A Review of the Environmental Safety of the Cry2Ab Protein

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INTRODUCTION

This document provides a comprehensive review of the information and data relevant to the environmental risk assessment of Cry2Ab, a protein encoded by genes isolated from *Bacillus thuringiensis* (Bt), and it presents a summary statement about the environmental safety of this protein when produced in genetically engineered (GE) cotton (*Gossypium hirsutum*) and maize (*Zea mays*) plants. All sources of information reviewed herein are publicly available and include dossiers presented to regulatory authorities, decision documents prepared by regulatory authorities, product descriptions prepared by product developers, and peer-reviewed literature.

Environmental risk assessments related to the planting of GE crops are conducted on a case-by-case basis, taking into account the biology of the plant, the characteristics of the transgenes and any encoded proteins, the phenotype conferred by the transgenes, the intended uses of the crop, and the nature of the receiving environment into which the plant will be introduced. These assessments, which consider both potential hazards and exposure levels, typically involve comparisons to an untransformed parental line or closely related isolines. The goal of these comparisons is the identification of potential risks the GE plant might present beyond those already deemed acceptable when similar, non-GE plants are grown in the environment. The consequences of these risks, if any, are then evaluated (OECD, 2007; Craig *et al.*, 2008).

Several regulatory authorities have performed environmental risk assessments on GE crop varieties producing Cry2Ab. Table I shows the current status[1] of regulatory approvals for the environmental release of cotton varieties containing Cry2Ab event MON15985 and maize varieties containing Cry2Ab event MON89034.[2] In some countries a separate regulatory approval may be given when an already approved event is combined with other GE events in a stack (Que *et al.*, 2010; Storer *et al.*, 2012). The table shows the date of the earliest approval given for the event.

Table 1. Regulatory approvals for the environmental release of GE cotton and maize varieties containing Cry2Ab (as of April 30, 2013).

Country	MON15985 Cotton	MON89034 Maize
Argentina		2010
Australia	2002	
Brazil	2009	2009
Burkina Faso	2008	
Canada		2008
Costa Rica	2009	
Honduras		2012
India	2006	
Japan	2004	2008
Mexico	2003	
Philippines		2009
South Africa	2003	2010
United States	2002	2008

Key words

Cry2Ab, insecticidal crystalline proteins, binary toxin, *Bacillus thuringiensis*, insect resistance, genetically engineered, environmental risk assessment

1 Some countries' regulations may require periodic renewal of GE crop registrations. For example, the current status of USEPA registrations can be found at http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm.

2 Many regulatory authorities have also approved Cry2Ab cotton and maize for food and feed use. Additional information can be found at http://cera-gmc.org/index.php?action=gm_crop_database.

ORIGIN AND FUNCTION OF THE Cry2Ab PROTEIN

Bacillus thuringiensis and the Cry2Ab Insecticidal Protein

Bacillus thuringiensis is a rod-shaped, gram-positive bacterium capable of forming long-lived endospores. It is often referred to as a soil bacterium, although it is ubiquitous in the environment (See, for example, Apaydin, Çınar, Turanli, Harsa, and Güneş, 2008; Martínez and Caballero, 2002; Seifinejad, Jouazni, Hosseinzadeh, and Abdmishani, 2008). The species has been studied extensively and used commercially for many years due to its ability to synthesize proteins with pesticidal properties (Hofte and Whiteley, 1989; Cannon, 1996; Schnepf *et al.*, 1998; OECD, 2007; van Frankenhuyzen, 2009). Preparations of natural isolates of *B. thuringiensis* were first used as a commercial insecticide in France in 1938, and *B. thuringiensis* subspecies *kurstaki* has been registered with USEPA since 1961 (USEPA, 1998). Microbial preparations of *B. thuringiensis* are currently approved for use around the world including in Australia, Canada, the European Union, and the United States (Kumar *et al.*, 1996; Schnepf *et al.*, 1998; USEPA, 1998; Baum *et al.*, 1999; Health Canada, 2008; APVMA, 2013; DGSANCO, 2013).

Since its discovery, Cry2Ab has been given various names: P2, CryX, CryIIB, CryB2, and CryIIAb (Crickmore *et al.*, 1998). Collectively, "*cry2Ab*" refers to two separate, virtually identical genes, *cry2Ab1* (Widner and Whiteley, 1989) and *cry2Ab2* (Dankocsik *et al.*, 1990). The gene sequences differ at nucleotide 1035, where there is a cytosine in *cry2Ab1* and a thymine in *cry2Ab2*. The nucleotide difference is translationally silent, so that the genes each encode a protein with the same amino acid sequence. In this monograph, this protein, as expressed by both transgenic cotton and maize, will be referred to as "Cry2Ab."

Several hundred pesticidal substances have been isolated from Bt cultures (Cannon, 1996; Prieto-Samsónov *et al.*, 1997; Crickmore *et al.*, 2012), and these substances display tremendous variety in chemical structure, mode of action, and target specificity (Hofte and Whiteley, 1989; Boucias and Pendland, 1998; Schnepf *et al.*, 1998; OECD, 2007; Pigott and Ellar, 2007; van Frankenhuyzen, 2009; Vachon *et al.*, 2012). Insecticidal preparations derived from cultured cells of Bt bacteria may contain a complex mixture of the pesticidal substances produced by the particular Bt strain used (Tabashnik, 1992; Schnepf *et al.*, 2005). They include antifungal compounds, ß-exotoxin,[3] vegetative insecticidal proteins (Vip), and the δ-endotoxins, a group that includes the insecticidal Cry (crystalline) proteins, and the Cyt (cytolytic) proteins (Hofte and Whiteley, 1989; Schnepf *et al.*, 1998; OECD, 2007; Pardo-López *et al.*, 2013). These substances may interact with each other to influence the toxicity and activity spectrum of individual bacterial preparations (Schnepf *et al.*, 1998; OECD, 2007).[4] The Cry proteins have been studied extensively and used widely in agriculture for their ability to cause cell disruption in the digestive tracts of insect pests, resulting in the interruption of feeding and eventual insect death (Gill *et al.*, 1992; Cannon, 1996; Prieto-Samsónov *et al.*, 1997; Mendelsohn *et al.*, 2003; Gómez *et al.*, 2007; OECD, 2007; Pardo-López *et al.*, 2013).

In 1988, two closely related genes, both occurring on the same 110 MDa plasmid, were identified in *Bacillus thuringiensis* ssp. *kurstaki* strain HD-1 (Donovan *et al.*, 1988; Ahmad *et al.*, 1989). The two genes were eventually designated *cry2Aa* and *cry2Ab* (Crickmore e*t al.*, 1998)(See box for additional information). Each gene sequence corresponds to a peptide of 633 amino acids, and the two peptides share 87% amino acid identity. Since its initial discovery, genes encoding Cry2Ab proteins have been identified in Bt strains found throughout the world (Jain *et al.*, 2006; Jouzani *et al.*, 2008; Saadaoui *et al.*, 2010; Saleem and Shakoori, 2010; Alvarez and del Valle Loto, 2012). However the peptide corresponding to *cry2Ab* is typically not produced by these strains, and the native *cry2Ab* is therefore considered to be a pseudo- or cryptic gene (Hodgman *et al.*, 1993b; USDA, 2000; FSANZ, 2002; Health Canada, 2003; PDOA, 2003).

Lack of expression of the native *cry2Ab* is due to the absence of a functional promoter, and it was also determined that a molecular chaperone necessary for the formation of Cry2Ab crystals is missing from the bacterial strains bearing the cryptic *cry2Ab* (Crickmore *et al.*, 1994; Boucias and Pendland, 1998). Expression of Cry2Ab was accomplished in heterologous systems by replacing the regulatory sequences upstream of *cry2Ab* (Widner and Whiteley, 1989, 1990; Dankocsik *et al.*, 1990; Hodgman *et al.*, 1993b; Crickmore *et al.*, 1994; FSANZ, 2002; OGTR, 2002; Health Canada, 2003; CFIA, 2004).

