Study Title

Compositional Analyses of MON 801 Grain and Silage from the 1993 and 1994 Corn Field Trials

Authors

Patricia R. Sanders
Shari S. Patzer

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Performing Laboratories

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

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

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10(d)(1) (A), (B), or (C).

Company Monsanto Company

Company Agent Kent Croon Date 8/25/95

Title Mgr., Regulatory Affairs Signature Kent Croon

STATEMENT OF COMPLIANCE

This study meets the requirements for 40 CFR Part 160.

8/25/95
Submitter Date

8/25/95
Sponsor Date

Patricia R Sanders 8/25/95
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. Additional reviews conducted by the contract facility Quality Assurance Unit are reported in the contract facility report.

Date of Inspection	Type of Inspection	Date Reported to Study Director	Date Reported to Management
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Clyde L. Livingston 25 Aug 1995

Clyde L. Livingston
Quality Assurance Specialist
Monsanto Company

SIGNATURES OF APPROVAL

Study Number: HWI 6103-177
94-01-39-08

Title: Compositional Analyses of MON 801 Grain and Silage from the 1993 and 1994 Corn Field Trials

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

Study Director: Patricia R. Sanders

Principal Investigator: Shari S. Patzer

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

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

Study Initiation Date: June 15, 1994

Signatures of Approval

Patricia R Sanders Date: 8/25/95
Study Director

Ray Fuchs Date: 8/25/95
Sponsor

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ABBREVIATIONS

B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
CP4 EPSPS	5-Enolpyruvylshikimate-3-phosphate synthase isolated from *Agrobacterium species* strain designated CP4
ECB	European Corn Borer
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
fwt	fresh weight
g	gram
GOX	Glyphosate oxidoreductase protein
gox	Glyphosate oxidoreductase gene
HD-1	CryIA(b) insecticidal protein isolated from *B.t.k.*
HPLC	high performance liquid chromatography
IPC	insect protected corn
MSL	Monsanto St. Louis Report
NPTII	neomycin phosphotransferase II protein
ppm	parts per million
SOP	standard operating procedure
subsp.	subspecies

I. SUMMARY

Corn line MON 801 has been modified to express a protein from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 [Cry IA(b)] (Höfte and Whiteley, 1989). This protein, abbreviated as *B.t.k.* HD-1 in this report, has insecticidal activity against the European Corn Borer (ECB) insect pest. In addition to the *B.t.k.* HD-1 gene, genes encoding CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) (Padgette *et al.*, 1993) and glyphosate oxidoreductase (*gox*) (Padgette *et al.*, 1994) are also present. The CP4 EPSPS and *gox* genes are present to enable selection of cells in tissue culture that contain the *B.t.k.* HD-1 gene. The corn transformation vectors used to produce corn line MON 801 include the gene cassette containing a bacterial specific promoter and the coding region for neomycin phosphotransferase, NPTII. Neomycin phosphotransferase protein allows selection of bacteria containing the vector in media containing kanamycin. The *nptII* gene is under the control of a bacterial-specific promoter and, therefore, does not produce the NPTII protein in plant cells. The control line, MON 800, has background genetics representative of the test line, but has not been genetically modified and therefore, does not express the *B.t.k.* HD-1, CP4 EPSPS or GOX proteins.

The purpose of this study was to assess compositional parameters in grain from the insect protected corn (IPC) line MON 801 compared to grain from the parental corn line, MON 800. Grain of both lines was harvested from 1993 GLP field trials at 5 geographically distinct locations and analyzed for important corn grain components: proximates (moisture, protein, ash, fat), calories, crude fiber, carbohydrate, starch, fatty acid profile, sugar profile, amino acid composition, tocopherols, phytic acid, minerals (calcium and phosphorus) and aflatoxin contamination.

Compositional data on the test line, MON 801, was comparable to the control line, MON 800, and within the published literature ranges. The protein levels in the test line were significantly higher than the control line but within published literature ranges. The levels of five amino acids in the test line were significantly different from the values for the control line but within published literature ranges. There were no statistically significant differences between the values for test and control lines for fat, ash, calories, moisture, fatty acids, starch, crude fiber, sugars, phytic acid, alpha tocopherol, calcium and phosphorus. Based on these data, we conclude that there are no meaningful compositional differences between the insect protected corn line, MON 801 and the control line, MON 800.

In addition, proximate analyses were performed on corn grain and silage of the MON 801 event backcrossed into a commercial genotype (Halsey *et al.*, 1995). The test and control lines were similar for all the parameters determined (protein, fat, ash, carbohydrates, calories, moisture, crude fiber, calcium and phosphorus).

II. INTRODUCTION

A. Background

Corn line MON 801 has been modified to express a protein from *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 [Cry IA(b)] (Höfte and Whiteley, 1989). This protein, abbreviated as *B.t.k.* HD-1 in this report, has insecticidal activity against the European Corn Borer (ECB) insect pest. In addition to the *B.t.k.* HD-1 gene, genes encoding CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) (Padgette *et al.*, 1993) and glyphosate oxidoreductase (*gox*) (Padgette *et al.*, 1994) are also present. The CP4 EPSPS and *gox* genes are present to enable selection of cells in tissue culture that contain the *B.t.k.* HD-1 gene. The corn transformation vectors used to produce corn line MON801 include the gene cassette containing a bacterial specific promoter and the coding region for neomycin phosphotransferase, NPTII. Neomycin phosphotransferase protein allows selection of bacteria containing the vector in media containing kanamycin. The *nptII* gene is under the control of a bacterial-specific promoter and, therefore, does not produce the NPTII protein in plant cells. The control line, MON 800, has background genetics representative of the test line, but has not been genetically modified and therefore, does not express the *B.t.k.* HD-1, CP4 EPSPS or GOX proteins.

B. Purpose

The purpose of this study was to assess compositional parameters in grain from the insect protected corn (IPC) line MON 801 compared to grain from the parental corn line, MON 800. Grain of both lines was harvested from 1993 GLP field trials at 5 geographically distinct locations and analyzed for important corn grain components: proximates (moisture, protein, ash, fat), calories, crude fiber, carbohydrate, starch, fatty acid profile, sugar profile, amino acid composition, tocopherols, phytic acid, minerals (calcium and phosphorus) and aflatoxin contamination.

In addition, proximate analyses were performed on corn grain and silage of the MON 801 event backcrossed into a commercial genotype (Halsey *et al.*, 1995).

C. Protocol Amendments

The protocol for this study is contained within Appendix 1. There were two Amendments to this study. Amendment #1 added the proximate analysis of the corn grain and silage from field Study 94-01-39-17 to the study (Halsey *et al.*, 1995). Calcium and phosphorus analyses of all grain samples were added as well. Amendment #2 changed the Study Director designation from Hazleton Wisconsin, Inc. to Monsanto Company. These Amendments had no adverse effect on the study.

III. MATERIALS

A. Test Substance

The test substances for this study were grain and silage of the corn line MON 801 from two separate GLP field trials. The grain of the corn line MON 801 was produced in 1993 GLP field trials (EPA MRID #43533202). The batch of grain from each field site was identified with a 5-digit MON number as listed in Table 1.

