**UNITED STATES ENVIRONMENTAL PROTECTION AGENCY**
WASHINGTON, D.C. 20460

OFFICE OF PREVENTION,
PESTICIDES AND TOXIC
SUBSTANCES

**MEMORANDUM**

**SUBJECT:** EPA Review of Additional Product Characterization and Human Health Data in Support of the Section 3 Application for the Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton, Submitted by Dow AgroSciences [Reg. No. 068467-G; Decision Number 214150; DP Barcode: D290936; Case: 071326; 15 Studies with MRID#'s: 45808401-06, 45808408 45808416, 45808422, 45808424, 45922701, 45818601-04]

**TO:** Leonard Cole, Regulatory Action Leader
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

**FROM:** Sharlene R. Matten, Ph.D., Biologist
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**THROUGH:** John L. Kough, Ph.D., Senior Scientist
Microbial Pesticides Branch, Biopesticides and
Pollution Prevention Division (7511C)

**ACTION REQUESTED:** To review additional product characterization and human health data submitted by Dow AgroSciences to support their application for a Section 3 Registration for Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton

**CONCLUSION**

The additional product characterization and human health data, in conjunction with the previously reviewed data for the Experimental Use Permit EPA No. 68457-EUP-6, provide adequate information on product characterization and human health to support a tolerance exemption for the

modified Cry1F, Cry1Ac, and phosphinothricin acetyl tranferase (PAT) proteins in WideStrike cotton [Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006]. Additional information not found in this review can be found in the following memoranda:

- S. Matten to L. Cole entitled "Review of Analytical Methods for Cry1F Truncated Protein in Cotton Seed for Purposes of the Determination of the Exemption from the Requirement of a Tolerance for the Dow AgroSciences Experimental Use Permit for WideStrike Cotton", dated March 28, 2003;
- S. Matten to L. Cole entitled "Review of Analytical Methods for Cry1Ac Truncated Protein in Cotton Seed for Purposes of the Determination of the Exemption from the Requirement of a Tolerance for the Dow AgroSciences Experimental Use Permit for WideStrike Cotton", dated March 28, 2003; and
- S. Matten to L. Cole entitled "EPA Review of the Product Characterization and Human Health Data in Support of the Experimental Use Permit (EUP) Application for the Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton Submitted by Dow AgroSciences," dated March 26, 2003.

## RISK ASSESSMENT

*Product Characterization*

Product characterization data indicate that plant-produced and bacterially-produced Cry1F, Cry1F, and PAT proteins are biologically, biochemically, and immunologically equivalent. Southern blot data of restriction enzyme digests suggest that the Cry1Ac event, Cry1F event, and the stacked Cry1F/Cry1Ac cotton event all contain a single, unique, insertion of the transgenic DNA from the appropriate plasmids. The insert from pMYC3006 contains one intact copy of *cry1Ac* and one intact copy of *pat* (plant selectable marker gene, phosphinothricin acetyltransferase, PAT). The insert from pAGM281 contains one intact copy of *cry1F* and one intact copy of *pat*. An additional hybridizing fragment of *pat* was integrated into the cotton genome from pAGM281 coding for an 85 amino acid protein. This partial PAT (pPAT) was further characterized to be a 255 bp open reading frame (ORF) formed from the 231 bp partial *pat* gene adjacent to 24 bp of 3' T-DNA Border B region. Reverse transcriptase (RT)-PCR showed that the pPAT transcript was produced from *B.t.* Cry1F cotton 281-24-236, but not from *B.t.* Cry1Ac cotton 3006-23-210, and that both *B.t.* Cry1F and Cry1Ac cotton produced full-length PAT transcripts. No significant open reading frames or homologies with sequences in GenBank were found (using a BLAST search) for the cotton genome at the insertion sites. Based on the segregation analyses, transgenic cotton lines carrying *cry1F* (synpro) or *cry1Ac* (synpro) alone, or stacked *cry1F* (synpro)*/ cry1Ac* (synpro) exhibited stable Mendelian inheritance of the insect resistance traits.

*Expression Levels*

The soluble, extractable Cry1F, Cry1Ac and PAT proteins were measured using ELISA methods with a limit of quantitation ranging from 0.001-0.4 ng protein/mg sample weight. Fresh sample weight was used for cottonseed, pollen, nectar and processed products; and dry sample weight was used for all other tissues. The Cry1Ac and Cry1F proteins were detected in all matrices except nectar, meal and oil. Mean Cry1Ac expression was approximately three- to twenty-times lower than Cry1F expression

in leaves, squares, flowers, whole plant, boll, and seed tissue, depending on the tissue. Pollen was the only tissue in which Cry1Ac expression was higher than Cry1F expression. Expression levels of individual Cry1F and Cry1Ac proteins were similar for the single event and stacked cotton lines. PAT proteins were detected in the cotton samples from the Cry1F event and the Cry1F/Cry1Ac stacked event, but generally not detected in the Cry1Ac event samples. Varying expression of Cry1F and Cry1Ac proteins in different plant parts may cause differential selection pressure for insect resistance.

Highest Cry1Ac mean expression was observed in young leaves and squares, 1.82 ng Cry1Ac/mg tissue and in flowers, 1.83 ng Cry1Ac/mg tissue. Mean Cry1Ac expression was 1.31 ng Cry1Ac/mg tissue in terminal leaves, and 0.55 ng Cry1Ac/mg tissue in seeds. Mean Cry1Ac expression in root tissue ranged from N.D. to 0.2 ng Cry1Ac/mg tissue. Mean Cry1Ac expression in pollen was 1.45 ng Cry1Ac/ mg pollen.

Highest Cry1F mean expression was observed in young leaves 6.81 ng Cry1F/mg tissue and terminal leaves, 8.19 ng Cry1F/mg tissue. Mean Cry1F expression was 4.88 ng Cry1F/mg tissue in squares, 5.44 ng Cry1F/mg tissue in flowers, 3.52 ng Cry1F/mg tissue in bolls, and 4.13 ng Cry1F/mg tissue in seeds. Mean Cry1F expression in root tissue was 0.5 to 0.9 ng Cry1F/mg tissue. Mean Cry1F expression in pollen was less than the limit of quantitation, <0.15 ng Cry1F/ mg pollen.

For purposes of the dietary risk assessment, the maximum levels of expression in cottonseed (cotton processed fraction) were 0.46 and 3.1 ng protein/mg tissue fresh weight for Cry1Ac and Cry1F proteins in the stacked Cry1F/Cry1Ac/PAT product, respectively (p. 44, Table 4, MRID 458084-08).

*Toxicity and Allergenicity*
Based upon the human health data provided, there does not appear to be a significant risk of toxic effects and/or allergenic effects to humans or animals due to exposure to the Cry1F (synpro), Cry1Ac (synpro), or PAT proteins. Based on review of the data, there is a reasonable certainty of no harm to humans and animals posed by these proteins.

