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149

DATA EVALUATION REPORT

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STUDY TYPE: Protein Stability

MRID NO: 447343-04

TEST MATERIAL: Cry9C Protein

PROJECT NO: CM-97B-01

SPONSOR: AgrEvo USA Company, Wilmington, DE

TESTING FACILITY: AgrEvo USA Company, Pikeville, NC

TITLE OF REPORT: Determination of the Stability of PAT and Cry9C Protein in Processed Grain of Transgenic Field Corn in Fractionated Agricultural Commodities

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STUDY COMPLETED: January 13, 1999

CONCLUSION: Results of ELISA tests, using antibodies specific for PAT and CRY9C proteins suggest that these proteins are present in transgenic corn plants, in relatively small amounts as a percentage of total protein. However, these results are somewhat questionable due to the results obtained from control corn grown in Illinois. The control corn, grown in a location adjacent to the transgenic corn, also showed positive results for both the PAT and Cry9C proteins. Although the amount of protein in the controls is small, compared to the transgenic lines, this result is surprising. From the data provided, it not possible to determine why the controls grown in Illinois provided these results. It appears that there was possibly contamination during the processing of the corn samples. There was no Cry9C protein detected in the control whole corn samples, yet the protein was detected in several of the processed samples from this same corn. Therefore, the results provided for both the control and test samples become somewhat questionable. Although it is likely that the Cry9C and PAT proteins are present in relatively small amounts in the transgenic plant line, further justification/explanation should be provided to address the issue of positive reactions in the control samples, and what impact this has upon the data for the transgenic corn fractions.

CLASSIFICATION: SUPPLEMENTARY. This submission can be upgraded to ACCEPTABLE with submission of an adequate justification or supplemental data for the results in the control plants.

GOOD LABORATORY PRACTICE: The field study portion of this study was not performed in accordance with Good Laboratory Standards guidelines.

I. STUDY DESIGN

Test Material: Protein Reference Samples -

- PAT Protein (Phosphinothricin-N-Acetyltransferase)
- Cry9C Protein (Insecticidal Crystal Protein 9C)

Both proteins, and the antibodies specific for each protein were supplied by Plant Genetic Systems.

Plant Samples -

Illinois Trial - Transgenic glufosinate resistant and corn-borer resistant *Bt* field corn containing the *bar* and *cry9C* genes (CHB351), and near isogenic non-transgenic, non-resistant corn. The trial was harvested at maturity on 10/31/97.

North Carolina Trial - Non-transgenic, non-resistant corn plants (Pioneer Hybrid 3394). The trial was harvested at maturity on 10/7/97.

Methods:

- A. Processing - Processing was carried out under GLP at the Food Protein Research and Development Center, (Texas A & M). SOP numbers 8.6 (revision 08) and 8.5 (revision 09) were followed (attached). Samples of the whole corn were removed and frozen for analysis before processing. Wet milled commodities produced were: hulls (bran), steepwater concentrate, gluten, starch, crude oil, refined oil, and solvent extracted germ (presscake). Dry milled commodities produced were: hulls (bran), grits, meal, flour, crude oil, refined oil, and solvent extracted germ.

Illinois Trial: Approximately 369 pounds of control corn and 42 pounds of transgenic corn were processed. Samples were frozen immediately after processing and sent frozen to AgrEvo laboratories for analysis.

North Carolina Trial: Approximately 580 pounds of control corn were processed. Samples were immediately frozen after processing and sent to AgrEvo labs for analysis.

2. PAT and Cry9C Protein Analysis -

The presence and amount of both the Cry9C and PAT proteins was determined by ELISA using PAT or Cry9C-specific antibodies in samples of grain and processed fractions from transgenic and non-transgenic corn plants. The ELISA test for each protein is capable of detecting both intact and degraded proteins. The test and validation samples were generated by studies CM97B01 and BK97B04, respectively.

Seed, grits, hulls, and solvent extracted germ were ground in the presence of dry ice before extraction for ELISA assay. Further processing was not necessary for meal, crude and refined oil and flour. Non-transgenic and transgenic samples were ground on different days. Standards and non-transformed samples fortified with pure Cry9C or PAT protein prior to extraction were included with each set of assays.

A representative sample (approximately 1 g) was mixed with the extraction buffer (10 ml) in a 50 ml centrifuge tube, shaken for 30 minutes (@ 4° C, 700 rpm) and

2014

then centrifuged at 4190 g for 10 minutes. The supernatant was transferred to a clean tube and the cycle of centrifugation and separation was repeated to produce a clear supernatant (duplicate extracts were prepared for each sample).

The total extractable protein (TEP) was determined for each sample extract. Duplicate 10 μ l aliquots of the sample extract were placed onto microtiter plates, followed by addition of 200 μ l of Bradford Reagent. The samples were incubated for 15-20 minutes on a shaker and the OD was measured (595 nm)

3. Limit of Detection -

A set of eight standards ranging from 0 to 30 ng/ml of PAT or Cry9C were included in duplicate on each respective ELISA plate. The limit of detection (LOD) for each matrix using the optical density (OD) of the control samples based on the 0.95 confidence level in one tail t-distribution:

$$OD_{LOD} = OD_{mean} + [(t_s \times SD) / (n-1)^{0.5}]$$

OD_{LOD} = optical density corresponding to the LOD

OD_{mean} = mean OD of the zero dose replicates

N = number zero dose replicates

T_s = t critical value for a one-sided test at $p = 0.95$ and
df = n-1

p = probability or confidence level

df = degree of freedom

The ELISA reading above this limit of detection can be assumed to represent a 95% probability of being greater than zero dose reading.

4. Limit of Quantitation -

The LOQ (limit of quantitation) is given by the lowest concentration of the standard (0.47 ng/ml) or the LOD when this value is greater than the lowest concentration of standard (Table 1). Values below LOQ are reported as non-detectable (ND).

3814

Table 1. Limits of Quantitation of PAT and Cry9C Proteins in Processed Commodities of Field Corn as Detected by ELISA

Process	Commodity	PAT ELISA LOQ (ng/ml)	Cry9C ELISA LOQ (ng/ml)
	Whole Corn	2.01	0.47
Dry Mill	Composite Grits	2.50	0.47
	Meal	0.47	0.47
	Flour	0.47	0.47
	Hull Material	0.47	0.47
	Solvent Extracted Germ	6.40	0.47
	Crude Oil	0.80	0.47
	Refined Oil	0.47	0.47
Wet Mill	Steepwater Concentrate	0.82	0.47
	Hull Material	0.47	0.47
	Gluten	0.47	0.47
	Starch	0.47	0.47
	Solvent Extracted Germ	0.47	0.47
	Crude Oil	0.47	0.47
	Refined Oil	0.47	0.47

E. Validation -

The PAT and Cry9C ELISA procedures were validated for whole corn and processed corn samples using the PAT and Cry9C standards. Due to what AgrEvo believes was apparent contamination of the control sample from this study (CM97B01 - grown in Champaign County, Illinois), determination of LOD and LOQ's and validation were carried out using the control samples from another study (BK97B04) which was conducted in Wayne County, North Carolina.

Non-transgenic control samples were separately fortified at 0.9 ng/ml and 30 ng/ml with either PAT ELISA in four replicates, or with Cry9C ELISA in six replicates. The fortified samples were processed in extraction buffer prior to the extractions. Each replicate was analyzed using duplicate wells.

4914