Grain and silage samples (MON 801), representing a commercial genotype, were produced in the 1994 GLP field trial, Study 94-01-39-17 (Halsey *et al.*, 1995). The silage samples were collected as "green chop" at soft dough stage and frozen at ≈ -20°C. The sample identifiers are listed in Table 1.

B. Control Substance

The control substances for this study were grain and silage of the corn line MON 800 from two separate Monsanto GLP field trials. The grain of corn line MON 800 was produced in 1993 GLP field trials (EPA MRID #43533202). The batch of grain or silage from each field site was identified with a 5-digit MON number. The grain and silage identifiers as well as site locations are listed in Table 1.

In addition, grain and silage of the control line (MON 819) was harvested from Study 94-01-39-17 (Halsey *et al.*, 1995), a 1994 GLP field trial located at the Monsanto Research Farm in O'Fallon, MO. Line purity was maintained by planting each line in an isolated area to prevent cross-pollination by other corn plants.

C. Test and Control Substance Characterization

This study was conducted using grain harvested from Study 93-01-39-02. The test and control corn grain was harvested at 5 locations: Jerseyville, IL (JV), Monmouth, IL (MN), Winterset, IA (WS), Windfall, IN (WN) and York, NE (YK). Line purity was maintained in two ways during the field trial: 1) ear shoots were bagged to prevent open pollination; and 2) each plant was self-pollinated by hand. After line verification, equal quantities of seed from each of 3-4 replicated plots per site were pooled and assigned a unique identifier as listed in Table 1 (EPA MRID #43533202).

Grain collected from the 1993 and 1994 field sites was tested for the presence of the *B.t.k.* HD-1 protein before being used as the test substance in this study. Before the seed from each plot was pooled in Study 93-01-39-02, a *B.t.k.* HD-1 ELISA was performed on an aliquot of each seed sample to confirm line identity. Control grain collected from the field was tested for the absence of the *B.t.k.* HD-1 protein before being used as the control substance in this study. Chain of custody documentation supports proper identification and handling of the grain samples. The results are included in the study data files of Study 93-01-39-02.

In Study 94-01-39-17, a single three acre block of each test and control line was planted in isolated areas in the 1994 field test and the harvested silage and grain was assayed for the presence (test line) or absence (control) of the *B.t.k.* HD-1 protein. Chain of custody documentation supports proper identification and handling of the grain samples. The results are included in the study data files of Study 94-01-39-17.

D. Collection, Storage, and Transfer of Test and Control Substances

Grain from Study 93-01-39-02 was harvested in bulk for each plot of each line and shipped to Monsanto Company in October 1993. Harvested grain was stored at approximately 6°C until initiation of the pooling procedure (on 4/27/94) to prepare grain pools for this study. These storage conditions were appropriate to maintain seed quality (Wych, 1988). The grain was ground to a fine powder and shipped on dry ice to Hazleton Wisconsin, Inc. on 6/7/94 and then stored at approximately -20°C during this study.

The corn silage from Study 94-01-39-17 was harvested on 9/9/94 and representative samples stored at approximately -20°C. The grain from this study was harvested 11/16/94 and stored in grain storage bins at ambient temperature until used for analysis (4/95).

E. Reference Substance

A reference substance was not used in this study. Appropriate analytical standards were used in each assay as reference standards for the analytical procedure. The reference standards are described with the method for each analysis where appropriate.

F. Stability of the Test, Control and Reference Substances

Stability of test and control grain was ensured by storing the samples under conditions appropriate to maintain seed quality (Wych, 1988). The silage samples were stored at ≈ -20°C immediately following harvest. Stability of the silage (green chop) samples was not addressed but the test and control samples were stored under identical conditions. The analytical standards were known to be stable for the experimental period; assay parameters were monitored for each procedure to ensure integrity of analyses. Samples of the test and control substances were archived under Monsanto Study 93-01-39-02 and Study 94-01-39-17 until final notification of disposition by the Sponsor.

IV. METHODS

A. Test System

The panel of analytical biochemical methods employed in this study was considered the test system. These are standard published analytical methods which are currently used to evaluate nutritional quality and composition

parameters in corn for commercial purposes. The method references are listed in Appendix 2.

B. Proximate Analysis of Grain and Silage Samples

The same methods were used for both grain and silage (green chop) samples except for fat, as described below.

Moisture (M100). The sample was dried in a vacuum oven at 100°C and dried 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 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 sample was hydrolyzed in a water bath using hydrochloric acid. The fat was extracted using ether and hexane. The extract was washed with a dilute alkali solution and filtered through a sodium sulfate column. The remaining extract was evaporated, dried and weighed (AOAC methods 922.06 and 954.02, 1990). This method was used for the silage samples. There was no analytical reference substance for these analyses.

Fat (FSOX). The 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 samples. 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.

Calories (CALC). Calories were calculated using the Atwater factors with the fresh weight-derived data and the following equation (USDA Agricultural Handbook No. 8, 1975):

calories (kcal/100g) = (4 * % protein) + (9 * % fat) + (4 * % carbohydrates)

There was no analytical reference substance for these analyses.

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 Fiber (CFIB). Crude fiber 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.

C. 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 #60H0635 and 52H0717), Cysteic Acid Monohydrate (Sigma Chemical, lot #50H2616), Methionine Sulfone (Sigma Chemical, lot #49F0113).

D. Fatty Acid Analyses (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. Sugar Profile (SUGN)

The sugars were extracted from the grain samples in deionized water, treated with a hydroxylamine hydrochloride solution in pyridine containing phenyl-β-D-glucoside as the internal standard. The resulting oximes were converted to silyl derivatives with hexamethyldisilazane (HMDS) and trifluoracetic acid (TFA) treatment and analyzed by gas chromatography using a flame ionization detector (Mason and Slover, 1971; Brosbt, K., 1972). The reference substances were obtained from Sigma Chemical: fructose (lot #109F0752), glucose (lot #41H0313), sucrose (lot #90H0679), maltose (lot #51H0476), lactose (lot #29F-0105), and galactose (lot #43H03631).

F. Starch Analysis (STCH)

Grain samples were extracted with alcohol to remove the carbohydrates other than starches. The starches were gelatinized, hydrolyzed with enzymes and titrated as reducing sugars (AOAC methods 979.10, 906.03 and 929.09, 1990). There was no analytical reference substance for these analyses.

G. Tocopherols (EFD2)

The grain samples were saponified to release the tocopherols, which were then extracted with organic solvent, followed by quantitation on an high performance liquid chromatography (HPLC) silica column using fluorescence detection (Cort *et al.*, 1983; Speek *et al.*, 1985; McMurray *et al.*, 1980). The reference substances for this method were alpha tocopherol (USP, lot K and L) (Kodak lot #4254104144), gamma-tocopherol (Sigma Chemical lot #13H0807, 43H0475, and 43H0475)

H. Phytic Acid Analysis (A1XX)

The grain samples were weighed, extracted with 0.5N hydrochloric acid, filtered and treated with ferric chloride. The amount of phosphorus was determined colorimetrically at a wavelength of 660 nm by comparison of the sample, reacted with the color reagent to form a blue complex, to standards prepared in an identical manner. Phytic acid values are determined by calculation:

$$\frac{\text{ppm phosphorus X } 0.0001}{0.2816} = \%\ \text{phytic acid}$$

(McChance and Widdewson, 1935; Fiske and Subbarrow, 1925). The reference substance for this method were 10,000 ppm phosphorus solution (SPEX lot #D-261P and E-87P).