The Cry1F and Cry1Ac proteins are classified as Toxicity Category III: $LD_{50}$> 700 mg/kg body weight for Cry1Ac, $LD_{50}$> 600 mg/kg body weight for Cry1F and $LD_{50}$> 375 mg Cry1F/kg body weight and $LD_{50}$>350 Cry1Ac mg/kg body weight for the stacked Cry1F/Cry1Ac proteins. The Cry1Ac, Cry1F, and PAT proteins are not stable to digestion in simulated gastric fluid (<1 min), nor do they share any significant sequence similarity to known toxins or allergens using an eight amino acid step-wise comparison. In addition, Cry1 and PAT proteins have not been implicated in toxic and/or allergenic reactions in humans or animals. Based on the submitted data, Cry1F, Cry1Ac, and PAT proteins have no demonstrated toxicity and none of these proteins have characteristics to indicate they could be allergens.

*Dietary Exposure [Information taken from Appendix C, MRID 45872301]*
Humans may consume cotton products as a food (cottonseed oil) or as food ingredients (cottonseed fatty acids, cooked and partially defatted cottonseed flour, and decorticated ground cottonseed kernels). Consumption data from the USDA Continuing Survey of Food Intake by Individuals (CSFII)

1994-98 as used within DEEM (version 7.73, Novigen Sciences, Inc., Washington, DC) contains information on daily dietary consumption of cottonseed oil (food code 290) and cottonseed meal (food code 291). These data indicate mean dietary consumption of cotton products in the US diet is 2 g/day (95$^{th}$ percentile, 5 g/day). Cottonseed oil is the exclusive contributor to diet at the higher percentiles of consumption.

In a worst case scenario, the estimate of human dietary exposure conservatively assumes dietary consumption of cotton products where 100% of diet contains Cry1Ac and Cry1F protein at the maximum levels expressed in cottonseed (whereas, the dominant food consumed is cottonseed oil where actual protein content is nil). The worst case upper bound on combined Cry1Ac and Cry1F protein intake in the human diet is <10 ng/g (ppb) of daily consumption. The exposure estimate does not account for the rapid digestibility of Cry1Ac and Cry1F protein (non-detectable in <1 min). Assuming the average estimated lifetime dietary intake of 2141 g food/day for the US population (USDA, 1998), the combined daily intake of Cry1Ac and Cry1F protein from consumption of cotton products is < 10 ppb (1.1 and 7.2 ng/g diet, respectively).[1] This estimate is ca 15-fold high, since the only food ingredients consumed are oil and a minimal amount of cottonseed flour (i.e., meal), both of which have ND protein residues (< 0.1 ng/mg). Actual exposure is therefore nil (ca 0.5 ppb).

In processed fractions, there is no detectable Cry1Ac and Cry1F protein in either toasted meal or refined oil.

*Dietary Risk*

Human consumption of cotton products is limited. Typically, cotton by-products occur as blended items and comprise a minor component of daily dietary intake. The worst case exposure scenario described above indicates that the combined daily intake of Cry1Ac and Cry1F protein from consumption of cotton products is < 10 ppb (1.1 and 7.2 ng/g diet, respectively) based on uncorrected expression data. The actual dietary exposure to these proteins from consumption of cotton products in food will be nil. Dow's analysis of cottonseed processed products indicates that there is no detectable Cry1Ac and Cry1F protein in either toasted meal or refined oil (<0.1 ng/mg (Level of Quantitation, p.23; p.44, Table 5, MRID 458084-08). Both of these Cry proteins are readily broken down in simulated gastric fluid (<1 min). Therefore, the dietary risk posed by combined daily intake of Cry1Ac and Cry1F proteins, based on a demonstrated lack of oral toxicity and actual exposure to cottonseed processed products in the human diet, is negligible.

*Animal Dietary Exposure and Risk [Information taken from Appendix D, MRID 45872301]*

Animals may consume cottonseed, cottonseed meal, cottonseed hulls, or cotton gin by-products as a portion of their diet. Animal feeding of whole cottonseed (22% protein) is constrained by the level of gossypol and polyunsaturated oil content. Cottonseed is not fed to poultry. Swine are only fed cottonseed on an infrequent basis because of gossypol toxicity. Cottonseed may be 10 to 20% of the total ration feed to sheep or beef steers because of gossypol toxicity. Cotton seedmeal (41% protein) can be used as a protein supplement for dairy cows (35% of the finished feed because of gossypol

[1] Human exposure example calculation for Cry1Ac (dietary intake basis):

0.46 ng Cry1Ac/mg CP X 5g CP consumed/day X 1000 mg/g X day/2141g diet = 1.1 ng Cry1Ac consumed/g diet

toxicity). Cottonseed hulls (4 to 12% protein) can be used as roughage in a variety of cattle diets, but are rationed to <25% of the diet depending on the age and type of cattle (beef vs. dairy). Cotton gin by-products contain from 4 to 8% protein. The actual use of cotton gin by-products in U.S. beef feed is about 5% of the diet. EPA assumes cotton gin by-products constitute 20% of the cattle diet for a conservative overestimate of dietary feed exposure.

The worst case exposure from consumption of Cry1Ac and Cry1F residues in cotton products is that of dairy cattle consuming 100% of their total protein as cottonseed meal (CSM). This, of course, is an unrealistic scenario based on the previously mentioned gossypol toxicity limitations for cottonseed. A high end estimate of CSM (41% protein) consumption is 2.3 kg/day assuming a 500 kg mature dairy cow in early gestation producing 20 kg 4% fat milk daily. The CSM is assumed to contain 0.92 and 6.2 ng/g of Cry1Ac and Cry1F protein, respectively (reflecting a doubling of the concentration of protein observed in cottonseed). Daily dietary intakes of Cry proteins by this diet are 1.8 and 11.9 ng Cry protein/kg BW/day for Cry1Ac and Cry1F, respectively. Subsequent analysis of cottonseed processed products indicates that there is no detectable (i.e., < 0.1 ng/mg) Cry1Ac and Cry1F protein in toasted meal, so the worst case exposure estimates used in the animal exposure assessment are ca 10- to 20-fold high (Level of Quantitation, p.23; p.44, Table 5, MRID 458084-08). Based on the toxicity and the exposure analyses, the animal dietary risk from consumption of Cry1Ac and Cry1F residues in cottonseed-based feeds is negligible.

**Exemptions for the Requirement of a Tolerance, Cry1F, Cry1Ac, and PAT**

An existing tolerance exemption, CFR 40 Section 180.l155, exists for *Bacillus thuringiensis* subsp. *kurstaki* Cry1Ac and the genetic material necessary for its production in all plants. An existing tolerance exemption, CFR 40 Section 180.1151, exists for phosphinothricin acetyltransferase (PAT) and the genetic material necessary for its production in all plants. The PAT in Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton is covered by the existing tolerance exemption, CFR 40 Section 180.1151. Similarly, data provided by Dow AgroSciences are adequate to support the existing tolerance exemption for Cry1Ac. A tolerance exemption, CFR 40 Section 180.1217, exists for *Bacillus thuriengiensis* Cry1F protein and the genetic material necessary for its production in corn. The data submitted would support extending this tolerance exemption to include cotton. The analytical methods for the Cry1Ac and Cry1F proteins are acceptable.