The peptide encoded by *cry2Aa* is toxic to both lepidopteran (*Heliothis virescens*, *Lymantria dispar*, and *Manduca sexta*) and dipteran (*Aedes aegypti*, *Musca domestica*) species (Knowles *et al.*, 1986; Donovan *et al.*, 1988; Widner and Whiteley, 1989; Hodgman e*t al.*, 1993a; Kondo *et al.*, 1995), a property shared by other proteins in the Cry2 group. Originally, the peptide encoded by *cry2Ab* was thought to be toxic only to lepidopteran species, such as *Manduca sexta* (Widner and Whiteley, 1989, 1990), like the Cry1 group of proteins (Crickmore *et al.*, 1998). It was later determined that the sequences of Cry2Aa and Cry2Ab differ in 18 amino acids present in a 76-amino acid segment, and the different toxicity spectra for the two proteins was attributed to the sequence differences (Ahmad and Ellar, 1990; Widner and Whiteley, 1990; Liang and Dean, 1994). However, more recent research indicates that Cry2Ab is active on at least one dipteran species and is properly placed in the Cry2 protein group (McNeil and Dean, 2011). In addition, Cry2Ab shares other properties of the Cry2 group: the Cry1 and Cry2 proteins are antigenically distinct (Höfte *et al.*, 1988; Gill *et al.*, 1992), and while Cry1 proteins are soluble at pH 9, Cry2 proteins, including Cry2Ab,

3 Also called *thurigiensin* (OECD, 2007; Liu *et al*., 2010).

4 Therefore, the activity spectrum of sprays made from Bt bacterial cultures may differ from the activity spectrum of individual Bt proteins produced by a GE plant (OECD, 2007).

generally require a more alkaline environment, pH 12, for complete solubilization (Gill *et al.*, 1992).

Mechanism of Cry2Ab Insecticidal Activity

Besides having different activity spectra, another key difference between Cry1-type Bt toxins and Cry2-type is that Cry1 proteins are synthesized initially as protoxins, greater than 130 KDa in size, while Cry2-type toxins, including Cry2Ab, are synthesized as smaller proteins, approximately 70 kDa in size. Both Cry1 and Cry2 proteins require proteolytic processing in the insect gut to release the active toxin (Hernández-Rodríguez *et al.*, 2008). In other respects, the mode of action for Cry2Ab is similar to that of the Cry1 group of toxins: once consumed by the target insect, the toxin dissolves, is activated by midgut proteases, and binds to specific membrane receptors present in susceptible insects (Hernández-Rodríguez *et al.*, 2008). After specific interaction with the receptor, the toxin is thought to insert itself into the membrane and cause the formation of pores, resulting in ionic disequilibrium and cell lysis, similar to the case of Cry1 proteins (Gill *et al.*, 1992; Prieto-Samsónov *et al.*, 1997; Gómez *et al.*, 2007).

Cry2Ab is either toxic to, or inhibits the growth of, several lepidopteran species—*Diatraea saccharalis*, *Earias insulana*, *Heliothis virescens*, *Helicoverpa zea*, *Helicoverpa armigera*, *Lymantria dispar*, *Manduca sexta*, *Ostrinia nubilalis*, *Pectinophora gossypiella*, P*seudoplusia includens*, *Spodoptera exigua*, *Spodoptera frugiperda*, and *Trichoplusia ni*—and the *cry2Ab* gene is used both singly and in combination with other Bt toxin genes to control a range of cotton and maize insect pests (Widner and Whiteley, 1989, 1990; Dankocsik *et al.*, 1990; Adamczyk *et al.*, 2001; Stewart *et al.*, 2001; Tabashnik *et al.*, 2002; Avilla *et al.*, 2005; Aguilar-Medel *et al.*, 2007; Dennehy *et al.*, 2007; Sivasupramaniam *et al.*, 2008; Brevault *et al.*, 2008; Ibargutxi *et al.*, 2008; ICAC, 2008; Ali and Luttrell, 2011; Wangila *et al.*, 2012; Siebert *et al.*, 2012)

Considerable attention has been devoted to understanding the nature of Cry2Ab membrane binding in order to determine whether other toxins share the same binding site. For example, the binding of Cry2Ab to the midgut brush border of *Helicoverpa zea* can be displaced by Cry2Ab or by other proteins of the Cry2A family, but not by Cry1Ac, a Bt toxin that is frequently used in combination with Cry2Ab in insect-resistant varieties of cotton and maize (Hernández-Rodríguez *et al.*, 2008). The lack of dilution of Cry2Ab binding by Cry1Ac suggests that the two proteins do not share a common component for binding, a factor that could contribute to the development of cross-resistance (USEPA, 2002). Additional studies using insects selected to be resistant to specific Bt toxins show that Cry1Ac-resistant *Helicoverpa zea* and *Helicoverpa armigera* are susceptible to Cry2Ab, which researchers have concluded reduces the probability that cross-resistance will develop (Luo *et al.*, 2007; Anilkumar *et al.*, 2008; Brévault *et al.*, 2009). Conversely, laboratory-selected *Helicoverpa armigera* and *Helicoverpa punctigera* highly resistant to Cry2Ab remained susceptible to Cry1Ac, and the authors showed that binding of Cry2Ab to the insects' midgut membrane was greatly reduced whereas binding of Cry1Ac remained unaltered (Caccia *et al.*, 2010).

Modifications to the Genes Encoding Cry2Ab in GE Cotton and Maize

Cotton: The *cry2Ab* sequence used to create maize event 15985 was modified from that of the wild-type *cry2Ab* gene to use plant-preferred codons, for better expression in cotton (Perlak *et al.*, 1991). In addition, a *NcoI* restriction site was introduced at the N'-terminal end of the gene to facilitate cloning, resulting in an additional aspartic acid residue at position 2 of the peptide. Lastly, the N-terminal chloroplast transit peptide, from the *Arabidopsis thaliana epsps* gene, comprising 79 amino acids, was inserted immediately preceding the *cry2Ab* gene. Once the peptide is targeted to the chloroplast, the transit peptide is cleaved, leaving three amino acids at the N'-terminal end of the peptide. The final protein product contains 633 amino acids, plus the four additional amino acids discussed above, and is approximately 71 kDa (USDA, 2000, 2002; FSANZ, 2002; OGTR, 2002). The protein is not glycosylated post-translation (OGTR, 2006).

Table 2. Genetic elements used in the production of GE insect-resistant cotton and maize varieties (USDA, 2000, 2003, 2004a)

Cotton 15985		Maize 89034	
Genetic Element	**Function**	**Genetic Element**	**Function**
e35S	Cauliflower mosaic virus promoter with a duplicated enhancer region to promote gene expression (0.6 kb)	P-FMV	Figwort mosaic virus 35S promoter
PetHSP70-leader	5' untranslated leader sequence of the petunia heat shock protein 70 gene to increase levels of gene expression (0.1 kb)	I-Hsp 70	First intron of the maize heat shock protein 70 gene to increase levels of gene expression (0.1 kb)
CTP2	N-terminal chloroplast transit peptide, from *Arabidopsis thaliana epsps* gene, to facilitate movement of the protein into chloroplasts (0.2 kb)	TS-SSU-CTP	Chloroplast transit peptide region of maize ribulose 1,5-biphosphate carboxylase small subunit with the first intron
cry2Ab2	Synthetic *cry2Ab* gene with plant-preferred codons (Widner and Whiteley, 1989), based on sequence of native *cry2Ab* from *Bacillus thuringiensis* ssp. *kurstaki* (1.9 kb)	*cry2Ab2*	Synthetic *cry2Ab* gene with plant-preferred codons (Widner and Whiteley, 1989), based on sequence of native *cry2Ab* from *Bacillus thuringiensis* ssp. *kurstaki* (1.9 kb)
T-*nos*	3' untranslated region of the nopaline synthase gene from *Agrobacterium tumefaciens* to terminate transcription and direct polyadenylation of the mRNA (0.3 kb)	T-*nos*	3' untranslated region of the nopaline synthase gene from *Agrobacterium tumefaciens* to terminate transcription and direct polyadenylation of the mRNA (0.3 kb)

Maize: The *cry2Ab* sequence used to create maize event 89034 was modified from that of the wild-type *cry2Ab* gene to use plant-preferred codons, for better expression in maize (Murray *et al*., 1989). In addition, a *NcoI* restriction site was introduced at the N'-terminal end of the gene to facilitate cloning, resulting in an additional aspartic acid residue at position 2 of the peptide. Lastly, the maize ribulose 1,5-biphosphate carboxylase small subunit, comprising 79 amino acids, was inserted immediately preceding the *cry2Ab* gene. Once the peptide is targeted to the chloroplast, the transit peptide is cleaved from the N'-terminal end of the peptide. The final protein product contains 633 amino acids, plus the additional aspartic acid discussed above, and is approximately 71 kDa (JBCH, 2006b; USDA, 2006, 2008; USFDA, 2007; CFIA, 2008). The protein is not glycosylated post-translation (FSANZ, 2008).

Descriptions of the genetic elements used in the production of Cry2Ab cotton and maize events are provided in Table 2.