I. Aflatoxin (AHMF)

The levels of aflatoxins B_1, B_2, G_1, and G_2 were determined in grain samples from each 1993 field site. The sample was wetted with dilute hydrochloric acid and extracted with chloroform. A portion of the extract was applied to a silica gel column. The column was washed first with benzene/acetic acid to remove major interferences present in feeds, then with ether/hexane which removes several fluorescent non-aflatoxin compounds. Aflatoxins were eluted with methylene chloride/acetone and concentrated with a rotary evaporator. The extracts were then separated by HPLC and compared to a known standard (Third International Congress of Food Science and Technology, 1994; J. A. O. A. C. no. 1:26.052-26.060, 1988; J. A. O. A. C. no. 1:26.031, 1988; J. A. O. A. C. no. 1:26.074, 1988). The reference substance for this method was Aflatoxin Mix-M (Supelco, lot #LA39657 and lot #LA43174).

J. Inorganic Components

Calcium (CAA). The grain and silage samples were dried, pre-charred, and ashed overnight at 500° to 550°C. The samples were treated with nitric acid, dried, reashed and solubilized in 4% hydrochloric acid. The amount of calcium was determined at a wavelength of 422.7 nm by comparison of test sample to the signal of the standard solution. All solutions contain 1% lanthanum and 5% hydrochloric acid (AOAC method 965.09, 968.08 and 985.35). The reference substance for this method was 1000 ppm calcium solution (Fisher, lot #940982-24).

Phosphorus (PTA). The grain and silage samples were dried, pre-charred, and ashed overnight at 500° to 550°C. The samples were treated with nitric acid, dried, reashed and solubilized in 4% hydrochloric acid. The amount of phosphorus was determined colorimetrically at a wavelength of 420 nm by comparison of test sample to the signal of the standard solution, each reacted with molybdovanadate solution (AOAC method 965.17 and 962.11). The reference substance for this method was 10,000 ppm phosphorus solution (SPEX, lot #E-87P).

K. Data Reduction and Statistical Analysis

All data reduction and statistical analyses were performed using the SAS statistical program (SAS Institute, 1990). The statistical analysis report is contained in Appendix 3.

V. RESULTS AND DISCUSSION

The values reported from the analyses at Hazleton Wisconsin, Inc. were converted to percent dry weight of the sample using the measured moisture content. A paired t-test was used to examine differences between the values for the test line, MON 801 and the control line, MON 800 from the 1993 field sites. For each analyte, the differences between the values for test and control line at each of five locations were calculated. These differences were then used in the SAS MEANS procedure (t-test option) to test whether the mean differences were equal to zero (Appendix 3). The mean differences from control were statistically significant at the 5% level if the p-value was less than 0.05.

For many analytes, values were reported as "<X", where X is the limit of quantitation for the assay. All analytes showing one or more locations as "<X" were excluded from statistical analysis.

The samples from Study 94-01-39-17 were not subjected to statistical analyses since only one sample was analyzed per line.

A. Proximate Analysis of Corn Grain

The levels of the major components of corn grain (protein, fat, ash, carbohydrate, calories and moisture) were determined for grain harvested from the 5 GLP field site in 1993 (EPA MRID #43533202). Table 2a summarizes the results of these analyses. The mean protein and carbohydrate levels for the test line, MON 801 were statistically significantly different from the control line, MON 800. The mean value for protein in MON 801 was 13.07% compared to 12.03% for MON 800. However, the protein values for both lines were within the ranges reported in the literature (Jugenheimer, 1976) (Table 2b). The higher protein levels measured in MON 800 and MON 801 corn grain is likely due to the parental genotype used in the study, High Type II crossed once with FRB73. Commercial hybrids typically have lower protein levels, 6.0-12.0% (Table 2b). Considerable genetic variability of

protein content in hybrids has been reported (Dudley and Lambert, 1992).

Ciba Seeds reported similar protein values for their inbred and hybrid lines, with ranges for the control lines from 10.0-12.8% protein, and 10.8-13.6% protein for the transgenic *Bt* lines (Lotstein, 1995). DEKALB Genetics Corp. reported protein values of 12.1% for the B16 hybrid and 9.2% for the unconverted hybrid. The range of protein derived from analysis of commercial DEKALB hybrids grown throughout the midwest in 1993 and 1994 was 6.8 to 13.4% (Flick, 1995).

When the locus in MON 801 was crossed into a commercial germplasm (eg. the test samples used in the 1994 field trial) the level of protein in the test and control grain samples was within the range expected for commercial corn lines (6.0-12.0%, Watson, 1987) (Table 6). The level of protein (8.14%) in the unreplicated test sample (MON 80191) for the 1994 field test was lower than the level (9.10%) in the control samples (MON 81991) and levels of both were considerably lower than the 1993 results. This indicates that the difference in protein levels was not associated with the locus containing the *B.t.k.* HD-1 gene.

The carbohydrate values were derived by calculation : 100 - protein - ash - moisture - fat = carbohydrate. The higher protein levels for MON 801 in the 1993 field tests resulted in a statistically significant lower carbohydrate value.

The other characteristics reported: fat, ash, calories and moisture were not statistically significant between the test line, MON 801, and the control line, MON 800.

B. Amino Acid Composition of Corn Grain

Amino acid composition was completed on corn grain samples and the results presented in Table 3. The reported values for each amino acid (mg/g) were converted to percent of total protein on a dry weight basis and the statistical analyses performed. The test line, MON 801 contained five amino acids which were statistically different from the values for the control line, MON 800. However, the values for all five amino acids were within the range reported in the literature (Watson, 1982).

C. Fatty Acid Profile of Corn Grain

The fatty acid composition was determined for the test line, MON 801, and the control line, MON 800, grain from the replicated samples of the 1993 field sites and the results are summarized in Table 4. Statistical analysis was completed on the fatty acids which gave detectable values in the assay (Table 4). No statistically significant differences between the fatty acid composition of the test and control line was observed in a paired t-test analysis at the 95% confidence level. The fatty acids which were not detectable in the assay were: caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, heptadecanoic, eicosadienoic, eicosatrienoic and arachidonic.

D. Carbohydrates in Corn Grain

Some carbohydrate components, namely starch, crude fiber, sugars and phytic acid were evaluated for the test line, MON 801 and the control line, MON 800 for grain from the 1993 field sites. The results are presented in Table 5. In addition to fructose, glucose and sucrose, the sugars galactose, lactose and maltose were also assayed but the values were below the limit of quantitation of the assay. No statistically significant differences between the carbohydrate components of the test and control line was observed in a paired t-test analysis at the 95% confidence level.

E. Tocopherol Analyses of Corn Grain

Tocopherols are naturally present in corn seed oil and have vitamin E potency (Watson, 1982). The alpha, beta and gamma tocopherols were measured and the results summarized in Table 5. The mean values for alpha and beta were not significantly different between the test and control samples. The gamma tocopherol means were significantly different but this tocopherol is one-tenth as active as the alpha tocopherol (Watson, 1982) and therefore, not an important component of corn grain.