**FOLLOW-UP TO EUP REVIEW RECOMMENDATIONS OF THE PRODUCT CHARACTERIZATION AND HUMAN HEALTH REVIEW FOR THE CRY1F (SYNPRO)/CRY1Ac (SYNPRO) CONSTRUCT 281/3006 (WIDESTRIKE COTTON)**

Under the EUP, there were four interim studies that were "supplemental, pending the final report." Final data reports were provided and these studies have now been ungraded to acceptable.

The four interim studies were:

455423-22 Field Expression of Cry1F (synpro), Cry1Ac (synpro), and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed, and

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| | Cottonseed Processed Products - Interim Report |
| 455423-01 | Characterization of DNA Inserted into Transgenic Cry1F Cotton Event 281-24-236 - Interim Report |
| 455423-02 | Molecular Characterization of Cry1F (synpro)/Cry1Ac (synpro) Stacked Transgenic Cotton Events 281-48-81 and 3006-210-23 - Interim Report |
| 456079-03 | Molecular Characterization of Cry1Ac (synpro) Transgenic Cotton Events 3006-48-81 and 3006-210-23 - Interim Report |

The four final studies are:

| | |
|---|---|
| 458084-08 | Field Expression of Cry1F (synpro), Cry1Ac (synpro), and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products; and Compositional Analysis of Cottonseed and Cottonseed Processed Products |
| 458084-01 | Molecular Characterization of Cry1F (synpro) Transgenic Cotton Event 281-24-236 |
| 458084-02 | Molecular Characterization of Cry1Ac (synpro) Transgenic Cotton Event 3006-210-23 |
| 458084-03 | Molecular Characterization of Cry1F (synpro)/Cry1Ac (synpro) Stacked Transgenic Cotton Event 281-24-236/3006-210-23 |

The biochemical equivalency of Cry1Ac study, "Biochemical Characterization of Cry1Ac derived from Transgenic Cotton and *Pseudomonas fluorescens*," MRID 455423-06, was originally classified as "supplemental, upgradeable with additional data." A new study, "Purification and Characterization of Cry1Ac Delta-Endotoxin from Transgenic Cotton Event 3006-210-23," MRID 458084-04, has now been reviewed and classified as "acceptable."

**DATA REVIEW RECORD FOR PRODUCT CHARACTERIZATION AND HUMAN HEALTH DATA**

**Active Ingredient(s):** Cry1Ac (synpro) Protein (*Bacillus thuringiensis* subsp. *kurstaki* HD-73) and Cry1F (synpro) Protein (*Bacillus thuringiensis* var. *aizawai* strain PS811 (NRRL B-18484)) as Expressed in Cotton and the DNA which encodes for these Proteins

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|---|---|
| **Product Name:** | Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton |
| **Company Name:** | Dow AgroSciences, Indianapolis, IN |
| **ID No:** | 068467-G |
| **Chemical Number:** | 071326 |
| **Decision Number:** | 214150 |
| **DP Barcode:** | D290936 |

**MRID Nos:**

Product Characterization and Identity

458084-08 Field Expression of Cry1F (synpro), Cry1Ac (synpro), and Phosphinothricin Acetyltransferase (PAT) Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products; and Compositional Analysis of Cottonseed and Cottonseed Processed Products

458084-01 Molecular Characterization of Cry1F (synpro) Transgenic Cotton Event 281-24-236

458084-02 and 459227-01 Molecular Characterization of Cry1Ac (synpro) Transgenic Cotton Event 3006-210-23

458084-03 Molecular Characterization of Cry1F (synpro)/Cry1Ac (synpro) Stacked Transgenic Cotton Event 281-24-236/3006-210-23

458186-01 Cloning and Characterization of DNA Sequences in the Insert and Flanking Border Regions of B.t. Cry1F Cotton 281-24-236

458186-02 Cloning and Characterization of DNA Sequences in the Insert and Flanking Border Regions of B.t. Cry1Ac Cotton 3006-210-23

458186-03 Expression of the Partial PAT Open Reading Frame in B.t. Cry1F Cotton Event 281-24-236

458084-04 Purification and Characterization of Cry1Ac Delta-Endotoxin from Transgenic Cotton Event 3006-210-23

458084-05 Characterization of Phosphinothricin Acetyltransferase (PAT) from Recombinant *Escherichia coli* and Transgenic Cotton

458084-06 Biological Equivalency of Event 3006-210-23 Cotton- and *Pseudomonas*-Expressed Cry1Ac B.t. Insecticidal Crystal Protein

Human Toxicity and Allergenicity

458186-04 Comparison of the Putative Amino Acid Sequence of the Partial Phosphinothricin (PAT) ORF in Cry1F Cotton Event 281-24-236 to Known Protein Allergens

458084-16 *In Vitro* Simulated Gastric Fluid Disgestibility Study of Recominant Phosphinothricin Acetyltransferase (PAT)

Analytical Methods

458084-24 Development and Characterization of Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Cry1Ac

| | |
|---|---|
| | Protein |
| 458084-22 | Independent Laboratory Validation of Method GRM 02.11, "Determination of Cry1Ac Insecticidal Crystal Protein in Cotton Tissues by Enzyme-Linked Immunosorbent Assay" |

**BACKGROUND:**

Dow AgroSciences has submitted the above additional product characterization and human health data to support their application for a Section 3 registration for Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton. Dow AgroSciences previously submitted product characterization and human health data to support their application for the Experimental Use Permit, EPA No. 68457-EUP-6. These data have been reviewed and are included as part of the support for the Section 3 registration of Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Contstruct 281/3006 Cotton. Additional information not found in this review can be found in the following memoranda:

- S. Matten to L. Cole entitled "Review of Analytical Methods for Cry1F Truncated Protein in Cotton Seed for Purposes of the Determination of the Exemption from the Requirement of a Tolerance for the Dow AgroSciences Experimental Use Permit for WideStrike Cotton", dated March 28, 2003;
- S. Matten to L. Cole entitled "Review of Analytical Methods for Cry1Ac Truncated Protein in Cotton Seed for Purposes of the Determination of the Exemption from the Requirement of a Tolerance for the Dow AgroSciences Experimental Use Permit for WideStrike Cotton", dated March 28, 2003; and
- S. Matten to L. Cole entitled "EPA Review of the Product Characterization and Human Health Data in Support of the Experimental Use Permit (EUP) Application for the Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281/3006 Cotton Submitted by Dow AgroSciences," dated March 26, 2003.