Expression of Cry2Ab in GE Insect-Resistant Cotton and Maize

Transgene expression levels in a GE plant can be influenced by several factors related to the genetic transformation process, including the types of promoter and terminator sequences employed, as well as the chromosomal location where the transgene has been incorporated into the genome. Expression levels may also be influenced by the type of tissue sampled, the age of the plant at the time the sample was taken, and the environmental conditions under which the plant was growing (Siebert *et al*., 2009).

Data from enzyme-linked immunosorbent assays (ELISA), showing levels of Cry2Ab protein expression in GE cotton and maize events have been made publicly accessible via regulatory dossiers and decision documents associated with regulatory authorization processes. Samples were collected from several tissue types, and at multiple growth stages, from plants grown in several different locations to produce data representative of the typical range of protein expression. Protein expression data may be used to estimate the potential exposure of various organisms in the environment to Cry2Ab when cotton and maize plants producing Cry2Ab are cultivated. Currently available protein expression data for Cry2Ab by cotton event 15985 and by maize event 89034 used alone and when stacked with other GE events are presented in Annex I.

NON-TARGET ORGANISM TESTING AND IMPACTS OF EXPOSURE TO THE Cry2Ab PROTEIN

The Cry2Ab toxin has insecticidal properties against certain lepidopteran insect species when expressed in cotton and maize plants. The toxin targets herbivorous lepidopteran insect pests, which would otherwise cause feeding damage to the crop. Organisms in the environment that are not pests of cotton or maize but are directly or indirectly exposed to Cry2Ab expressed in transgenic cotton or maize plants are called non-target organisms (NTOs).

Assessments of impacts to NTOs include the review of data submitted to regulators by the product developer to demonstrate that NTOs exposed to the Cry2Ab, either directly or indirectly, are not harmed. The NTO risk assessment typically begins with a determination of the organisms that are likely to be directly or indirectly exposed to Cry2Ab. Particular consideration is often given to NTOs having beneficial environmental functions, such as pollinators or the natural enemies of agricultural pests. Regulatory authorities may give special attention to NTOs that have been designated as threatened or endangered species or species of recognized cultural value. These species, or valid surrogates for these species, are then tested to determine if exposure to Cry2Ab could cause significant adverse impacts (Romeis *et al*., 2013).

Assessments of the potential impacts to NTOs, and the regulatory decisions informed by the assessments, have been grounded in the well-documented and long history of evaluation of chemical insecticidal formulations including microbial formulations of *B. thuringiensis* (USEPA, 2007; Romeis *et al*., 2008, 2013; Carstens *et al*., 2012; Sanvido *et al*., 2012). The "tiered" approach for assessing the impacts of chemical pesticides on NTOs has been used effectively for many years, and tiered testing has also been determined by scientists and regulators to be appropriate for the assessment of potential impacts of GE crops on NTOs (Dutton *et al*., 2003; EFSA, 2006; Garcia-Alonso *et al*., 2006; Raybould, 2006; USEPA, 2007, 2011b; Romeis *et al*., 2008, 2013; Duan *et al*., 2010). Tier 1 studies involve the exposure of NTOs or surrogate species to high concentrations of the pesticide, under controlled laboratory conditions. These studies identify those species that are significantly affected by the pesticide. Such effects, when found, may require further analysis at a higher tier level. Early tier tests also identify NTOs that are unaffected by the pesticidal protein and for which higher tier testing is therefore unnecessary. Higher level tier testing may also be appropriate when the results of early tier tests are inconclusive. Testing at higher tiers typically involves increasing levels of complexity and increasingly realistic assay conditions (EFSA, 2006; Garcia-Alonso *et al*., 2006; USEPA, 2007, 2011b; Romeis *et al*., 2008).

Routes of Environmental Exposure

Direct exposure occurs when NTOs feed on living crop tissues expressing Cry2Ab or on crop residues, either above or belowground. Indirect exposure results from the predation by one organism on another organism that has had direct exposure to Cry2Ab (Tian *et al*., 2012). In addition to direct consumption of parts of the GE cotton or maize plant, regulatory authorities may consider other routes of potential indirect exposure to the Cry2Ab toxin: exposure to the toxin in pollen, exposure to toxin deposited in the soil by decomposing plant material, and exposure to predator species consuming herbivores that have been feeding on the GE maize plants (USDA, 2000, 2002, 2006, 2008; OGTR, 2002, 2006; FSANZ, 2002, 2008;

PDOA, 2003; USEPA, 2003, 2011a; JBCH, 2004, 2005, 2006a; b, 2008a; b, 2009, 2011; EFSA, 2008; CTNBio, 2009a; b, 2010a; b, 2011, 2012; AMAGP, 2012). Regulators may consider protein expression data to determine potential routes and levels of exposure. For example, plant tissues producing little or no Cry2Ab are unlikely to pose a hazard to NTOs. (See Annex I for Cry2Ab expression level data in the tissues of approved cotton and maize varieties.) Published data as well as data submitted to regulatory authorities indicate that Cry2Ab is quickly degraded once released from decomposing plant tissue and is not likely to persist or accumulate in the soil environment (USDA, 2000, 2002, 2006, 2008; USEPA, 2002, 2003; Head, 2007; CFIA, 2008; Cheeke, 2013).

Ecotoxicological Testing of Cry2Ab on Non-Target Organisms

For many years, ecotoxicological testing to determine the effects of chemical pesticides on NTOs has been conducted on a variety of well-characterized test organisms. Data from these tests have been shown to effectively assess the environmental risks of chemical pesticides and to inform regulators' decisions regarding the safe development and use of pesticides. Analogous testing using many of the same organisms has been successfully used to assess impacts from the environmental release of transgenic crops expressing one or more Bt proteins (Dutton *et al.*, 2003; Garcia-Alonso *et al.*, 2006; Raybould, 2007; USEPA, 2007; Romeis *et al.*, 2008; Gealy *et al.*, 2010; Carstens *et al.*, 2012).

Because Cry2Ab is toxic to several lepidopteran species, regulatory authorities examine data regarding impacts of Cry2Ab on non-target lepidopterans, such as the monarch butterfly or other lepidopteran species of local importance. Regulators may also request and evaluate impact data on beneficial species, such as pollinators, predators, and decomposers, as well as representative soil dwelling species, to demonstrate that there are no significant impacts to these species from exposure to Cry2Ab (Mattila *et al.*, 2005; Head, 2007; Wolfenbarger *et al.*, 2008; Duan *et al.*, 2008; Gatehouse *et al.*, 2011; Hendriksma *et al.*, 2011, 2012; Prischl *et al.*, 2012; Schuppener *et al.*, 2012; Cheeke, 2013; Dohrmann *et al.*, 2013). Test organisms have included A*pis mellifera* (honeybee); *Hippodamia convergens* (ladybird beetle) *Chrysoperla carnea* (green lacewing); *Danaus plexippus* (monarch butterfly); *Nasonia vitripennis* (parasitic wasp); *Folsomia candida* (springtail); *Daphnia magna* (crustacean); and *Eisenia foetida* (earthworm). Test organisms were exposed to levels of Cry2Ab many times higher than the highest exposure levels predicted from the observed tissue concentrations of Cry2Ab in GE cotton and maize plants (See Annex II). After evaluating these test results, regulators have concluded that no significant adverse effects were observed (USDA, 2000, 2002, 2006, 2008; FSANZ, 2002, 2008; OGTR, 2002, 2006; USEPA, 2002, 2003, 2009, 2011a; Health Canada, 2003; PDOA, 2003; CFIA, 2004, 2008; JBCH, 2004, 2005, 2006a; b, 2008a; b, 2009, 2011; EFSA, 2008; EC, 2011; CTNBio, 2009a; b, 2010a; b, 2011, 2012; EC, 2009, 2010; UMGAP, 2012).

Additionally, vertebrate toxicological testing of the Cry2Ab protein and nutritional equivalence testing of cottonseed meal and maize grain from Cry2Ab varieties have been conducted on *Mus musculus* (mouse); *Ictalurus punctatus* (catfish); *Gallus domesticus* (chicken); *Rattus norvegicus* (rat); *Bos taurus* (cattle); and *Colinus virginianus* (northern bobwhite quail) (See Annex II). From these test data, scientists and regulators have concluded that the Cry2Ab protein is not toxic to animals or to humans (USDA, 2000, 2002, 2006, 2008; FSANZ, 2002, 2008; OGTR, 2002; USEPA, 2002, 2003, 2009, 2011a; Health Canada, 2003; PDOA, 2003; Hamilton *et al.*, 2004; JBCH, 2004, 2005, 2006a; b, 2008a; b, 2009, 2011; Taylor *et al.*, 2007a; b; CFIA, 2008; EFSA, 2008; Li *et al.*, 2008; Drury *et al.*, 2008; CTNBio, 2009a; EC, 2011; CTNBio, 2009b, 2010a; b, 2011, 2012; EC, 2009, 2010; Singhal *et al.*, 2011; Sissener *et al.*, 2011; Weber *et al.*, 2011; UMGAP, 2012; Lundry *et al.*, 2013).