F. Inorganic Components in Corn Grain

The calcium and phosphorus levels were determined for the test line, MON 801, and the control line, MON 800 and the results summarized in Table 5. No statistically significant differences between calcium and phosphorus levels of the test and control line were observed in a paired t-test analysis at the 95% confidence level.

G. Aflatoxin Analyses of Corn Grain

Aflatoxins are a group of mycotoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* that may contaminate food and feed products (Jorgensen and Price, 1981). The test and control samples were assayed for B1, B2, G1 and G2 aflatoxins. All results were below the limit of detection of the assay (Appendix 3).

H. Analysis of Grain and Silage from Study 94-01-39-17

Limited compositional analyses were performed on grain and silage samples from the test line, MON 801 and control line, MON 819 planted in the Monsanto GLP field Study 94-10-39-17. The test line was a commercial hybrid genotype which had been backcrossed to contain the MON 801 locus. Additional analyses were performed by Corning Hazleton which were not requested in the protocol. Those results are not presented in this report but the data is included in the study file. The results reported are on a dry weight basis for grain and fresh weight basis for silage as summarized in Table 6. The protein values for this commercial genotype are lower than those observed in Study 93-01-39-02 (Table 2a). The analyte values are similar for the test and control lines, for both grain and silage.

VI. CONCLUSIONS

Compositional data on the test line, MON 801, was comparable to the control line, MON 800, and within the published literature ranges. The protein levels in the test line were significantly higher than the control line but within published literature ranges. The levels of five amino acids in the test line were significantly different from the values for the control line but within published literature ranges. There were no statistically significant differences between the values for test and control lines for fat, ash, calories, moisture, fatty acids, starch, crude fiber, sugars, phytic acid, alpha tocopherol, calcium and phosphorus. Based on these data, we conclude that there are no meaningful compositional differences between the insect protected corn line, MON 801 and the control line, MON 800.

VII. ACKNOWLEGEMENTS

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VIII. REFERENCES

The references for the analytical methods are listed in Appendix 2.

Dudley, J.W. and R.J. Lambert. 1992. Ninety Generations of Selection for Oil and Protein in Maize. *Maydica* 37:1-7.

EPA MRID #43533202. Ream, J. E., Sanders, P.R., Davies, S.R., Elswick, E., Groth, M.E., and Ledesma, B.E. 1995. Evaluation of European Corn Borer Resistant Corn Lines in 1993 U.S. Field Test Locations. Monsanto Technical Report MSL-13860, St. Louis.

Flick, Christopher E. 1995. Petition for Determination of Nonregulated Status: Glufosinate Resistant Corn Line B16. USDA Petition # 95-145-01p.

Halsey, Mark E., Sanders, Patricia R., and Dodson, Hope C. 1995. 1994 Large Scale Production of European Corn Borer Resistant and Control Corn Plants. Monsanto Technical Report MSL-14271, St. Louis. Study 94-01-39-17.

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

Jorgensen, K.V. and Price, R.L. 1981. Atmospheric Pressure-Ambient Temperature Reduction of Aflatoxin B1 in Ammoniated Cottonseed. *J. Agric. Food Chem.* 29:585-588.

Jugenheimer, Robert W. 1976. Corn: Improvement, Seed Production, and Uses. pg 227. John Wiley & Sons, New York.

Lotstein, Richard. 1995. Petition for Determination of Nonregulated Status of Ciba Seeds' Genetically Engineered to Express the CryIA(b) Protein from *Bacillus thuringiensis* subspecies *kurstaki*. USDA Petition #94-319-01p.

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

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

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.

Watson, Stanley A. 1987. Structure and Composition. Pg 53-82. *In* Corn: Chemistry and Technology, S.A. Watson and P.E. Ransted (eds). American Association of Cereal Chemists, Inc., Minnesota.

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

Wych, Robert D. Production of Hybrid Seed Corn. 1988. pg 603. In *Corn and Corn Improvement*, 3rd ed.; G.F. Sprague and J. W. Dudley, (eds). American Society of Agronomy, Inc. Wisconsin.

Table 1. Summary of Test and Control Substance Identifiers

	MON 800 Control Line	MON 801 Test Line
A. Study 93-01-39-02[a] Grain		
Field Location		
Jerseyville, IL	MON 80081	MON 80181
Monmouth, IL	MON 80082	MON 80182
Winterset, IA	MON 80084	MON 80184
Windfall, IN	MON 80085	MON 80185
York, NB	MON 80086	MON 80186
B. Study 94-01-39-17[b]		
Grain	MON 81991	MON 80191
Silage	MON 81990	MON 80190

a : EPA MRID #43533202, Ream *et al.*, 1995.
b : Study 94-01-39-17, Halsey *et al.*, 1995.

Table 2a. Summary of Proximate Analysis of Corn Grain

Characteristic	MON 800 Control Mean[b]	MON 800 Control Range[c]	MON 801 Test Mean[b]	MON 801 Test Range[c]
Protein[a]	12.03	11.2-12.9	13.07[d]	12.6-13.6
Fat[a]	4.03	3.8-4.2	4.01	3.9-4.1
Ash[a]	1.62	1.5-1.8	1.56	1.4-1.7
Carbohydrate[a]	82.23	81.7-83.0	81.31[d]	80.8-81.7
Calories/100g[a]	413.7	412.6-415.7	413.8	413.4-414.6
Moisture %	14.58	13.0-15.8	14.75	13.9-15.8

a : Percent dry weight of sample.
b : Value reported is mean of five samples, one from each field site.
c : Range denotes the lowest and highest individual values across sites for each line.
d : Values are significantly different from control line, MON 800, at the 95% confidence level (paired t-test).

Table 2b. Literature References

Component	Literature Mean	Literature Range	Literature Reference
Protein %	9.5	6.0-12.0	Watson, 1987
	12.3	9.7-16.1	Jugenheimer, 1976
Fat (oil) %	4.3	3.1-5.7	Watson, 1987
Ash %	1.4	1.1-3.9	Watson, 1987
Moisture %	16.0	7-23	Watson, 1987

Table 3. Amino Acid Composition of Corn Grain[a]