Mycogen Brand Cry1F (synpro)/Cry1Ac (synpro) Construct 281-3006 Cotton expresses both the Cry1F (synpro) and Cry1Ac (synpro) insect control proteins. These transgenic cotton plants also express phosphinothricin acetyl transferase (PAT) that confers tolerance to the herbicide glufosinate ammonium. A synthetic *cry1F* (synpro) transgene optimized for plant codon usage was transformed into cotton. The expressed Cry1F (synpro) protein effectively controls certain lepidopteran pests, e.g., *Heliothis virescens* (tobacco budworm). Similarly, a plant optimized *cry1Ac* (synpro) transgene was transformed into cotton. The expressed Cry1Ac (synpro) protein effectively controls certain lepidopteran pests, e.g., *Heliothis virescens* (tobacco budworm) and *Helicoverpa zea* (cotton bollworm), and *Pectinophora gossypiella* (pink bollworm). Cotton lines carrying the single events of Cry1F and Cry1Ac were developed through a series of backcrosses and self-pollinations. Cotton lines containing both the Cry1F and Cry1Ac traits (i.e., stack) were developed by crossing a backcrossed BC3F1 line containing the Cry1F event to a backcrossed BC3F1 line containing one of the Cry1Ac events. The stacked event to be commercialized is 281-24-236/3006-210-23.

**SUMMARY OF DATA SUBMITTED:**

Summaries and discussion of each review supporting the product characterization and human health safety of these products are provided below.

**Product Characterization**

**458084-08 Field Expression of Cry1F (synpro), Cry1Ac (synpro), and PAT Proteins in Transgenic Cotton Plants, Cottonseed, and Cottonseed Processed Products**

The purpose of this study is to provide quantitative data on the amount of Cry1F and Cry1Ac proteins in several different transgenic cotton events. An interim report (MRID 455423-22) provided some quantitative data on the amount of Cry1F and Cry1Ac proteins in several different transgenic cotton events. This final report provides quantitative data on the expression of Cry1F and Cry1Ac proteins in different cotton plant tissues: in young leaves, terminal leaves, squares, pollen, and nectar. It also contains data from the compositional analysis of cottonseed. Expression levels were measured in several different transgenic cotton lines: Cry1F alone (Event 281-24-236), Cry1Ac alone (Event 3006-210-23), and Cry1F/Cry1Ac stacked (Event 281-24-236/3006-210-23), as well as the herbicide resistant selectable marker that expresses the protein phosphinothricin acetyltransferase (PAT). Field expression trials were conducted in 2001 at six sites located in the major cotton-producing states of Arizona, California, Mississippi, North Carolina and two in Texas. Samples were collected at various times during plant development including young leaves, terminal leaves, squares, flowers, bolls, whole plant, root, pollen, nectar, cottonseed and cottonseed process fractions consisting of kernel, hulls, meal and oil.

The soluble, extractable Cry1F, Cry1Ac and PAT proteins were measured using ELISA methods with a limit of quantitation ranging from 0.001-0.4 ng protein/mg sample weight. Fresh sample weight was used for cottonseed, pollen, nectar and processed products; and dry sample weight was used for all other tissues. The Cry1Ac and Cry1F proteins were detected in all matrices except nectar, meal and oil. In expressed tissues, average values ranged from non-detected (ND) to 25.3 ng/mg. Cry1Ac expression levels in transgenic cotton tissues were typically several fold lower than Cry1F (with the exception of pollen). In expressed tissue, average values ranged from ND to 1.83 ng/mg.

Highest Cry1F mean expression was observed in young leaves 6.81 ng Cry1F/mg tissue and terminal leaves, 8.19 ng Cry1F/mg tissue. Mean Cry1F expression was 4.88 ng Cry1F/mg tissue in squares, 5.44 ng Cry1F/mg tissue in flowers, 3.52 ng Cry1F/mg tissue in bolls, and 4.13 ng Cry1F/mg tissue in seeds. Mean Cry1F expression in root tissue was 0.5 to 0.9 ng Cry1F/mg tissue. Mean Cry1F expression in pollen was less than the limit of quantitation, <0.15 ng Cry1F/ mg pollen.

Mean Cry1Ac expression was approximately three to twenty times lower than Cry1F expression in leaves, squares, flowers, whole plant, boll, and seed tissue, depending on the tissue. Highest Cry1Ac mean expression was observed in young leaves and squares 1.82 ng Cry1Ac/mg tissue and flowers,

1.83 ng Cry1Ac/mg tissue. Mean Cry1Ac expression was 1.31 ng Cry1Ac/mg tissue in terminal leaves, and 0.55 ng Cry1Ac/mg tissue in seeds. Mean Cry1F expression in root tissue ranged from N.D. to 0.2 ng Cry1Ac/mg tissue. Mean Cry1Ac expression in pollen was 1.45 ng Cry1Ac/ mg pollen. Pollen was the only tissue in which Cry1Ac expression was higher than Cry1F expression. There was no statistical analysis of the expression data to determine whether Cry1F expression was overall significantly greater than Cry1Ac expression (except for pollen). Expression levels of individual Cry1F and Cry1Ac proteins were similar for the single event and stacked cotton lines. PAT proteins were detected in the cotton samples from the Cry1F single event and the Cry1F/Cry1Ac stacked event, but generally not detected in the Cry1Ac single event samples. Varying expression of Cry1F and Cry1Ac proteins in different plant parts may cause differential selection pressure for insect resistance. Adamczyk *et al*. (2001) found that larval survival of corn earworms and larval development of fall armyworms were correlated to the differential expression of Cry1Ac in various plant parts of commercial varieties of Bollgard® cotton. Expression differences may be due to parental background and environmental X genetic interactions. The insect resistance management strategy (product durability plan) for WideStrike cotton (MRID# 458084-15) is reviewed separately. The field efficacy data (MRID# 458084-07) is also reviewed separately.

Statistical analysis (ANOVA, $p<0.05$) of the data from compositional analysis of cottonseed resulted in only one instance where the transgenic treatment was different from the control. Crude fiber content from the stacked line was slightly lower than the control but within reported literature ranges thus the difference was not considered meaningful.

**CLASSIFICATION: ACCEPTABLE**

**45808401** **Molecular characterization Cry1F (synpro) transgenic cotton event 281-24-236**

Southern blot data of restriction enzyme digests suggest that there is a single, unique, insertion of intact *cry*1F and *pat* genes and their regulatory elements ubi, mas, and ORF25 in transgenic cotton event 281-24-236. The integration site also contains an additional hybridizing fragment of the *pat* gene and of the *pat* gene promoter. Expression of the partial PAT open reading frame in Bt Cry1F cotton event 281-24-236 is discussed in MRID# 458186-03. The transgenes and regulatory elements were stable across four generations. The bacterial erythromycin resistance gene $ery^R$ on the plasmid vector pAGM281 was not integrated in transgenic cotton event 281-24-236. These conclusions are the same as seen in the Interim Report, MRID# 455423-01.