The results from Tier 1 tests discussed above indicate that no higher tier testing should be necessary from a regulatory standpoint, because no adverse effects were noted;[5] however, studies of the effects of Cry2Ab maize on natural populations of NTOs and soil microorganisms have been performed (Schuppener *et al.*, 2012; Dohrmann *et al.*, 2013). For the organisms studied, these field tests found no significant differences between populations in fields where Cry2Ab maize was grown and fields where a non-GE maize variety was grown.

The potential for harm to NTOs from exposure to Cry2Ab has been considered in risk assessments conducted by several regulatory authorities. Data collected from laboratory and field trials of GE cotton and maize producing Cry2Ab and submitted to regulators have established that the Cry2Ab protein is active specifically against the subset of lepidopteran pests which feed on the aboveground parts of cotton and maize plants and are harmless to vertebrate species and other NTOs (OGTR, 2002, 2006; USDA, 2002, 2006, 2008; USEPA, 2011a, 2003; CFIA, 2008; EFSA, 2008; CTNBio, 2009b; JBCH, 2009).

Regulatory authorities have determined that adverse effects on NTOs are unlikely for several reasons. First, Cry2Ab has a narrow spectrum of pesticidal activity. Second, Tier I laboratory assays, employing a range of invertebrate species present in cotton and maize agricultural ecosystems, or surrogates for those species, have shown that Cry2Ab causes no significant observable effects in these species. Third, Tier I studies have demonstrated that Cry2Ab has no observable effect on representative vertebrate and aquatic species. Fourth, the levels of Cry2Ab used in these Tier I assays were much higher than those measured in GE cotton and maize tissues growing in the field. Fifth, field studies of maize varieties producing Cry2Ab showed no significant adverse effects on the species studies (Schuppener *et al.*, 2012; Dohrmann *et al.*, 2013). Sixth, when compared to insect control via Cry2Ab, traditional insect control using chemical pesticides signifi-

5 Conducting field studies is considered case-by-case, based on the level of potential hazard and exposure, and goals may be adjusted as information and experience accumulate (USEPA, 2007).

cantly alters species diversity and harms non-target species (Gatehouse *et al.*, 2011). Together, these findings indicate that Cry2Ab is unlikely to have adverse effects on natural populations of organisms, except for the target lepidopteran crop pests it is meant to control.

ESTABLISHMENT AND PERSISTENCE IN THE ENVIRONMENT OF MAIZE PLANTS EXPRESSING CRY2Ab

Biology of the Plant Species

The biology of the non-GE plant species in the receiving environment is typically the starting point for environmental risk assessments of GE plants (OECD, 2003, 2007, 2008). Information about the biology of the non-GE plant can be used to assess whether a GE variety of the plant may become weedy, invasive, or otherwise harmful to the environment. It can also provide details on significant interactions between the plant and other organisms that may be important when considering potential harms. By considering the biology of the host plant, a risk assessor can identify potential hazards that may be associated with the expression of the novel protein (*e.g.*, Cry2Ab) and then be able to assess the likelihood of these hazards. For example, whether the plant is an annual or perennial species or whether the plant is self pollinated or wind pollinated can bear on the assessment of the likelihood of the GE plant establishing and persisting outside of cultivation (OECD, 1992, 2003, 2007, 2008; EFSA, 2006).

Phenotypic Data

Information about the phenotype of GE plants expressing Cry2Ab is collected from laboratory, greenhouse, and field trial studies and is presented in regulatory submissions to (1) identify any intentional changes to the phenotype that might impact the environmental safety of the plant and (2) to identify any unintended changes to the biology of the plant that might impact environmental safety. Phenotypic data in regulatory submissions and peer reviewed publications have focused on characteristics of the plant that might contribute to its survival or persistence (*i.e.*, potential weediness), or those that may negatively affect agricultural performance (*e.g.*, disease susceptibility and yield data). The phenotypic observations take into account the desired phenotype resulting from the transgenic trait, in this case insect predation resistance mediated by Cry2Ab. Some of the collected data are quantitative (*e.g.*, plant height or percent seed germination) while other data are qualitative and observational (*e.g.*, symptoms of disease susceptibility). Any differences between GE cotton or maize plants expressing Cry2Ab and controls were within the reported range for non-GE cotton and maize varieties (USDA, 2000, 2002, 2006, 2008; OGTR, 2002, 2006; CFIA, 2008). Collectively, regulators have determined that the phenotypic data do not support the hypothesis that the expression of Cry2Ab had any unintended impact on the gross morphology or phenotypic characteristics of cotton or maize plants, besides conferring resistance to lepidopteran insect pests.

Weediness in Agricultural Environments

Cotton: Cultivated cotton can grow as a perennial in areas lacking a cold season, but it lacks weedy or aggressive characteristics, and it is not generally considered to be an economically important agricultural weed. Researchers and regulators have evaluated the potential for insect-resistant GE cotton varieties to become weeds, including cotton producing the Cry2Ab protein, and they have found that there are no characteristics of insect-resistant cotton that would increase its potential to become an agricultural weed, because any volunteer cotton plants would be readily controlled using conventional weed management techniques (USDA, 2002, 2000; Carpenter *et al.*, 2002; Eastick, 2002; OGTR, 2002, 2006; CFIA, 2004; JBCH, 2004, 2005; Eastick and Hearnden, 2006; CTNBio, 2010b).

Maize: Maize is not generally regarded as a weed, possessing few of the characteristics that increase the likelihood of a plant to become a weed, such as seed dormancy, shattering, and competitiveness (Baker, 1965, 1974). There are no data indicating that expression of Cry2Ab results in altered seed dormancy, over-wintering capacity, or other characteristics that would alter the prevalence of volunteer maize in subsequent growing seasons. Following-season maize volunteers producing Cry2Ab would not be expected to present any unusual weed management challenges and can be dealt with in the same manner as conventional volunteers of maize (Carpenter *et al.*, 2002; USDA, 2006, 2008; CFIA, 2008; EFSA, 2008; JBCH, 2008a; b, 2009; CTNBio, 2009a, 2010a; b; Raybould *et al.*, 2011).

Weediness in Non-Agricultural Environments

The primary mechanisms by which Cry2Ab may be introduced into a non-agricultural environment are through the movement of propagules outside of cultivated areas (Lee and Natesan, 2006), and regulators evaluate how such introductions may result in a GE plant becoming weedy or invasive.

Cotton: While all plants may exhibit weedy characteristics under certain conditions, commercial varieties of cotton are not considered to pose a significant weed risk in non-agricultural environments. Selective breeding has resulted in modern cotton varieties' dependence on human intervention, and factors such as water stress and cold severely limit the ability of commercial varieties to survive in non-agricultural environments. Although insect resistance mediated through the Cry2Ab protein may provide some fitness advantage to an escaped GE cotton plant, researchers and regulators have determined that such an advantage would be insufficient to allow GE cotton expressing Cry2Ab to be competitive or persist in a non-agricultural environment (USDA, 2002, 2000; Carpenter *et al.*, 2002; Eastick, 2002; OGTR, 2002, 2006; CFIA, 2004; JBCH, 2004, 2005; Eastick and Hearnden, 2006; CTNBio, 2010b).

Maize: As a result of extensive selective breeding, commercial maize varieties are severely restricted in their ability to persist in non-agricultural environments without human intervention, and maize is not

considered to be an invasive or aggressive weed outside of agricultural systems (Carpenter *et al.*, 2002). Agronomic data show that Cry2Ab does not have a significant impact on traits associated with weediness. Although release from natural control factors (including insect herbivores) has been offered as a partial explanation for the success of invasive species (Mack, 1996; Keane and Crawley, 2002; Mason *et al.*, 2004; Blumenthal, 2005), regulatory decisions have determined that it is unlikely that resistance to lepidopteran pests would allow maize producing Cry2Ab to become invasive in non-agricultural environments (Carpenter *et al.*, 2002; USDA, 2006, 2008; CFIA, 2008; EFSA, 2008; JBCH, 2008a; b, 2009; CTNBio, 2009a, 2010a; b; Raybould *et al.*, 2011).

