

The compositional analysis data on the grain is presented in Table C.12, expressed as ranges. The range represents the minimum and maximum levels measured across the MON 802 hybrids or control hybrids at a given location. Many of the nutritional characteristics measured in these studies are known to vary widely across hybrids and environments (Perry, 1988). Therefore, it is most informative to analyze a range of values for nutritional characteristics when reporting results of such studies.

A. Proximate analysis on grain from progeny of maize line MON 802

The results of proximate analyses as performed by AOAC methodology on grain of maize line MON 802 are summarized in Table C.12. The ranges for all parameters are similar for the control hybrid and MON 802 hybrids, within and between the field sites. The measured ranges are similar to the published literature ranges (Watson, 1987).

Table C.12. Proximate Analyses on Grain from Progeny of Maize Line MON 802

	Italy site Ranges[b]	
	Control	MON 802
Protein[a]	8.6-10.1	8.6-10.0
Ash[a]	1.4-1.5	1.4-1.5
ADF[a,c]	2.6-3.8	2.6-3.5
NDF[a,d]	8.3-10.5	8.5-10.7
Total fat[a]	2.8-3.7	2.6-3.4
Dry Matter %	88.3-89.1	87.4-88.2

[a]: Percent dry weight of sample.
[b]: The range denotes the lowest and highest individual values across the six hybrids.
[c]: Acid detergent fibre.
[d]: Neutral detergent fibre.

B. Amino acid composition of grain from progeny of maize line MON 802

The amino acid composition of grain from progeny of maize line MON 802 and the control is summarized in Table C.13. The range of values for each amino acid are similar for the control and MON 802 hybrids. The values are also similar to those reported in the literature (Watson, 1982).

Table C.13. Amino Acid Composition of Grain from Progeny of Maize Line MON 802

	Italy site Range[a] % of Total Protein[b]	
Amino Acid	**Control**	**MON 802**
Nutritionally essential		
Methionine	1.9-3.8	2.1-4.2
Cystine	2.2-2.4	2.3-2.7
Lysine	3.2-3.3	3.3-3.9
Tryptophan	0.9-1.0	0.9-1.0
Threonine	3.6-3.9	3.6-3.9
Isoleucine	3.3-3.6	3.4-3.8
Histidine	2.9-3.2	3.0-3.3
Valine	4.6-5.0	4.6-5.3
Leucine	12.3-13.3	12.5-13.1
Arginine	4.5-4.7	4.4-5.7
Phenylalanine	5.0-5.4	5.0-5.5
Glycine	3.8-3.9	3.8-4.4
Nonessential		
Alanine	7.6-7.8	7.6-8.2
Aspartic acid	6.9-7.1	6.7-7.5
Glutamic acid	19.6-20.8	19.7-20.9
Proline	9.3-9.9	9.1-10.0
Serine	4.9-5.4	4.9-5.4
Tyrosine	3.8-4.1	3.3-4.0

[a]: Range denotes the lowest and highest individual values across 4 hybrids at each site.
[b]: Values are expressed as percent of total protein.

In summary, the compositional data was comparable between the MON 802 hybrids and the control hybrids. This is evidenced by the overlap in the range of values for each component. These data demonstrate that the composition of the grain of MON 802 maize hybrids are equivalent to the control hybrids grown commercially.

(c) Expression Levels in the Tissues of Progeny of Line MON 805 from the 1995 European Field Trials

Forage and grain samples were collected from progeny of MON 805 planted in a field trial in Italy. The six Insect-Protected Roundup Ready maize hybrids were developed through crossing of the MON 805 event into commercial hybrids. Non-modified versions of the same hybrids were used as the controls. The CryIA(b), CP4 EPSPS and GOX protein levels were assessed in the maize samples using validated ELISAs (Table B.8).

Table B.8. Summary of CryIA(b), CP4 EPSPS and GOX Protein Levels in Tissues of Progeny from Maize Line MON 805 Grown in the 1995 European Field Trials[1]

		Protein (µg / g fwt)		
		CryIA(b)	CP4 EPSPS	GOX
Forage[2]	range[3]	1.95-3.01	2.52-4.61	0.70-18.47
Grain[4]	range	1.18-2.70	0.64-1.77	4.53-8.65

[1]: There were six hybrids planted at one field site. All values are expressed as µg / g fresh weight of tissue.
[2]: Two plants of each hybrid were pooled and analyzed.
[3]: The range is the minimum and maximum values from the analysis of six samples, one of each hybrid.
[4]: The range was determined from the analysis of pooled grain samples, one sample from each of six hybrids.