**CLASSIFICATION: ACCEPTABLE**

**MRIDs. 458084-02 and 459227-01** **Molecular characterization of Cry1Ac (synpro) transgenic cotton event 3006-210-23**

[**Note that these two reports are identical except that MRID 45922701 has corrected some**

**minor typographic errors present in MRID 45808402. MRID 45922701 supercedes MRID 45808402.**]

Southern blot data from restriction enzyme digests suggest that there is a single, unique, insertion of intact *cry*1Ac and *pat* genes in transgenic cotton event 3006-210-23. The presence of the regulatory elements ubi, mas, and ORF25 was confirmed in event 3006-210-23, the restriction digests indicating apparently one intact copy of each. The transgenes *cry*1Ac and *pat* were stable across four generations. The stability of the regulatory elements mas, ubi, and ORF25 was not evaluated. The bacterial erythromycin resistance gene $ery^R$ on the plasmid vector pMYC3006 was not integrated in transgenic cotton event 3006-210-23. These conclusions are the same as seen in the Interim Report, MRID# 456079-03.

**CLASSIFICATION: ACCEPTABLE**

**458084-03** **Molecular Characterization of Cry1F(synpro)/Cry1Ac(synpro) Stacked Transgenic Cotton Line 281-24-236/3006-210-23**

Southern blot data of restriction enzyme digests suggest that the stacked transgenic cotton event 281-24-236/3006-210-23 contains a single, unique, insertion of the transgenic DNA from pAGM281 and pMYC3006. The insert from pAGM281 contains one intact copy of *cry1F* and one intact copy of *pat* (plant selectable marker gene, phosphinothricin acetyltransferase). The insert from pMYC3006 contains one intact copy of *cry1Ac* and one intact copy of *pat*. The *Pac* I digest did not show any bands when hybridized with *cry*1F (Figure 5, lanes 4-5) possibly due to poor resolubilization prior to gel loading that led to poor gel visualization of higher molecular weight fragments. *Pac* I fragments were easily observed elsewhere in this package (see Figure 3, lanes 21-23, MRID# 458084-01) for the Cry1F Event 281-24-236. An additional hybridizing fragment of *pat* was integrated into the cotton genome from pAGM281, but it was not possible from the data in this study to determine how it was integrated. Expression of the partial PAT open reading frame in Bt Cry1F cotton event 281-24-236 is discussed in MRID# 458186-03. The data confirm that the bacterial erythromycin resistance gene, $ery^R$, on plasmid pMYC 3006 and on plasmid PAGM281 was not integrated in transgenic cotton event 281-24-236/3006-210-23. These conclusions are consistent with the findings presented in the Cry1F Interim Report, MRID# 455423-01, and Cry1F Final Report, MRID# 458084-01; Cry1Ac Interim Report, MRID# 456079-03 and Cry1Ac Final Report, MRID# 459228-01; and Cry1F/Cry1Ac Stacked Interim Report, MRID# 455423-02.

**CLASSIFICATION: ACCEPTABLE**

**MRID 458186-01** **Cloning and characterization of DNA sequences in the insert and flanking border regions of *B.t.* Cry1F cotton 281-24-236**

DNA sequencing of cloned *B.t.* Cry1F cotton 281-24-236 transgenes and their 5' and 3' border

regions yielded results consistent with previously conducted Southern restriction analysis (described in MRID 45808401). The transgene insert was shown to contain an intact transgene cassette from pAGM281 (with *cry*1F, *pat*, their respective promoters [*(4OCS) ) mas 2'* and *UbiZm1*], and ORF25 polyA terminator), albeit with two single base changes within the *UbiZm1* promoter region. Sequencing also defined the partial *pat* gene expression cassette (complete *UbiZm1* promoter and 231 bp *pat* fragment) and determined its location as adjacent to the 3' end of the intact insert but in opposite orientation to the intact PAT expression cassette. The cotton genomic regions flanking the transgenes were also defined (2073 bp at 5' end and 2902 bp at 3' end) and shown by PCR analysis to indeed be cotton DNA. PCR analysis determined the location of the 281-24-236 transgene insertion site in the cotton genome, although it appeared that 53 bp of cotton DNA from the original locus was missing. No significant ORFs or homologies with sequences in GenBank were found (using a BLAST search) for the cotton genome insertion site. A 255 bp ORF formed from the 231 bp partial *pat* gene adjacent to 24 bp of 3' T-DNA Border B region coding for a possible 85 amino acid protein is further characterized in MRID 45818603.

*Explanation on the integration of the partial PAT.*
Dow AgroSciences provided the following explanation for integration of the partial PAT into Bt Cry1F Cotton 281-24-236 (Gatti, 2003).

> "In *Agrobacterium* mediated transformation, a single-stranded T-DNA from the Ti plasmid is wrapped with VirE2 proteins, plus a VirD2 protein covalently bound to its 5' terminus, forming a transport (T) complex. This complex is transferred into the plant cell and integrated into plant genomic DNA. Therefore, DNA sequences (gene of interest) harbored between T-DNA Border B (Right Border) and T-DNA Border A (Left Border) are transferred and integrated into chromosomes. The mechanism of T-DNA integration has been widely studied (Gelvin 2000; Tzfira et al. 2003; Valentine 2003). The observation of the random nature of T-DNA integration has led to the suggestion that the likely mechanism is through non-homologous end joining (NHEJ). Generally, *Agrobacterium*-mediated transformation gives rise to a larger percentage of simple insertions containing one intact copy of the T-DNA. However, complex multicopy loci containing intact and rearranged T-DNA sequences have also been documented and locus structure can vary widely (Kohli et al. 2003). The integration of a second partial T-DNA encompassing the UbiZm1 promoter and a portion of the *pat* gene at the same locus as the intact T-DNA insert in Cry1F cotton event 281-24-236 is not uncommon according to the nature of T-DNA integration."
>
> This rationale is acceptable.
>
> **Note:** The study report (MRID 45818601) originally had several typographic errors: (1) Table 2 on p. 21 incorrectly listed the primer set used to examine the specific 3' end insertion junction sequence as 281_9/ 281_9; it should be 281_9/ 281_10, (2) Tables 2 and 3 on pp. 31-32 had numerous inconsistencies between the length of stated primers, their location, and their DNA sequence length, (3) the first line of p. 18 refers to *B.t.* Cry1Ac cotton, which should be *B.t.* Cry1F cotton; and (4) the description of conventional PCR (p. 13) did not list the concentration of dNTPs in the reaction. These errors have now been corrected (Gatti, 2003) (See specific remarks for

MRID 45818601 in Appendix 1 and Tables 2 and 3 in Confidential Appendix 1).