Amino Acid	MON 800 Control % of Total Protein Mean[c]	MON 800 Control % of Total Protein Range[d]	MON 801 Test % of Total Protein Mean[c]	MON 801 Test % of Total Protein Range[d]	Literature Range[b] %
Nutritionally essential					
Methionine	2.3	2.0-2.6	2.2	2.0-2.3	1.0-2.1
Cystine	2.2	1.9-2.3	2.1	1.9-2.2	1.2-1.6
Lysine	3.2	2.9-3.4	2.8	2.6-3.2	2.0-3.8
Tryptophan	0.6	0.5-0.6	0.5[e]	0.5-0.6	0.5-1.2
Threonine	4.1	4.0-4.2	4.0	3.9-4.2	2.9-3.9
Isoleucine	3.7	3.7-3.8	3.5[e]	3.5-3.6	2.6-4.0
Histidine	3.2	3.0-3.3	3.0	2.8-3.1	2.0-2.8
Valine	4.7	4.5-4.8	4.3[e]	4.2-4.5	2.1-5.2
Leucine	13.7	13.6-13.8	14.2[e]	13.7-14.5	7.8-15.2
Arginine	4.7	4.4-5.0	4.3	4.1-4.6	2.9-5.9
Phenylalanine	5.3	5.2-5.4	5.5	5.4-5.6	2.9-5.7
Glycine	4.1	3.9-4.2	3.7[e]	3.4-4.1	2.6-4.7
Nonessential					
Alanine	7.9	7.8-8.1	8.0	7.9-8.2	6.4-9.9
Aspartic acid	7.1	6.8-7.3	6.9	6.7-7.3	5.8-7.2
Glutamic acid	20.4	19.9-20.9	20.8	20.2-21.4	12.4-19.6
Proline	9.2	9.0-9.4	9.2	9.0-9.4	6.6-10.3
Serine	5.7	5.5-6.0	5.9	5.8-6.1	4.2-5.5
Tyrosine	4.0	3.8-4.3	4.1	3.9-4.2	2.9-4.7

a : Values are expressed as percent of total protein.

b : Watson, 1982. Values are per cent of total protein [10.1% total protein (Nx6.25)].

c : Value reported is mean of five samples, one from each field site, Study 93-01-39-02.

d : Range denotes the lowest and highest individual values across sites for each line.

e : Significantly different from the control line, MON 800, at the 5% level (paired t-test).

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

Component	MON 800 Control		MON 801 Test		Literature[d]
	Mean[b]	Range[c]	Mean[b]	Range[c]	Range
Linoleic (18:2)	62.7	61.7-65.0	62.0	58.0-64.7	35-70
Oleic (18:1)	22.9	21.3-23.6	23.3	21.2-25.9	20-46
Palmitic (16:0)	10.5	10.2-10.8	10.5	10.2-10.9	7-19
Stearic (18:0)	2.0	1.6-2.1	2.2	1.8-3.1	1-3
Linolenic (18:3)	1.0	0.9-1.1	1.1	1.0-1.1	0.8-2
Arachidic (20:0)	0.40	0.4-0.4	0.42	0.4-0.5	01.-2
Eicosenoic (20:1)	0.3	0.3-0.3	0.3	0.3-0.3	not reported
Behenic (22:0)	0.18	0.1-0.2	0.14	0.1-0.2	not reported

a : Value of fatty acid is % of total lipid. Other fatty acids were below the limit of detection of the assay.
b : Values presented are means (five samples for each line).
c : Range denotes the lowest and highest individual value across sites for each line.
d : Watson, 1982.

Table 5. Analysis of Carbohydrates, Tocopherols and Inorganic Components of Corn Grain[a]

Component	MON 800 Control		MON 801 – Test		Literature[d] Range
	Mean[b]	Range[c]	Mean[b]	Range[c]	
A. Carbohydrates					
Starch %	69.9	68.8-71.5	67.7	63.7-70.2	64-78.0
Crude Fiber %	2.19	2.09-2.36	2.35	1.98-2.61	2.0-5.5
Sugars[e] g/100g					
Fructose	0.54	0.47-0.81	0.58	0.47-0.96	
Glucose	0.63	0.47-1.03	0.62	0.47-0.96	
Sucrose	0.63	0.40-0.94	0.75	0.59-0.89	
Phytic Acid %	0.50	0.45-0.56	0.54	0.51-0.57	0.7-1.0
B. Tocopherols					
Tocopherols mg/kg					
alpha	10.0	7.6-12.0	10.5	7.3-12.3	3.0-12.1
beta	8.7	7.9-9.3	9.2	8.4-10.7	
gamma	31.7	24.0-42.5	26.3	21.7-36.0[f]	
C. Inorganic Components					
Calcium %	0.0035	0.0030-0.0039	0.0037	0.0033-0.004	0.01-0.1
Phosphorus %	0.332	0.311-0.356	0.336	0.316-0.363	0.26-0.75

a : Values on a dry weight basis.
b : Value reported is mean of five samples, one from each field site.
c : Range denotes the lowest and highest individual values across sites for each line.
d : Watson, 1982. Literature values provided if available.
e : Galactose, lactose and maltose were also measured but values were below the limit of detection of the assay.
f : Significantly different from control line, MON 800, at the 95% confidence level (paired t-test).

Table 6. Summary of Analysis of Grain and Silage from Study 94-01-39-17[a]

Characteristic	Grain[b] MON 81991 Control	Grain[b] MON 80191 Test	Silage[c] MON 81990 Control	Silage[c] MON 80190 Test	Literature[d] Average as fed
Protein	9.10	8.14	2.50	2.50	2.2
Fat	3.19	3.41	1.10	1.30	0.8
Ash	1.37	1.31	1.40	1.50	1.5
Carbohydrate	86.35	87.08	31.7	37.4	15
Calories/100g	411	412	147	171	114
Moisture	12.1	12.15	63.13	57.94	74.4
Crude Fiber	1.82	1.82	7.5	7.3	6.6
Calcium	0.0033	0.0034	0.055	0.043	0.09
Phosphorus	0.29	0.28	0.072	0.079	0.06

a: Value reported is single analysis on one sample from one site. Statistical analysis was not performed.
b: Values are reported as percentages (except calories) on a dry weight basis. Calculation: DW (calculated) = (FW measured)/[(100-% moisture)/100].
c: Values are reported as percentages (except calories) on a fresh weight basis.
d: Watson, 1982.

Appendix 1:

Protocol

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
MADISON, WI 53707-7545

a CORNING Company

HWI 6103-177

Sponsor:

The Agricultural Group of Monsanto Company
St. Louis, Missouri

Study Title:

Precision Determination of Cornseed for
Composition/Quality Parameters

Date:

June 15, 1994

Performing Laboratory:

Hazleton Wisconsin, Inc.
3301 Kinsman Boulevard
Madison, Wisconsin 53704

Laboratory Project Identification:

HWI 6103-177

Phone 608-241-4471 Fax 608-241-7227
EXPRESS-MAIL DELIVERY: 3301 KINSMAN BLVD. MADISON, WI 53704

STUDY IDENTIFICATION

Precision Determination of Cornseed for
Composition/Quality Parameters

HWI Study No.	6103-177
Test Material	Cornseed
Sponsor Study Title	Quality and Toxicant Analysis of Cornseed from a European Corn Borer Resistant Corn Line Grown in the 1993 GLP Trials
Sponsor	The Agricultural Group of Monsanto Company 700 Chesterfield Parkway North St. Louis, MO 63198
Study Monitor	Patricia Sanders The Agricultural Group of Monsanto Company 700 Chesterfield Parkway North GG4K St. Louis, MO 63198 (314) 537-6412 Fax No. (314) 537-6880
Study Director	Shari S. Patzer Hazleton Wisconsin, Inc. P.O. Box 7545 Madison, WI 53707 (608) 241-4471 Fax No. (608) 241-7227
Study Location	Hazleton Wisconsin, Inc. 3301 Kinsman Boulevard Madison, WI 53704
Proposed Study Schedule	
Samples Received	June 8, 1994
Initial Sample Analysis	June 16, 1994
Completion of Sample Analysis	July 11, 1994