Movement of the Transgene to Sexually Compatible Relatives

The movement of transgenes from a GE plant to its wild relatives is pollen mediated, and the production of reproductively viable hybrids depends on several factors: whether the pollen donor is self-pollinated, the physical and temporal proximity of the GE plants to sexually compatible species, pollen mobility and viability, and the presence of appropriate pollinators (Chandler and Dunwell, 2008).

Cotton: The *Gossypium* genome is very complex and is organized into eight diploid species groups and one tetraploid species group, which includes *G. hirsutum*. Crosses within groups can occur, but crosses between groups are rare, and offspring display meiotic abnormalities and infertility, including crosses between *G. hirsutum* and members of the diploid species. Hybridization between *G. hirsutum* and the three wild tetraploid species (*G. mustelinum*, *G. darwinii*, and *G. tomentosum*) as well as crosses with feral populations of *G. barbadense* and *G. hirsutum* can be readily made experimentally and result in fertile offspring. Under the favorable conditions discussed above, spontaneous hybridizations can occur when commercial varieties of *G. hirsutum* are grown near natural populations of tetraploid species (OECD, 2008). However, the frequency of such crosses between transgenic *G. hirsutum* and sexually compatible wild relatives is considered to be no greater than crosses between traditionally bred varieties of *G. hirsutum* and wild species (USDA, 2000, 2002; Carpenter *et al.*, 2002; OGTR, 2002; CFIA, 2004; JBCH, 2004; OECD, 2008).

Maize: Maize is predominantly wind pollinated and does not have sexually compatible relatives that are considered invasive (Carpenter *et al.*, 2002; OECD, 2003). Maize freely hybridizes with wild teosintes, but gene introgression is thought to be limited (Castillo-Gonzalez and Goodman, 1997; OECD, 2003; Baltazar *et al.*, 2005). Wild teosinte populations are limited to Mexico, Guatemala, and a single population in Nicaragua, and while teosinte is considered a serious weed by some farmers in Mexico, it is used as a forage plant by other farmers, and it is also considered a culturally significant species (González and Corral, 1997; Mondragon-Pichardo and Vibrans, 2005). Crosses between teosinte and GE maize expressing Cry2Ab are not expected to occur more frequently than those between teosinte and traditionally bred maize varieties (Carpenter *et al.*, 2002; USDA, 2006, 2008; CFIA, 2008; EFSA, 2008; CTNBio, 2009a; JBCH, 2009).

COMPOSITIONAL ANALYSIS OF MAIZE PLANTS EXPRESSING CRY2Ab

A compositional analysis is required in many regulatory approval processes for GE plants intended to be used in food or feed. Compositional data can be used to identify unintended changes in the crop due to the presence of the transgene. The analysis typically compares the GE plant to the untransformed parent line or a closely related isoline, and the analytes measured depend on the crop and its intended uses. The analysis may use plants grown in a variety of locations and may include data from multiple growing seasons, because local environmental conditions may impact nutritional composition even in conventionally bred varieties. The goal of the analysis is to verify that the values obtained for the GE plant are within the range observed in traditional varieties grown under comparable conditions.

Seed and forage from Cry2Ab maize and seed from Cry2Ab cotton has undergone proximate analysis to determine levels of crude protein, crude fat, fiber, moisture, and ash. In addition, levels of select minerals, fatty acids, amino acids, and antioxidants have been determined. Some crop plants produce toxins or anti-nutritive compounds, and levels of these compounds are also measured to determine whether the presence of the transgenes has inadvertently resulted in elevated production of these substances. Maize is known to produce the anti-nutritive compounds phytic acid, raffinose, and trypsin inhibitor (OECD, 2003), and cotton produces the toxins gossypol and cyclopropenoid fatty acids (OECD, 2008). Levels of these substances produced by cotton and maize varieties expressing Cry2Ab were measured and compared with levels in conventional cotton and maize varieties. The data from publicly available sources are summarized in Annex III. All differences noted between the GE cotton event 15985 and the comparator varieties were within the normal range of variation, and these differences were deemed irrelevant to environmental safety (USDA, 2000; USFDA, 2002; FSANZ, 2002; Health Canada, 2003; PDOA, 2003; CFIA, 2004; Hamilton *et al.*, 2004; Li *et al.*, 2008; CTNBio, 2009b, 2012; Singhal *et al.*, 2011). A similar comparison for maize event 89034 and comparator varieties revealed no differences relevant to environmental safety (USDA, 2006; Taylor *et al.*, 2007b; USFDA, 2007; CFIA, 2008; Drury *et al.*, 2008; EFSA, 2008; FSANZ, 2008; CTNBio, 2010a, 2011; Weber *et al.*, 2011; Lundry *et al.*, 2013).

CONCLUSION

The Cry2Ab protein produced by insect-resistant GE cotton and maize plants is derived from the common soil bacterium *Bacillus thuringiensis* and is specifically toxic to Lepidoptera. Toxicity testing with a range of representative non-target organisms demonstrated that Cry2Ab produced no observable effects at concentrations sig-

nificantly higher than the expected environmental concentrations of Cry2Ab. Field data suggest that cultivation of GE maize plants expressing Cry2Ab does not affect the abundance of non-target arthropods. Cry2Ab in plants can be toxic to non-target Lepidoptera, but regulatory risk assessments for approved products have concluded that the risk is low, due to the lack of exposure to the toxin in the environment, especially when compared to other insect-control practices. The weight of evidence from analyses of phenotypic and compositional data demonstrates that Cry2Ab expression in approved cotton and maize varieties does not alter the gross physiology of the crop plants and indicates that these plants are not more likely to become weedy or invasive than conventional cotton and maize varieties.

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ANNEX I: SUMMARY OF CRY2Ab PROTEIN EXPRESSION DATA

The tables that follow present summary data from applicant dossiers and regulatory decisions documents. Whenever possible, the data and accompanying statistics are presented as they appeared in the cited document to facilitate cross-referencing. Additional information on data collection and sampling methodologies can be found in the referenced sources.

Summary Data for Cotton

Table I.1. Mean levels of Cry2Ab protein in cotton event 15985, the parental control DP50B, and the non-transgenic control DP50 (USDA, 2000; FSANZ, 2002; OGTR, 2002; USEPA, 2003; CFIA, 2004; JBCH, 2005).

Cotton Line	Cry2Ab (µg/g fresh weight)
Leaf[1]	
15985	23.8 ± 6.3[2] (10.1 – 33.3)[3]
DP50B	< 2.65
DP50	< 2.65
Seed[4]	
15985	43.2 ± 5.7 (31.8 – 50.7)
DP50B	< 2.31
DP50	< 2.31
Whole plant[5]	
15985	8.80 ± 1.20 (7.28 – 10.46)
DP50B	< 1.24
DP50	< 1.24
Pollen[5]	
15985	< 0.25
DP50B	< 0.25
DP50	< 0.25

1 Leaf tissue n = up to 6 plants/plot from each site, 8 sites, taken at 4 – 6 weeks post planting.
2 Mean and standard deviation were calculated from samples, one from each of 8 field sites except for tissues collected from a single site.
3 Range is the minimum and maximum value from samples across sites.
4 Bulk seed samples were collected for each line from each plot.
5 The sample of whole plant and pollen was up to 16 plants per line.

Table I.2. Levels[1] of Cry2Ab protein detected in leaf samples of transgenic cotton line 15985 and a control line at various sampling dates during the 1998 and 1999 growing seasons (USDA, 2000; OGTR, 2002; USEPA, 2003).

Days After Planting	15985		DP50B	
	1998	1999	1998	1999
28	21 ± 4.9	7.1 ± 1.6	Not Detected	Not Detected
55	40.1 ± 6.5	14.3 ± 5.3	Not Detected	Not Detected
85	19.7 ± 2.7	17.0 ± 1.2	Not Detected	Not Detected
108	16.7 ± 0.6	11.9 ± 2.9	Not Detected	Not Detected

1 Mean (µg/g fresh weight) ± standard deviation.

Summary Data for Maize

Table I.3. Summary of Cry2Ab2 protein levels in tissues from MON89034 (USDA, 2006; FSANZ, 2008).