**CLASSIFICATION: ACCEPTABLE**

**MRID 458186-02** **Cloning and characterization of DNA sequences in the insert and flanking border regions of *B.t.* Cry1Ac cotton 3006-210-23**

DNA sequencing of cloned *B.t.* Cry1Ac cotton 3006-210-23 transgenes and their 5' and 3' border regions yielded results consistent with previously conducted Southern restriction analysis (described in MRID 45808402 and 45922701). The transgene insert was shown to contain an intact transgene cassette from pMYC3006 (with Cry1Ac, *pat*, their respective promoters [*UbiZm1* and *(4OCS) ) mas 2'*], and ORF25 polyA terminator). The cotton genomic regions flanking the transgenes were also defined (535 bp at 5' end and 482 bp at 3' end) and shown by PCR analysis to indeed be cotton DNA. PCR analysis determined the location of the 3006-210-23 transgene insertion site in the cotton genome and showed that 16 bp of cotton DNA from the original locus were missing. No significant open reading frames or homologies with sequences in GenBank were found (using a BLAST search) for the cotton genome insertion site.

**Notes:** The study report (MRID 45818602) originally had several typographic errors: (1) The length of the 5' and 3' cotton flanking regions is unclear because different values are presented in MRID 45818602. The length of the 5' region is given alternatively as 535 bp (Abstract p. 8), 534 bp (p. 16, 17) and 533 bp (p. 4 of Confidential Attachment) and of the 3' end as 482 bp (Abstract p. 8; p. 17; pp. 8-9 of the Confidential Attachment) and 481 bp. (p. 15), (2) Tables 1, 2, and 3 on pp. 2-3 of the Confidential Attachment have numerous inconsistencies between the length of the primers, their location, and their stated DNA sequence, (3) the description of conventional PCR (p. 13-14) does not list the concentration of dNTPs in the reaction. These errors have now been corrected (Gatti, 2003) (See specific remarks for MRID 45818602 in Appendix 1 and Tables 2-4 in Confidential Appendix 1).

**CLASSIFICATION: ACCEPTABLE**

**MRID 458186-03** **Expression of the Partial PAT Open Reading Frame in B.t. Cry1F cotton Event 281-24-236**

Previously conducted DNA sequencing and restriction digest analysis of *B.t.* Cry1F cotton event 281-24-236 revealed the presence of an intact transgene from pAGM281 (containing the *cry*1F and *pat* genes, their respective promoters, and ORF25 polyA) as well as a partial *pat* gene expression cassette (complete *UbiZm1* promoter and 231 bp *pat* fragment) (MRID 45818601). This study examines transcription of the partial and complete *pat* genes in transgenic cotton 281-24-236 and partial PAT protein (pPAT) expressed in *E. coli* transformed with the partial *pat* gene from transgenic cotton 281-24-236.

RT-PCR showed that partial PAT (pPAT) transcript was produced from *B.t.* Cry1F cotton 281-24-236, but not from *B.t.* Cry1Ac cotton 3006-23-210, and that both *B.t.* Cry1F and Cry1Ac cotton produced full-length PAT transcripts. This is consistent with previous sequencing and restriction analysis studies (MRID 45808401, 45818601). *B.t.* Cry1F cotton had pPAT transcript levels much lower (at least 8- to16-fold based on the figure in the report) than those of PAT. The reason was not discussed in the studies, but it may be from RNA silencing due to the presence of incomplete mRNAs. Based on the N-terminal sequencing, MALDI-TOF MS mass estimation, and peptide mass fingerprint analysis, the cloned pPAT ORF in *E. coli* expressed a pPAT protein as predicted by the pPAT ORF sequence in *B.t.* Cry1F cotton 281-24-236. Western blot analysis of pPAT-enriched *E. coli* extract using PAT-specific rabbit polyclonal primary antibody judged to be very sensitive and goat anti-rabbit IgG and horseradish peroxidase confirmed the presence of both the pPAT and PAT proteins, as well as revealing the presence of immunoreactive aggregates. Previous studies with various tissues from transgenic cotton detected only full-length PAT in Western Blots. Because these results show the antibody can detect pPAT, it is unclear whether pPAT is actually present in transgenic cotton plants.

**Notes.** The study report (MRID 45818603) originally had a couple of typographic errors: (1) Table 1 on p. 46 has several inconsistencies between the length of stated primers, their location, and their DNA sequence length, and (2) the description of RT-PCR and of conventional PCR (p. 14) does not list the concentration of dNTPs in the reactions. These errors have now been corrected, see below, and Table 2 of the Confidential Appendix (Gatti, 2003).

1. Page 14. In SuperScript™ One-Step RT-PCR kit, 2X reaction buffer contains dNTP. In a 50 ml reaction, 25 ml of 2X buffer was included, resulting the final concentration of 0.2 mM of each dNTP.
2. Page 14. In Conventional PCR, the final concentration of each dNTP in 50 ml reaction was 0.2 mM (4ml of 2.5 mM of each dNTP was included in 50 ml).
3. Primer positions in Table 1. of Confidential Attachment were re-checked and errors were corrected (see Table 2)

**CLASSIFICATION: ACCEPTABLE**

**MRID 458084-04 Purification and characterization of Cry1Ac delta-endotoxin from transgenic cotton event 3006-210-23**

Cry1Ac protein was purified from leaves of transgenic cotton 3006-210-23. The protein was biochemically identical to Cry1Ac obtained by tryptic digestion of recombinant Cry1Ac from *P. fluorescens*. Both proteins were truncated from the 131 kDa synthetic protoxin Cry1Ac(synpro) to a mass of ~65 kDa, and were immunoreactive with the anti-Cry1Ac monoclonal antibody. No full-length protoxin was purified due to its susceptibility to protease cleavage and/or its low solubility. The truncated cotton-derived protein had no detectable post-translational glycosylation. N-terminal sequencing showed that the first 28 amino acids of the protoxin were cleaved, since the

N-terminal sequence matched residues #29-45 of full-length Cry1Ac(synpro) [XETGYTRPIDXSLXLTQF]. A large piece of the protoxin C-terminus was also cleaved proteolytically. MALDI-TOF MS of the Cry1Ac tryptic peptide mass fingerprint identified all 29 predicted peptides as well as additional peptides likely due to protein over-digestion or degradation.

**CLASSIFICATION: ACCEPTABLE.**

**MRID 458084-05 Characterization of Phosphinothricin Acetyltransferase (PAT) from Recombinant *Escherichia coli* and Transgenic Cotton**

Biochemical and immunological analyses were performed to characterize phosphinothricin acetyltransferase (PAT) protein expressed in a recombinant microbe (*E. coli*) and transgenic cotton plant materials. PAT is an inert ingredient, a selectable genetic marker, that has an existing tolerance exemption, 40 CFR 180.1151. Based on the SDS-PAGE and Western blot analyses, the microbe-derived PAT and the plant-derived PAT proteins are biochemically equivalent. Dow AgroSciences has provided sufficient data to indicate that the PAT protein is supported by the existing tolerance exemption.