The level of CryIA(b) protein in progeny of MON 805 ranged from 1.95-3.01 µg/g fwt in forage tissue, and 1.18-2.70 µg/g fwt in harvested grain. The CryIA(b) protein levels are similar for MON 805 plants derived from backcrosses to B73/Mo17 and commercial hybrids (Table B.8).

In summary, the level of CryIA(b), CP4 EPSPS and GOX proteins in MON 805 plants is similar when plants are grown in different geographics and when the genes are present in different genetic backgrounds. The CryIA(b) protein level remains consistently high to provide season long control of the targeted insect pests. The CP4 EPSPS and GOX proteins levels are sufficient to confer Roundup tolerance to the plants.

iv. Compositional analyses of progeny from maize line MON 805

Grain samples were collected from a field trial conducted in Italy in 1995 for the compositional analysis of hybrids of maize line MON 805. Six maize hybrids were developed through crossing of the MON 805 event. Nonmodified versions of the same hybrids were used as controls.

Grain from three to five plants of each insect-protected Roundup Ready maize hybrid and control hybrid was pooled by site, ground to a fine powder and analyzed by Corning Hazleton, Inc. The samples were analyzed by AOAC methodology (Association of Official Analytical Chemists) for protein, ash, acid detergent fiber (ADF), neutral detergent fiber (NDF), fat, moisture content, and amino acid composition according to published methods (AOAC, 1990).

The compositional analysis data on the grain is presented in Table C.12, expressed as ranges. The range represents the minimum and maximum levels measured across the MON 805 hybrids or control hybrids at a given location. Many of the nutritional characteristics measured in these studies are known to vary widely across hybrids and environments (Perry, 1988). Therefore, it is most informative to analyze a range of values for nutritional characteristics when reporting results of such studies.

A. Proximate analysis on grain from progeny of maize line MON 805

The results of proximate analyses as performed by AOAC methodology on grain of maize line MON 805 are summarized in Table C.12. The ranges for all parameters are similar for the control hybrid and MON 805 hybrids, within and between the field sites. The measured ranges are similar to the published literature ranges (Watson, 1987).

Table C.12. Proximate Analyses on Grain from Progeny of Maize Line MON 805

	Italy site Ranges[b]	
	Control	MON 805
Protein[a]	8.6-10.1	7.8-9.9
Ash[a]	1.4-1.5	1.4-1.6
ADF[a,c]	2.6-3.8	2.9-3.8
NDF[a, d]	8.3-10.5	9.6-11.8
Total fat[a]	2.8-3.7	2.9-3.6
Dry Matter %	88.3-89.1	87.8-88.4

[a]: Percent dry weight of sample.
[b]: The range denotes the lowest and highest individual values across the six hybrids.
[c]: Acid detergent fibre.
[d]: Neutral detergent fibre.

B. Amino acid composition of grain from progeny of maize line MON 805

The amino acid composition of grain from progeny of maize line MON 805 and the control is summarized in Table C.13. The range of values for each amino acid are similar for the control and MON 805 hybrids. The values are also similar to those reported in the literature (Watson, 1982).

Table C.13. Amino Acid Composition of Grain from Progeny of Maize Line MON 805

	Italy site Range[a] % of Total Protein[b]	
Amino Acid	Control	MON 805
Nutritionally essential		
Methionine	1.9-3.8	1.9-3.3
Cystine	2.2-2.4	2.1-2.3
Lysine	3.2-3.3	3.1-3.6
Tryptophan	0.9-1.0	0.8-1.1
Threonine	3.6-3.9	3.4-3.9
Isoleucine	3.3-3.6	3.2-3.6
Histidine	2.9-3.2	2.8-3.3
Valine	4.6-5.0	4.4-5.1
Leucine	12.3-13.3	12.2-13.8
Arginine	4.5-4.7	4.1-4.8
Phenylalanine	5.0-5.4	5.0-5.5
Glycine	3.8-3.9	3.7-4.0
Nonessential		
Alanine	7.6-7.8	7.4-8.5
Aspartic acid	6.9-7.1	6.3-7.4
Glutamic acid	19.6-20.8	19.2-21.6
Proline	9.3-9.9	9.0-9.8
Serine	4.9-5.4	4.8-5.6
Tyrosine	3.8-4.1	3.7-3.9

[a]: Range denotes the lowest and highest individual values across six hybrids.
[b]: Values are expressed as percent of total protein.

In summary, the compositional data was comparable between the MON 805 hybrids and the control hybrids. This is evidenced by the overlap in the range of values for each component. These data demonstrate that the composition of the grain of MON 805 maize hybrids is equivalent to the control hybrids grown commercially.