Tissue Type	Growth State	Cry2Ab2 Mean (SD) [Range], n = 15	
		µg/g fresh wt.	µg/g dry wt.
Young leaf	2 – 4 Leaves	29 (6.8) [19 – 43]	180 (59) [94 – 270]
Pollen	Silking	0.34 (0.084) [0.21 – 0.47]	0.64 (0.091) [0.49 – 0.79]
Silk	Silking	8.2 (3.6) [3.3 – 16]	71 (35) [33 – 160]
Forage	Early dent	12 (4.0) [6.5 – 18]	38 (14) [15 – 55]
Forage root	Early dent	4.1 (1.4) [2.2 – 6.5]	21 (5.9) [14 – 33]
Grain	Maturity	1.1 (0.31) [0.67 – 1.8]	1.3 (0.36) [0.77 – 2.1]
Stover	After harvest	22 (3.6) [17 – 29]	62 (15) [46 – 97]
Senescent root	After harvest	5.3 (2.0) [2.4 – 9.1]	26 (8.8) [13 – 43]

Table I.4. Cry2Ab protein expression levels in tissues from MON89034 (µg/g dry wt.) (EFSA, 2008).

Year/Location	Grain	Forage	Pollen	Forage root	Stover
2004/Argentina	0.95	45	0.56	31	44
2005/USA	1.3	38	0.64	21	62

Table I.5. Cry2Ab protein expression levels in tissues from MON89034 (µg/g dry wt.) (CFIA, 2008).

Tissue	Cry2Ab
Leaves	130 – 180
Root	21 – 58
Whole Plant	38 – 130
Grain	1.3
Pollen	0.64

Table I.6. Cry2Ab2 protein expression levels in overseason tissues of MON89034 (USDA, 2006).

		Days After Planting (DAP)											
		21 – 29		28 – 43		41 – 53		56 – 68		100 – 120		130 – 160	
Tissue		µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW	µg/g DW	µg/g FW
Leaf	Mean (SD)	180 (59)	29 (6.8)	170 (34)	32 (5.3)	130 (34)	29 (5.4)	160 (44)	37 (12)	N/A	N/A	N/A	N/A
	Range	94 – 270	19 – 43	110 – 230	23 – 44	85 – 200	23 – 41	48 – 210	11 – 56	N/A	N/A	N/A	N/A
Whole Plant	Mean (SD)	130 (51)	13 (4.6)	79 (18)	7.5 (1.8)	40 (9.9)	4.2 (0.94)	39 (16)	5.9 (2.6)	38 (14)	12 (4.0)	62 (15)	22 (3.6)
	Range	52 – 230	5.2 – 21	45 – 110	4.0 – 9.7	22 – 61	2.4 – 5.8	5.0 – 67	0.7 – 11	15 – 55	6.5 – 18	46 – 97	17 - 29
Root	Mean (SD)	56 (17)	6.4 (1.6)	58 (18)	7.6 (4.2)	35 (17)	5.0 (2.7)	26 (7.7)	4.2 (1.2)	21 (5.9)	4.1 (1.4)	26 (8.8)	5.3 (2.0)
	Range	33 – 100	4.4 – 10	25 – 86	2.5 – 15	17 – 74	2.2 – 12	15 – 45	3.2 – 7.6	14 – 33	2.2 – 6.5	13 – 43	2.4 – 9.1

ANNEX II: SUMMARY OF CRY2Ab ECOTOXICITY DATA

Table II.1. Summary of toxicity testing of Cry2Ab (USDA, 2006; FSANZ, 2008).

Test Animal	Treatment	Results
CD-1 mice, 10 male and 10 female per treatment	Cry2Ab2, 2198 mg/kg total, administered by gavage, in 2 doses, 4 hours apart. Animals observed daily, body weights, and food consumption measured at days 0, 7, and 14.	No treatment-related mortality; no significant differences in body weight, cumulative body weight, or food consumption between the control groups and the Cry2Ab2-treated group. No treatment-related gross pathological findings were observed at necropsy on day 14.
CD-1 mice, 10 male and 10 female per treatment	Vehicle (2 mM carbonate-bicarbonate buffer, 2 mM reduced glutathione, pH 10.5), administered by gavage, in 2 doses, 4 hours apart. Animals observed daily, body weights, and food consumption measured at days 0, 7, and 14.	
CD-1 mice, 10 male and 10 female per treatment	Bovine serum albumen, 2442 mg/kg total, administered by gavage, in 2 doses, 4 hours apart. Animals observed daily, body weights, and food consumption measured at days 0, 7, and 14.	

Table II.2. Summary of guideline hazard tests for rffects of Cry2Ab (FSANZ, 2002).

Species	Study	Treatment	Results
Mouse	Acute Oral Toxicity Study (885.3050)	Diet contained 67, 359, and 1450 mg Cry2Ab2/kg body weight.	No significant adverse effects > 1450 mg/kg body weight
Northern bobwhite quail	Avian Testing (885.4050)	Up to 10% Cry2Ab2 cottonseed meal in diet, fed for 5 days.	NOEC = 100,000 ppm
Rat	90-day Feeding Study	Either 11% or 33% (w/w) of MON89034 grain in diet, fed for 90 days.	No test substance related deaths or health effects noted.
Channel catfish	Freshwater Fish Testing	Up to 20% Cry2Ab2 cottonseed meal in diet, fed for 8 weeks.	LC50 > 20% Cry2Ab2 cottonseed meal in diet
Earthworms	Earthworm Testing (850.6200)	Up to 330 mg Cry2Ab2/kg dry soil for 14 days.	14-day LC50 > 330 mg Cry2Ab2/kg dry soil
Honey Bee	Honey Bee Adult and Larval Testing (885.4380)	Diet fed to larvae contained up to 100 µg Cry2Ab2/mL. Diet fed to adults contained 68 µg Cry2Ab2/mL.	NOEC for larvae > 100 µg Cry2Ab2/mL. NOEC for adults > 68 µg Cry2Ab2/mL.
Green Lacewing	Dietary Toxicity Study with Green Lacewing Larvae (885.4340)		NOEC is > 1,100 ppm Cry2Ab2; LD50 > 4,500 ppm Cry2Ab2
Ladybird Beetle	Dietary Toxicity Study with the Ladybird Beetle (885.4340)		LC50 > 4,500 ppm Cry2Ab2
Collembola	Chronic Collembola Toxicity Study (885. 4340)	Diet contained up to 69.5 µg Cry2Ab2/g.	NOEC > 69.5 µg Cry2Ab2/g diet

Table II.3. No observed effect concentrations (NOECs) of Cry2Ab2 for non-target organisms (USDA, 2006).

Test Organism	**NOEC**
Collembola	70 µg/g
Earthworm	330 mg/kg dry soil
Honeybee larvae	100 µg/ml as a single dose
Honeybee adult	68 µg/ml
Minute pirate bugs	100 µg/g
Ladybird beetle	120 µg/g
Parasitic wasp	100 µg/ml
Mouse	2198 mg/kg
Quail	50% corn grain from MON 89034 in diet
Broiler	50% to 55% corn grain from MON 89034 in diet
Daphnia	100 mg/l pollen from MON 89034

Table II.4. Estimated margins of exposure (MOEs) to non-target arthropods for the Cry2Ab2 protein (USDA, 2006).

	Source	**MEEC[1]**	**NOEC**	**MOE[2]**
Collembola	Leaf	3.7 mg/kg dry soil[3]	≥ 70 µg/g	≥ 19
Earthworm	*B. t.*	3.7 mg/kg dry soil	≥ 330 mg/kg dry soil	≥ 89
Honeybee larvae	*B. t.*	0.47 µg/g fw (pollen level)	≥ 100 µg/ml as a single dose[4]	≥ 213
Minute pirate bugs	*E. coli*	0.47 µg/g fw (pollen level)	≥ 100 µg/g	≥ 213
Ladybird beetle	*E. coli*	0.47 µg/g fw (pollen level)	≥ 120 µg/g	≥ 255
Parasitic wasp	*E. coli*	0.47 µg/g fw (pollen level)	≥ 100 µg/ml	≥ 213

1 MEEC = Maximum Expected Environmental Concentration

2 Margins of Exposure (MOEs) were calculated based on the ratio of the NOEC to MEEC. The MOE was determined based on the expression level of the Cry2Ab2 protein in the tissue from MON89034 deemed most relevant to the NTO exposure.

3 The MEEC for Collembola and earthworm was calculated using the following parameter assumptions: 25,000 corn plants/acre; corn plant dry weight is 650 g/plant; the bulk density of soil is 1500 kg/cubic meter; soil depth is 0.15 m (about 6 inches); soil volume in a one-hectare 0.15 m layer is 1500 cubic meters. The Cry2Ab2 expression values were taken for leaves at the pre-tasseling stage and were 240 and 210 µg/g dwt, respectively.