Analyses performed were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and peptide mass fingerprinting by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. SDS-PAGE confirmed that the microbial PAT protein derived from *E. coli* and transgenic cotton had the expected molecular weight (23.3 kDa). Western blotting confirmed that both test materials contained protein immunoreactive to antibodies specific to the PAT protein at the expected molecular weight. Non-transgenic cotton did not contain immunoreactive proteins. Peptide mass fingerprinting (PMF) analysis by mass spectrometry demonstrated that the PAT microbial test substance matched 9 of 17 theoretical trypsin fragments of PAT protein. Low amounts of PAT derived from transgenic cotton prohibited PMF analysis. The biochemical and immunological methods employed determined the biological equivalency of PAT proteins derived from recombinant *E. coli* and transgenic cotton.

The *E. coli*-derived PAT protein and transgenic cotton-derived PAT protein had the same intact, full-length PAT protein as shown by SDS-PAGE and Western blot analysis. The predicted size of the PAT microbial protein is 23.3 kDa and the predicted size of the PAT protein in the transgenic cotton extract is 20.6 kDa. There were differences due to the addition of a His-tag and N-terminal leader sequence added to aid the purification and expression of the PAT protein in *E. coli*. Only one specific immunoreactive protein, matching PAT, was seen on the Western blot (Figure 2). No immunoreactive protein or alternate size proteins were seen in the transgenic samples and non-transgenic cotton samples did not show any PAT protein. The large volume of cotton protein added to each well, needed to visualize the PAT protein, caused broadening and distortion of the lanes especially in the SDS-PAGE gels (Figure 1) so that the identification of individual proteins is

difficult.

In the trypsin digest of microbe-derived PAT protein, 9 out of a possible 17 (53%) peptides were identified using the theoretical cleavage of peptide masses of PAT (Table 2). This covered greater than 55% of the protein sequence. The peptide fragments detected were between residues 13 and 207 of the microbe-derived PAT. A 53% match seems rather low. In contrast, tryptic peptide mass fingerprints of the truncated Cry1F from both transgenic cotton (Event 281) and *P. fluorescens* determined by MALDI-TOF MS revealed 23 to 25 peptides matched the theoretical deduced peptide masses of Cry1F (synpro) (MRID 455423-05). There were some unidentified peptides detected with MALDI-TOF MS, but other factors could contribute to the formation of non-match peptides, e.g., over digestion leading to non-specific cleavage, self-digestion products of trypsin, or random breakage of peptides during ionization. There was no MALDI-TOF MS analysis of the plant-derived PAT protein due to low amounts of the plant-derived PAT protein purified from the cotton tissue.

One cannot definitively conclude the biochemical equivalency of the microbe-derived and plant-derived PAT proteins using MALDI-TOF MS analysis because of low levels of PAT extracted from the transgenic cotton tissue. PAT is a very well-characterized protein. Additional MALDI-TOF MS analysis of the plant-derived PAT protein will not likely alter the conclusion that the microbe-derived and the plant-derived PAT proteins are biochemically and immunologically equivalent. Additional MALDI-TOF MS analysis with the plant-derived PAT protein might be useful, but is not deemed as essential.

**CLASSIFICATION: ACCEPTABLE. Data are sufficient to indicate that PAT protein is covered by the existing tolerance exemption, 40 CFR 180.1151. Additional analysis would be needed for a novel protein.**

**458084-06 Biological Equivalency of Event 3006-210-23 Cotton- and *Pseudomonas*-Expressed Cry1Ac B.t. Insecticidal Crystal Protein**

Studies were conducted to investigate the equivalency between the *Pseudomonas*- produced and 3006-210-23 BC3F6 cotton-produced insecticidal protein, Cry1Ac. The three insect pests chosen to test biological equivalency for this study were the tobacco budworm (*Heliothis virescens*), cotton bollworm (*Helicoverpa zea*) and beet armyworm (*Spodoptera exigua*). Comparison of potency estimates indicates that the plant-produced and microbe-produced proteins display equivalent insecticidal activities (Table 7). Tobacco budworm was the most sensitive species followed by cotton bollworm and lastly, beet armyworm. The low concentration of plant-derived Cry1Ac did not allow calculation of $LC_{50}$ values for either cotton bollworm or beet armyworm. Concentrations were not high enough to cause mortality of these insects. A $GI_{50}$ value was used to calculate valid point estimates of growth inhibition because the data from both plant and microbial sources best bracketed this value. Beet armyworm values for the plant-produced Cry1Ac were the most variable due to mortality in beet armyworm larvae exposed to low

concentrations and inconsistent weight gain data to support $GI_{50}$ estimates. The $LC_{50}$ values for the tobacco budworm were statistically the same, 1.2 ng ai/$cm^2$ and 1.3 ng ai/$cm^2$ for microbe-produced and plant-produced Cry1Ac, respectively, indicating equivalency. The microbe-produced and plant-produced Cry1Ac $GI_{50}$ confidence intervals did not overlap for tobacco budworm. There is an order of magnitude difference between the $GI_{50}$ confidence intervals for tobacco budworm potency calculated using the *Pseudomonas*-expressed Cry1Ac protein and the transgenic cotton-expressed Cry1Ac protein. Although the tobacco budworm $GI_{50}$ for the plant Cry1Ac appeared significantly lower than for the microbe-derived Cry1Ac, this is likely due to naturally occurring secondary plant components present in the cotton leaves that are known to inhibit growth. The $GI_{50}$s for the two proteins are statistically indistinguishable for the cotton bollworm and beet armyworm indicating equivalency. Rank ordering among the two protein sources did not differ. Results from diet-based bioassays are highly variable, especially for Cry proteins (Robertson et al. 1995, Luttrell and Knighten 1999). Because of this inherent variability, insect bioassays can only be used as a qualitative tool to assess biological equivalency. This study replaces DAS study #010082, MRID 45542304.

**CLASSIFICATION: ACCEPTABLE**

**Allergenicity Potential of PAT**

**MRID 458186-04 Comparison of the putative amino acid sequence of the partial phosphinothricin acetyltransferase (PAT) ORF in Cry1F cotton event 281-24-236 to known protein allergens**

Sequencing of the transgenic insert and flanking border of Cry1F cotton event 281-24-236 showed the presence of partial phosphinothricin acetyltransferase (PAT) open reading frame (ORF). The 231 base-pair (bp) partial PAT plus the 24 bp sequence form the adjacent 3'T-DNA Border B region constitute a 255 bp ORF (85 amino acids). The 77 amino acid sequence of partial PAT ORF along with the 8 amino acid carboxy terminus tail were compared to databases containing known allergen amino acid sequences to determine if they would be potential allergens. Compared to the known allergen databases, no identical 8 or more contiguous amino acid sequences or an identity of $35% over 80 amino acid residues were found in the partial PAT ORF. Therefore, the amino acid sequence PAT ORF of Cry1F cotton event 281-24-236 is not consistent with known protein allergens. The positive control sequence was identified in both comparisons.