1. Study	Precision Determination of Cornseed for Composition/Quality Parameters
2. Purpose	To analyze cornseed samples for proximate anlaysis (moisture, protein, fat, ash, calories and carbohydrates), crude fiber, amino acids, aflatoxins, starch, sugar, fatty acid profile, vitamin E and phytic acid according to Hazleton Wisconsin (HWI) approved methods.
3. Regulatory Compliance	This study will be conducted in accordance with the Food and Drug Administration Good Laboratory Practice Regulations for Nonclinical Laboratory Studies, 21 CFR 58.
4. Quality Assurance	The protocol, study conduct, data and final report will be audited by the Quality Assurance Unit in accordance with Hazleton Wisconsin Standard Operating Procedures (SOPs).
5. Test Material	
A. Test Material	
(1) Identification	Cornseed
(2) Purity	Responsibility of the Sponsor
(3) Stability	Responsibility of the Sponsor
(4) Storage	In a freezer set to maintain -20°C ±10°
(5) Characteristics	Information on synthesis methods, composition, or other characteristics that define the test material is the responsibility of the Sponsor.
(6) Reserve Samples	Responsibility of the Sponsor.

6. Experimental Design

A. Samples

(1) Source	Samples will be shipped to HWI by Monsanto Company.
(2) Number of Samples	Five test samples and five control samples.
(3) Identification	The control samples are as follows: MON 80081, MON 80082, MON 80084, MON 80085 and MON 80086. The test samples are as follows: MON 80181, MON 80182, MON 80184, MON 80185 and MON 80186.
(4) Storage	In a freezer set to maintain -20°c ±10°. Any remaining test material, including original sample receipt containers will be returned to the Sponsor after completion of the study.

B. Analytical Methods — Each of the samples will be assayed by the following HWI methods: PGEN, M100, FSOX, ASHM, CFIB, CALC, CHO, TAAP, AHMF, STCH, SUGN, FAPM, TTLC AND A1XX.

7. Statistical Evaluation — Statistical evaluation of the data will be performed by the Sponsor.

8. Reports — A final report including, but not limited to, those items listed below will be submitted. One copy of the draft report and two copies of the final report will be provided.

A copy of the protocol and any protocol amendments

A copy of the Laboratory Information Management Systems (LIMS) reports

Statistical analysis and test material stability, purity and characterization will be provided by the Sponsor as an attachment(s) to the final report.

9. Maintenance of Raw Data and Records

Original data or copies will be available at HWI to facilitate auditing the study during its progress and before acceptance of the final report. When the final report is completed, original paper data and magnetically encoded records, including those items listed below will be retained in the archives of HWI in accordance with 21 CFR 58.

Protocol, and any protocol amendments, computer printouts, chromatographs, worksheets, data sheets, original notes by investigators, forms specified by SOP, transfer forms and final report, original signed copy.

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

PROTOCOL APPROVAL

Patricia Sanders — 6/24/94
Patricia Sanders
Study Monitor
The Agricultural Group of Monsanto Company
Date

Shari S. Patzer — 6/15/94
Shari S. Patzer
Study Director
Hazleton Wisconsin, Inc.
Date

Kim Weber — 6/15/94
Representative
Quality Assurance Unit
Hazleton, Wisconsin, Inc.
Date

Monsanto
New Agricultural Products
Regulatory Sciences

Protocol Amendment
SOP Reference: GEN-POL-005

Study Number: 94-01-39-08 **Amendment #:** 2
Date change implemented: 04/21/95
Project: B.t.k. Corn (39)

Experiment/s affected by this amendment:

Page No/s. &/or Section/s: Pages 1 and 2

Originally stated:

Page 1 -

Study Title: Precision Determination of Cornseed for Composition/Quality Parameters

Laboratory Project Identification - HWI 6103-177

Page 2

Study Monitor -
Patricia Sanders
The Agricultural Group of Monsanto Company
700 Chesterfield Parkway North GG4K
St. Louis, MO 63198,
(314) 537-6412
Fax No. (314) 537-6880

Study Director
Shari S. Patzer
Hazleton Wisconsin, Inc.
P.O. Box 7545
Madison, WI 53707
(608) 241-7227

Page 3

The protocol, study conduct, data and final report will be audited by the Quality Assurance Unit in accordance with Hazleton Wisconsin Standard Operating Procedures (SOPs).

Page 4 - 5

A final report including, but not limited to, those items listed below will be submitted. One copy of the draft report and two copies of the final report will be provided.

Statistical analysis and test material, stability, purity and characterization will be provided by the Sponsor as an attachment(s) to the final report.

Monsanto
New Agricultural Products
Regulatory Sciences

Protocol Amendment
SOP Reference: GEN-POL-005

When the final report is completed, original paper data and magnetically encoded records, including those items listed below will be retained in the archives of HWI in accordance with 21 CFR 58.

This section is amended as follows:

Page 1 -

Study Title: Compositional Analyses of MON 801 Cornseed and Silage from the 1993 Corn Field Trials

Laboratory Project Identification - HWI 6103-177/Monsanto Study Number - 94-01-39-08

Page 2

Study Director -
Patricia Sanders
The Agricultural Group of Monsanto Company
700 Chesterfield Parkway North GG4K
St. Louis, MO 63198,
(314) 537-6412
Fax No. (314) 537-6880

Principal Investigator
Shari S. Patzer
Hazleton Wisconsin, Inc.
P.O. Box 7545
Madison, WI 53707
(608) 241-7227

Add -
Primary Testing Facility
The Agricultural Group of Monsanto Company
700 Chesterfield Parkway North GG4K
St. Louis, MO 63198

Page 3

The protocol, study conduct, data and analytical subreport will be audited by the Quality Assurance Unit in accordance with Hazleton Wisconsin Standard Operating Procedures (SOPs).

Page 4 - 5

The final report will be generated by the Monsanto Study Director which includes or references statistical analysis and test material, stability, purity and characterization. A quality control checked and Quality Assurance accepted analytical subreport generated by the HWI Principal Investigator will be submitted to the Monsanto Study Director to be used in preparation of the final report. The

Monsanto
New Agricultural Products
Regulatory Sciences

Protocol Amendment
SOP Reference: GEN-POL-005

Monsanto Quality Assurance unit will assume responsibility for audit of the final report.

When the analytical subreport is completed, original paper data and magnetically encoded records, including those items listed below will be retained in the archives of HWI in accordance with 21 CFR 58.

except the final report. The original signed final report will be retained in the Monsanto Archives.

Reason for amendment:

Reevaluation of responsibilities of the Study Monitor and Study Director by Monsanto Testing Facility Management.

This change will impact the Study in the following ways:

Hazleton personnel will generate the data, assure that the data has been verified and checked by their Quality Assurance Department and supply copies of all original data and protocols to Monsanto. Monsanto will assume responsibility for generation of statistical analyses and the final report. Monsanto Quality Assurance will do the final report review. The original final report will be archived at Monsanto and a copy supplied to Hazleton Wisconsin, Inc. There will be no adverse impact on the study.