4 The NOEC for the honeybee larval assay is based on the concentration of the dosing solution.

ANNEX III: SUMMARY OF COMPOSITIONAL ANALYSES OF GE PLANTS EXPRESSING CRY2Ab, INCLUDING ANALYSES OF TOXINS AND ANTI-NUTRIENTS

Summary Data for Cotton

Table III.1. Summary of compositional mean values for cottonseed oil samples (FSANZ, 2002).

Component (mg/100g)	**15985**	**DP50B**	**DP50**	**Codex**	**Commercial Range[1]**
Vitamin E	59.8	45.1	53.4		45.1-58.5
Fatty acid[2]					
Myristic (14:0)	1.32	0.980	1.06	0.4-2.0	0.923-1.45
Pentadecanoic	<0.100	<0.100	<0.100	-	<0.100
Palmitic (16:0)	23.9	25.2	25.3	17.0-31.0	22.7-26.3
Palmitoleic (16:1)	0.832	0.735	0.78	0.5-2.0	0.735-0.954
Heptadecanoic (17:0)	<0.100	<0.100	<0.100	-	<0.100
Stearic (18:0)	2.04	2.34	2.04	1.0-4.0	1.98-2.34
Oleic (18:1)	15.1	15.7	14.7	13.0-44.0	14.7-17.8
Linoleic (18:2)	55.6	53.7	54.9	33.0-59.0	51-54.9
Linolenic and gamma linoleic (18:3)	0.171	0.152	0.145	0.1-2.1	0.120-0.152
Arachidic (20:0)	0.176	0.244	0.178	<0.7	0.178-0.244
Behenic (22:0)	<0.100	0.103	<0.100	<0.5	<0.100-0.103
Lignoceric (24:0)	<0.100	<0.100	<0.100	<0.5	<0.100

1 Range includes data from three commercially available cotton varieties.
2 Fatty acid values expressed as a percentage total fatty acids.
3 Ranges adopted by the FAO/WHO Codex Alimentarius Committee on fats and oils.

Table III.2. Summary of proximate analysis of cottonseed[1] (USDA, 2000; FSANZ, 2002).

Component	**15985** Range	**DP50B** Range	**DP50** Range	**Non-transgenic Reference[2]**	**Commercial Reference[3]**
Protein	26.13 21.45-28.82	26.06 21.93-28.15	25.96 21.76-27.79	21.76-27.79	21.76-28.15
Fat	20.52 17.54-27.42	20.37 16.04-23.48	19.74 15.44-23.64	15.44-23.64	15.44-23.83
Ash	4.36 3.93-4.81	4.38 4.06-4.67	4.34 3.76-4.85	3.76-4.85	3.76-4.85
Fibre, crude	16.83 14.93-17.95	17.17 15.42-19.69	17.79 15.38-19.31	15.38-19.31	15.38-20.89
Carbohydrate	49.09 42.97-52.69	49.23 46.85-51.93	49.94 45.64-52.44	45.64-53.62	45.64-53.62
Calories	485.33 468.50-520.01	484.45 463.09-498.71	481.57 457.77-499.84	457.77-499.84	457.77-500.49
Moisture	5.99 4.34-7.59	6.05 4.22-7.28	6.03 3.97-7.26	3.97-7.49	3.97-8.47

1 All values (average and range) expressed as % dry weight except moisture which is % fresh weight.
2 Range includes data from four commercially available non-transgenic cotton varieties.
3 Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

Table III.3. Summary of fatty acid profiles (% of total) of cottonseed.[1] (USDA, 2000; FSANZ, 2002).

Fatty Acid (% of total fatty acids)	**15985** Range	**DP50B** Range	**DP50** Range	**Non-transgenic Reference[2]**	**Commercial Reference[3]**	**Codex Range**	**Literature Values[6]**
Mysistic[4, 5] (14:0)	**1.26** 0.88 – 2.94	**0.92** 0.74 – 1.91	**1.02** 0.77 – 2.15	0.77 – 2.40	0.64 – 2.40	0.4 – 2.0	0.68 – 1.16
Palmitic (16:0)	25.80 24.5 – 27.90	25.92 24.90 – 27.60	25.81 24.30 – 28.10	24.30 – 28.10	23.40 – 28.10	17.0 – 31.0	21.63 – 26.18
Palmitoleic[4] (16:1)	**0.56** 0.33 – 0.65	0.58 0.43 – 0.68	**0.63** 0.43 – 0.98	0.43 – 0.98	0.43 – 0.98	0.5 – 2.0	0.56 – 0.82
Stearic[4, 5] (18:0)	**2.63** 2.41 – 3.10	**2.38** 2.24 – 2.60	**2.30** 2.06 – 2.71	2.06 – 3.11	2.06 – 3.11	1.0 – 4.0	2.27 – 2.88
Oleic (18:1)	15.58 13.60 – 18.10	15.59 13.30 – 18.10	15.40 12.90 – 17.40	12.90 – 20.10	12.90 – 20.10	13.0 – 44.0	15.17 – 19.94
Linoleic[4, 5] (18:2)	**52.52** 47.70 – 55.50	**53.10** 49.00 – 55.80	**53.31** 49.50 – 57.10	46.00 – 57.10	46.00 – 57.10	33.0 – 59.0	49.07 – 57.64
Linolenic and gamma linoleic (18:3)	0.13 0.050 – 0.29	0.14 0.05 – 0.55	0.11 0.05 – 0.31	0.005 – 0.31	0.05 – 0.55	0.1 – 2.1	0.23
Arachidic[4] (20:0)	**0.30** 0.25 – 0.43	0.29 0.25 – 0.36	**0.27** 0.24 – 0.34	0.24 – 0.34	0.24 – 0.36	< 0.5	0.41
Behenic (22:0)	0.14 0.12 – 0.21	0.15 0.11 – 0.23	0.14 0.12 – 0.24	0.12 – 0.24	0.11 – 0.24	< 0.5	
Lignoceric (24:0)	0.14 0.05 – 0.26	0.12 0.05 – 0.26	0.14 0.05 – 0.29	0.05 – 0.29	0.05 – 0.29	< 0.5	

1 Average and range values given. Values represent samples taken from 8 U.S. field sites. Significant differences indicated in bold.
2 Range includes data from commercially available cotton varieties: DP50, DP51, DP20, and DP5409.
3 Range includes data from 10 commercially available transgenic and non-transgenic cotton varieties.
4 Statistically significant difference to DP50 control ($p \leq 0.05$).
5 Statistically significant difference to DP50B parent ($p \leq 0.05$).
6 Cherry and Leffler, 1984: Cherry, 1983. Phelps *et al.* 1965.

Table III.4. Amino acid levels in Cry2Ab2 cotton line 15985 that were significantly different from the controls[1] (USDA, 2000; FSANZ, 2002).

Amino Acid (% total AA)	**15985** Range	**DP50** Range	**Non-transgenic Reference[2]**	**Commercial Reference[3]**
Alanine	4.32 (4.20 – 4.48)	4.27 (4.15 – 4.41)	4.15 – 4.41	4.15 – 4.60
Cysteine	1.79 (1.68 – 2.03)	1.87 (1.67 – 1.99)	1.67 – 1.99	1.46 – 2.12
Isoleucine	3.58 (3.47 – 3.79)	3.53 (3.38 – 3.71)	3.38 – 3.71	3.38 – 3.78
Leucine	6.58 (6.45 – 6.86)	6.52 (6.43 – 6.65)	6.42 – 6.65	6.38 – 6.94
Valine	4.94 (4.77 – 5.34)	4.89 (4.72 – 5.22)	4.72 – 5.22	4.72 – 5.34

1 Levels of eighteen essential amino acids were measured; only the five amino acids listed here were present at levels significantly different from the control line DP50 ($p \leq 0.05$).
2 Range includes data from four commercially available non-transgenic cotton varieties.
3 Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

Table III.5. Mineral levels in Cry2Ab2 cotton line 15985 that were significantly different from the controls[1] (USDA, 2000; FSANZ, 2002).

Component	**15985** Range	**DP50** Range	**DP50B** Range	**Non-transgenic Reference[2]**	**Commercial Reference[3]**
Iron (mg/kg dw)	50.83 (43.92 – 57.56)	54.13 (42.57 – 72.15)	51.13 (41.84 – 60.76)	42.57 – 72.15	41.84 – 72.15
Phosphorous (% dw)	0.70 (0.58 – 0.83)	0.73 (0.63 – 0.86)	0.71 (0.61 – 0.88)	0.63 – 0.86	0.61 – 0.88

1 Levels of calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium, and zinc were measured; only the two minerals listed here were present at levels significantly different from the control line DP50 ($p \leq 0.05$).
2 Range includes data from four commercially available non-transgenic cotton varieties.
3 Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.