**CLASSIFICATION: ACCEPTABLE**

**MRID 458084-16 *In vitro* simulated gastric fluid digestibility study of recombinant phosphinothricin**

The PAT protein was rapidly digested, <0.5 minutes, in simulated gastric fluid, as demonstrated by

SDS-PAGE and Western blot analyses. According to the SDS-PAGE results, >98% (limit of detection is ~5.75 ng PAT/lane) of the PAT protein was digested within 30 seconds of addition to the simulated digestion mixture (Appendix A). The SDS-PAGE system had a demonstrated sensitivity of ~5.75 ng protein/lane (<2% of the protein added to the digestion mixtures, 0.46 :g) based on analysis of the neutralized PAT serial dilutions (lanes 14-17, Figure 3). In addition, the immunoblot results showed destruction of PAT by the failure of the primary antibody to recognize PAT protein in all digestion mixtures. The limit of detection of the Western blot was a concentration of 1.25 ng/lane (Figure 4, lane 17) which is <2% of the amount of PAT protein loaded in the digestion samples for the Western blot (0.1 :g). BSA, the positive control, was digested within 30 seconds after addition to the simulated digestion mixture while the negative control, $-lac, was resistant to digestion for >60 minutes.

The kinetic approach used to analyze the *in vitro* digestibility of proteins in simulated gastric fluid (SGF) is more quantitative than simply examining the disappearance of the PAT (or protein of interest) by SDS-PAGE or Western blot (Herman et al. 2003). Both digestibility and dye-binding characteristics of a protein contribute to the intensity of protein bands on SDS-PAGE ges. The disappearance of bands on gels is not necessarily a reliable method for comparing the digestibility among proteins since these chemical characteristics could confound appraisal of the endpoint. The kinetic approach is based on the classical enzyme theory to model the degradation of a protein in which the Michaelis-Menten rate law describes enzymatic reactions. If digestibility is described by first-order kinetics then one can easily calculate point estimates of decay such as half-life or $DT_{90}$. The kinetic analysis of decay, based on relative band density, is a more quantitative assessment of digestibility. However, this approach needs further standardization and validation using different proteins: other Cry proteins, known allergens, and known non-allergens to establish the digestion rate baselines. It is also important to bear in mind that there is a varying sensitivity of analytical techniques for detecting different proteins. Relative band density measurements (densitometry readings) were not part of the submitted study.

It is likely that the limit of detection is in fact lower than the 5.75 ng/lane neutralized PAT in the SDS-PAGE analysis (Figure 3, lane 17) and lower than the 1.25 ng/lane neutralized PAT in the Western blot analysis (Figure 4, lane 17) . Clear, although somewhat faint, bands are visible. Further serial dilutions would enhance the precision of the *in vitro* SGF study of recombinant PAT. The point at which >98% of the PAT protein is degraded is <0.5 minutes based on the limit of detection of the SDS-PAGE and Western blot analyses. This is not a calculated $DT_{98}$ as Dow has written, rather it is the upper limit of the time in which at least 98% of the PAT protein was digested in SGF. The Western Blot analysis clearly shows that no PAT-antibody bound to the PAT protein in any of the digestion mixtures indicating that the recognition sites for the PAT-antibody were destroyed by digestion in the SGF. The limit of detection of the Western blot was a concentration of 1.25 ng/lane (Figure 4, lane 17) which is <2% of the amount of PAT protein loaded in the digestion samples for the Western blot (0.1 :g). Using both the SDS-PAGE and Western Blot analyses, PAT is rapidly digested in SGF in <0.5 minutes. No detection limits were provided for BSA (positive control) or $-lactoglobulin (negative control). Other detection techniques will show

different sensitivities and limits of detection.

**CLASSIFICATION: ACCEPTABLE**

**Analytical Methods for Cry1Ac**

**458084-24 Development and Characterization of Enzyme-Linked Immunosorbent Assay (ELISA) for the Detection of Cry1Ac Protein**

The data in this report demonstrate the high quantitative performance of the ELISA assay for the detection of the Cry1Ac truncated protein. The assay had a reproducible sensitivity of 0.4 ng/mL with an assay range of 0.4 to 6 ng/mL Cry1Ac (truncated) protein. The absorbance variability of this assay is less than 10% and the resulting % error and accuracy are within ± 10%. The assay demonstrated no cross-reactivity to Cry1Ab, Cry1F, Cry2Ab, Cry9C, Cry34Ab1 (or PS149B1 14kD), Cry35Ab1 (or PS149B1 44kD), PAT and BAR, agriculturally relevant *Bt* proteins. The Cry1Ac assay kit was projected to be stable for approximately 1 year at 4°C based on extrapolations from the accelerated stability testing.

**CLASSIFICATION: ACCEPTABLE**

**458084-22 Independent Laboratory Validation of Method GRM 02.11, "Determination of Cry1Ac Insecticidal Crystal Protein in Cotton Tissues by Enzyme-Linked Immunosorbent Assay"**

An independent laboratory, BioLab Solutions, validated Dow AgroSciences LLC residue analytical method GRM 02.11 "Determination of Cry1Ac Insecticidal Crystal Protein in Cotton Tissues by Enzyme Linked Immunosorbant Assay" for accuracy, precision, and sensitivity. The LOQ of the method was confirmed as 0.375 µg/g (or 2.5 ng/mL) for Cry1Ac in cotton seed. Average recoveries from samples spiked with Cry1Ac protein averaged 73 and 80 percent at the 0.375 and 0.75 µg/g spike levels, respectively. The relative standard deviation (RSD) of replicate recovery measurement did not exceed 20 percent at or above the LOQ and interferences were negligible (<20% of the response of the Cry1Ac protein at the LOQ of 0.375 µg/g). The results of this assay validation indicate that the ELISA based assay is suitable for the analysis of Cry1Ac as found in cotton seed.

This method satisfies EPA Residue Chemistry Guidelines OPPTS 860.1340(c)(6) Residue Analytical Method and PR Notice 96-1. Recovery values for the fortified samples are of at least one set are within the range of 70%-120%, the relative standard deviation (RSD) of the replicate recovery measurements does not exceed ±20% at or above the LOQ, and any interferences are less than 20% of the LOQ. EPA's Analytical Laboratory located in Ft. Meade (Maryland) will have to independently validate Dow's residue analytical method GRM 02.11 "Determination of Cry1Ac Insecticidal Crystal Protein in Cotton Tissues by Enzyme Linked Immunosorbant Assay"

for accuracy, precision, and sensitivity.

**CLASSIFICATION: ACCEPTABLE**