Signatures of Approval

Study Director: Pat Sanders Date: 5/12/95

Sponsor / Testing Facilities Management Representative: [signature] Date: 5/12/95

Signatures of Acknowledgement

Principal Investigator: [signature] Date: 5/15/95
Hazleton Facility Mgt. [signature] Date: 5/15/95
Date:

Signature of Review by QA

QA Rep.: Clyde L. Livingston Date: 11 May 1995
QA Rep.: [signature] Date: 5/15/95
CC:

Appendix 2:

Method References

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

AOAC Method 979.10, 906.03, 929.09. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

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

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

AOAC Method 962.09. 1990. *In* Official Methods of Analysis, 15th Edition. Association of Official Analytical Chemists, Arlington, Virginia, modified.

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

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

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

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

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

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

Brosbt, K. 1972. Methods in Carbohydrate Chemistry, Volume 6, pages 3-8, Academic Press, New York, New York, modified.

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

Determination of High Performance Liquid Chromatography. 1988. *Journal of the Association of Official Analytical Chemists* 71(1), methods 26.052-26.060, modified.

Determination by One Dimensional Thin Layer Chromatography. 1988. *Journal of the Association of Official Analytical Chemists* 71(1), method 26.031, modified.

Determination by Two Dimensional Thin Layer Chromatography. 1988. *Journal of the Association of Official Analytical Chemists* 71(1), method 26.074, modified.

Fiske, C.H. and Subbarrow, Y. 1925. *Journal of Biochemical Chemistry* 66:375, modified.

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

Mason, B.S., and Slover, H.T. 1971. *Journal of Agricultural Food Chemistry* 19(3):551-554, modified.

McChance, R.A. and Widdewson, E.M. 1935. *The Biochemical Journal* 29:2694, modified.

McMurray, C.H., Blanchflower, W.J., and Rice, D.A. 1980. *Journal of the Association of Official Analytical Chemists* 63:1258-1261 modified.

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

Proceeding of the 3rd International Congress of Food Science and Technology, 1979. Pages 705-711, modified.

Speek, A.J., Schrijver, J., and Schreurs, W.H.P. 1985. *Journal of Food Science* 50:121-124 modified.

USDA Agriculture Handbook No. 8. 1975. Composition of Foods. *In* Agricultural Hadnbook No. 8. United States Department of Agriculture, Washington, D.C. pp. 159-165.

Appendix 3:

Statistical Analysis Report for

Compositional Data

Study 94-01-39-08

Statistical Comparison of IPC Corn Test and Control Line for Composition and Quality.

Data

The composition and quality data from a transgenic test line of IPC corn and a control line were obtained in Macintosh Microsoft® Excel® spreadsheets. These data were then transferred to a file on the VAX BB1T computer cluster for processing by the SAS statistical program (SAS Institute Inc., 1990). Each value in the data corresponds to an analytical response (possibly averaged) provided for each variable from material grown at each of 5 locations. For convenience the locations are coded 701, 702, 704, 705 and 706. The analytes were grouped into 8 categories: proximates, amino acids, fatty acids, aflatoxins, starch (1 analyte), sugars, minerals and tocopherols. To the extent possible, this grouping was retained throughout processing of the data.

For many analytes, values were given as "<X", where X is a reporting threshold, presumably the limit of quantitation (LOQ). For simplicity, all analytes showing one or more locations as "<X" were excluded from statistical analysis (Table I). It was requested for analysis purposes that some analytes be reexpresed on either a dry weight or per protein basis. These requested re-expressions are described in Table II.

Statistical Analysis

A paired t-test was used to examine differences between the transgenic and control corn lines. For each analyte, the differences between the test and control line values at each location were calculated. These differences were then used in the SAS MEANS procedure (t-test option) to test whether the mean differences were equal to zero. A summary of these tests for all analytes, including the control and test means, the mean difference, the difference as a percent of the control mean, the standard error of the difference, and the p-value for the paired t-test, are given in Table III. Other summary statistics for the means were computed in SAS for the test and control lines separately. These included the percent coefficient of variation, the standard error of the overall mean, and a 95 percent confidence interval for the overall mean. Since these statistics are not relevant to the comparison of the transgenic and control lines, they are not listed in this report. (These details can, however, be found in the SAS computer listing, which is part of the data package.)

The paired t-test results in Table III show that for several analytes, the mean differences from control were statistically significant at the 5% level (i.e., the observed significance level, or p-value, is less than 0.05). The only

proximate analytes showing a statistically significant test-control difference were carbohydrates (p=0.01789) and protein (p=0.00879). The test line was about 1% lower than control for carbohydrates and about 1% higher for protein. The test line showed an approximate 5 mg/kg decrease (about 17%) in gamma tocopherol from the control line (p=0.01639). The transgenic IPC corn line showed a statistically significant average increase over the control line for leucine (about 4%; p=0.02278). The test line also showed statistically significant decreases for the following amino acids: glycine (about 10%; p=0.03416), isoleucine (about 6%; p=0.00127), tryptophan (about 9%; p=0.02173), and valine (about 8%; p=0.01110). There were no statistically significant mean differences for any other analyte tested. It should be noted that with as many as 43 t-tests, one might expect on the average 5%, or about 2, of these to give a statistically significant result by chance alone. Thus, it is likely that some of these significant differences were 'false positives.'

References

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

Table I.

List of analytes excluded from statistical analysis because one or more values were reported as less than the limit of quantitation.

Analyte Category	**Analyte Excluded**
Sugars	Galactose
	Lactose
	Maltose
Aflatoxins	All
Fatty Acids	10:0 Capric
	12:0 Lauric
	14:0 Myristic
	14:1 Myristoleic
	15:0 Pentadecanoic
	15:1 Pentadecenoic
	16:1 Palmitoleic
	17:0 Heptadecanoic
	17:1 Heptadecenoic
	20:2 Eicosadienoic
	20:3 Eicosatrienoic
	20:4 Arachidonic
	8:0 Caprylic
	Gamma linolenic
Tocopherols	Delta

Table II.

Re-expressions of analyte values requested prior to statistical analysis.

Analyte Category	**Original Units**	**Analyzed Units**	**Re-expression Formula**
Proximates (except moisture)	% seed fresh wt.	% seed dry wt.	Y = X/(1-M/100)
Proximates (moisture only)	% seed fresh wt.	same	none
Calories	Cal./100g of seed fresh wt.	Cal./100g of seed dry wt.	Y = X/(1-M/100)
Sugars and Starch	% seed fresh wt.	% seed dry wt.	Y = X/(1-M/100)
Minerals	% seed fresh wt.	% seed dry wt.	Y = X/(1-M/100)
Fatty Acids	% total fatty acid weight	same	none
Amino Acids	mg/g of seed fresh wt.	% Total protein	Y = 10X/P
Tocopherols	mg/kg of seed fresh wt.	mg/kg of seed dry wt.	Y = X/(1-M/100)

KEY:

X = value in the original units
Y = value in the units desired for statistical analysis
M = Moisture as a percent of seed fresh weight
P = Percent protein expressed on a fresh weight basis.

Table III.

Paired t-test comparison of test line to control line.†

Analyte	Control Mean	Test Mean	Mean Diff.	% Diff.	SE Diff.	p-Value*
Amino Acids						
alanine	7.901	8.044	0.143	1.8	0.092	0.19512
arginine	4.652	4.337	-0.316	-6.8	0.161	0.12219
aspartic acid	7.067	6.940	-0.128	-1.8	0.182	0.52272
cystine	2.164	2.057	-0.106	-4.9	0.066	0.18373
glutamic acid	20.437	20.762	0.325	1.6	0.281	0.31154
glycine	4.086	3.690	-0.396	-9.7	0.125	0.03416
histidine	3.155	2.954	-0.201	-6.4	0.078	0.06207
isoleucine	3.736	3.528	-0.208	-5.6	0.026	0.00127
leucine	13.659	14.158	0.500	3.7	0.139	0.02278
lysine	3.153	2.837	-0.316	-10.0	0.162	0.12323
methionine	2.341	2.165	-0.176	-7.5	0.085	0.10708
phenylalanine	5.332	5.476	0.144	2.7	0.063	0.08239
proline	9.203	9.203	0.000	0.0	0.090	0.99933
serine	5.742	5.935	0.193	3.4	0.106	0.14316
threonine	4.088	3.995	-0.093	-2.3	0.081	0.31451
tryptophan	0.564	0.512	-0.053	-9.3	0.014	0.02173
tyrosine	3.997	4.050	0.052	1.3	0.113	0.66803
valine	4.672	4.310	-0.362	-7.8	0.081	0.01110
Fatty Acids						
16:0 palmitic	10.480	10.470	-0.010	-0.1	0.189	0.96027
18:0 stearic	1.980	2.220	0.240	12.1	0.196	0.28894
18:1 oleic	22.920	23.280	0.360	1.6	0.671	0.62019
18:2 linoleic	62.740	62.040	-0.700	-1.1	1.102	0.55975
18:3 linolenic	1.020	1.070	0.050	4.9	0.022	0.08901
20:0 arachidic	0.400	0.420	0.020	5.0	0.020	0.37390
20:1 eicosenoic	0.300	0.300	0.000	0.0	0.000	–
22:0 behenic	0.180	0.140	-0.040	-22.2	0.024	0.17781
Minerals						
calcium	0.003466	0.003660	0.000194	5.6	0.000120	0.18066
phosphorus	0.3322	0.3360	0.0038	1.1	0.0101	0.72894

Table III (Continued).

Analyte	Control Mean	Test Mean	Mean Diff.	% Diff.	SE Diff.	p-Value*
Proximates						
ash	1.615	1.560	-0.055	-3.4	0.062	0.42876
calories	413.7	413.8	0.1	0.0	0.7	0.87915
carbohydrate	82.23	81.31	-0.92	-1.1	0.24	0.01789
crude fiber	2.19	2.35	0.16	7.3	0.11	0.22697
moisture	14.580	14.750	0.170	1.2	0.197	0.43733
phytic acid	0.4977	0.5390	0.0414	8.3	0.0267	0.19586
protein	12.03	13.07	1.04	8.6	0.22	0.00879
total fat	4.029	4.012	-0.017	-0.4	0.082	0.84340
Sugars and Starch						
fructose	0.537	0.576	0.039	7.2	0.031	0.27728
glucose	0.630	0.623	-0.007	-1.0	0.041	0.88096
sucrose	0.633	0.750	0.117	18.5	0.099	0.30341
starch (enzymatic)	69.928	67.688	-2.240	-3.2	1.144	0.12181
Tocopherols						
alpha	10.01	10.45	0.45	4.5	0.37	0.29847
beta	8.73	9.19	0.46	5.3	0.30	0.19470
gamma	31.65	26.34	-5.31	-16.8	1.33	0.01639

† For clarity of presentation, the number of decimal places reported for means, differences and standard errors was one more than the number of decimal places in the original data provided. The same number of decimal places was used for each analyte within a category whenever it was practical to do so. For all analytes, percent differences were reported to a single decimal place.

* The observed significance level from a paired t-test of mean difference equal to zero. Values of $p<0.05$ indicate statistical significance at the 5% level.

Study 94-01-39-08

Report Submitted By:

Anthony J. Carella

8-14-95

Anthony J. Carella, M.S., ASQC CQE
Statistician
Trilogy Consulting Corporation

Date

HAZLETON
WISCONSIN
POST OFFICE BOX 7545
MADISON, WI 53707-7545

a CORNING Company

Protocol Amendment

Precision Determination of Cornseed for Composition/Quality Parameters

HWI Study No. 6103-177

Sponsor

The Agricultural Group of Monsanto Co.
700 Chesterfield Parkway North
St. Louis, MO 63198

Analytical Testing Laboratory

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

Study Monitor

Patricia Sanders
The Agricultural Group of Monsanto Co.
700 Chesterfield Parkway North GG4K
St. Louis, MO 63195
(314) 537-6412

Study Director

Shari S. Patzer
Hazleton Wisconsin, Inc.
P.O. Box 7545
Madison, WI 53707
(608) 241-4471

Amendment No. 1 to the Protocol
Effective 4/6/95

This amendment modifies the following portions of the protocol:

1. Page 2, Test Material. To include the corn silage as a test material, include the following.

   and corn silage.

2. Page 3, Purpose. To include the analysis of the corn silage and include the calcium and phosphorus analysis, replace "To analyze cornseed samples for proximate analysis (moisture, protein, fat, ash, calories, carbohydrates), crude fiber, amino acids, aflatoxins, starch, sugar, fatty acid profile, vitamin E and phytic acid" with the following.

   To analyze cornseed and corn silage samples for proximate analysis (moisture, protein, fat, ash, calories, carbohydrates), crude fiber, amino acids, aflatoxins, starch, sugar, fatty acid profile, calcium, phosphorus, vitamin E and phytic acid.

3. Page 3, Identification. To include the corn silage as a test material, include the following.

   and corn silage.

4. Page 4, Number of Samples. To include the analysis of the corn silage, the additional cornseeds and the calcium and phosphorus analysis, replace "Five test samples and five control samples" with the following.

   Six test cornseed samples, six control cornseed samples, one test corn silage sample and one control corn silage sample.

5. Page 4, Identification. To include the analysis of the corn silage and the additional cornseeds, replace "The control samples are as follows: MON 80081, MON 80082, MON 80084, MON 80085, and MON 80086. The test samples are as follows: MON 80181, MON 80182, MON 80184, MON 80185, and MON 80186. " with the following.

   The cornseed control samples are as follows: MON 80081, MON 80082, MON 80084, MON 80085, MON 80086 and MON 81991. The test cornseed samples are as follows: MON 80181, MON 80182, MON 80184, MON 80185, MON 80186 and MON 80191. The corn silage control sample is MON 81990 and the corn silage test sample is MON 80190.

6. Page 4, Analytical Methods. To include the calcium and phosphorus analysis, and to include distribution calculations for fatty acids, include the following.

   CAA, PTA, FAC.

Protocol Amendment Approval

Patricia Sanders — 4/17/95

Patricia Sanders
Study Monitor
The Agricultural Group of Monsanto Co.

Date

Shari S. Patzer — 4/13/95

Shari S. Patzer
Study Director
Hazleton Wisconsin, Inc.

Date

Jonathan C. Kellogg — 4/13/95

Representative
Quality Assurance Unit
Hazleton Wisconsin, Inc.

Date