Table III.6. Summary of toxicant analyses of cottonseed oil and meal samples[1] (USDA, 2000; FSANZ, 2002).

	15985	**DP50B**	**DP50**	**Commercial Range[2]**	**Literature[3]**
Oil					
Free gossypol	< 0.005	< 0.005	< 0.005	< 0.005	< 0.01
Total gossypol	< 0.005	< 0.005	< 0.005	< 0.005	< 0.01
Cyclopropenoid fatty acid					
Malvalic (C-17)	0.378	0.384	0.377	0.294 – 0.405	0.22 – 1.44
Sterculic (C-18)	0.205	0.227	0.217	0.216 – 0.298	0.08 – 0.56
Dihydrosterculic (C-19)	0.165	0.169	0.146	0.146 – 0.202	—
Meal					
Free gossypol	0.037	0.042	0.041	0.025 – 0.068	—
Total gossypol	0.986	1.05	1.04	0.933 – 1.43	

1 Gossypol values expressed as % of fresh weight; cyclopropenoid fatty acid values as % of total fatty acids.
2 Range includes data from five commercially available cotton varieties.
3 Cherry and Leffler, 1984; Phelps *et al.* 1965.

Table III.7. Summary of toxicant analyses[1] (USDA, 2000; FSANZ, 2002).

Component	15985 Range	DP50 Range	DP50B Range	Reference Range[2]	Commercial Range[3]
Total gossypol	1.00 (0.79 – 1.29)	0.96 (0.72 – 1.23)	0.97 (0.78 – 1.24)	0.72 – 1.23	0.71 – 1.24
Cyclopropenoid fatty acids					
Malvalic (C-17)[4, 5]	0.45 (0.26 – 0.71)	0.39 (0.17 – 0.61)	0.39 (0.22 – 0.51)	0.17 – 0.61	0.17 – 0.61
Sterculic (C-18)[4, 5]	0.30 (0.21 – 0.58)	0.24 (0.13 – 0.43)	0.25 (0.16 – 0.44)	0.13 – 0.56	0.13 – 0.66
Dihydrosterculic (C-19)[4, 5]	0.18 (0.12 – 0.22)	0.16 (0.12 – 0.19)	0.15 (0.11 – 0.17)	0.12 – 0.22	0.11 – 0.22

1 Gossypol measured as % dry weight; cyclopropenoid fatty acids measures as % of total fatty acids.
2 Range includes data from four commercially available non-transgenic cotton varieties.
3 Range includes data from ten commercially available transgenic and non-transgenic cotton varieties.
4 These values from cotton event 15985 are significantly different from DP50 ($p \leq 0.005$).
5 These values from cotton event 15985 are significantly different from DP50B ($p \leq 0.005$).

Summary Data for Maize

Table III.8. Combined site compositional analysis of forage from MON89034 corn compared to a non-transgenic counterpart (USDA, 2006; FSANZ, 2008).

	MON89034	Control	Difference (MON89034 minus Control)			
Component[1]	**Mean ± S.E.[2]** (Range)	**Mean ± S.E.** (Range)	**Mean ± S.E.** (Range)	**95% CI** **Lower, Upper**	**p Value**	**Reference Range** (99% Tolerance Interval)
Acid detergent fibre	28.95 ± 1.69 (22.60 – 35.85)	27.26 ± 1.69 (19.93 – 35.59)	1.69 ± 1.18 (-6.22 – 10.45)	-0.81, 4.19	0.17	26.72 – 38.94 (16.76, 43.76)
Neutral detergent fibre	39.69 ± 1.32 (33.99 – 46.82)	37.60 ± 1.32 (31.44 – 43.96)	2.09 ± 1.40 (-3.47 – 7.47)	-0.88, 5.05	0.155	33.70 – 46.74 (25.94, 55.67)
Calcium	0.20 ± 0.019 (0.16 – 0.24)	0.19 ± 0.019 (0.13 – 0.28)	0.0066 ± 0.011 (-0.036 – 0.063)	-0.017, 0.031	0.569	0.11 – 0.29 (0.016, 0.38)
Phosphorus	0.25 ± 0.011 (0.22 – 0.32)	0.21 ± 0.011 (0.15 – 0.25)	0.040 ± 0.014 (-0.0019 – 0.13)	0.011, 0.069	0.010	0.14 – 0.25 (0.071, 0.32)
Ash	3.70 ± 0.27 (2.51 – 4.67)	3.90 ± 0.27 (2.59 – 5.10)	-0.20 ± 0.21 (-1.72 – 0.97)	-0.65, 0.25	0.356	3.40 – 5.45 (1.93, 6.31)
Carbohydrates	86.90 ± 0.43 (84.93 – 89.13)	86.69 ± 0.43 (84.36 – 89.57)	0.21 ± 0.53 (-4.23 – 4.41)	-0.91, 1.33	0.697	84.88 – 88.39 (83.05, 90.74)
Moisture	72.20 ± 1.35 (68.50 – 75.40)	71.53 ± 1.35 (65.90 – 76.80)	0.67 ± 0.52 (-3.50 – 4.20)	-0.44, 1.77	0.220	64.90 – 77.40 (57.62, 86.45)
Protein	7.82 ± 0.27 (6.34 – 8.98)	7.70 ± 0.27 (6.06 – 8.87)	0.13 ± 0.26 (-2.32 – 2.35)	-0.43, 0.68	0.635	6.58 – 8.82 (4.78, 10.38)
Total Fat	1.57 ± 0.24 (0.63 – 3.17)	1.71 ± 0.24 (0.77 – 2.91)	-0.13 ± 0.23 (-2.28 – 1.95)	-0.59, 0.32	0.558	0.58 – 3.11 (0, 4.54)

1 % dry weight, except for moisture
2 Abbreviations: S.E. = Standard Error; CI = Confidence Interval

Table III.9. Combined site amino acid analysis of grain for MON89034 corn compared to a non-transgenic counterpart (USDA, 2006; FSANZ, 2008).

	MON89034	Control	Difference (MON89034 minus Control)			
Amino Acid[1]	**Mean ± S.E.[2]** (Range)	**Mean ± S.E.** (Range)	**Mean ± S.E.** (Range)	**95% CI Lower, Upper**	**p Value**	**Reference Range** (99% Tolerance Interval)
Alanine	0.77 ± 0.039 (0.64 – 0.89)	0.78 ± 0.039 (0.67 – 0.89)	-0.0070 ± 0.019 (-0.13 – 0.089)	-0.046, 0.032	0.709	0.67 – 0.96 (0.48, 1.08)
Arginine	0.48 ± 0.013 (0.38 0 0.52)	0.47 ± 0.013 (0.41 – 0.51)	0.011 ± 0.012 (-0.090 – 0.062)	-0.014, 0.036	0.361	0.37 – 0.49 (0.33, 0.56)
Aspartic Acid	0.68 ± 0.029 (0.56 – 0.78)	0.67 ± 0.029 (0.60 – 0.76)	0.0038 ± 0.015 (-0.11 – 0.078)	-0.028, 0.036	0.804	0.57 – 0.77 (0.43, 0.90)
Cysteine	0.23 ± 0.0057 (0.20 – 0.26)	0.23 ± 0.0057 (0.21 – 0.25)	0.0023 ± 0.0038 (-0.022 – 0.023)	-0.0057, 0.010	0.554	0.20 – 0.24 (0.18, 0.27)
Glutamic Acid	1.97 ± 0.097 (1.63 – 2.29)	1.99 ± 0.097 (1.70 – 2.26)	-0.012 ± 0.049 (-0.33 – 0.24)	-0.11, 0.091	0.809	1.71 – 2.41 (1.25, 2.75)
Glycine	0.38 ± 0.0087 (0.32 – 0.41)	0.38 ± 0.0087 (0.36 – 0.41)	0.0042 ± 0.0071 (-0.067 – 0.035)	-0.011, 0.019	0.566	0.32 – 0.40 (0.28, 0.46)
Histidine	0.31 ± 0.011 (0.25 – 0.35)	0.31 ± 0.011 (0.28 – 0.34)	0.0027 ± 0.0055 (-0.050 – 0.030)	-0.0090, 0.014	0.632	0.26 – 0.33 (0.22, 0.38)
Isoleucine	0.36 ± 0.018 (0.30 – 0.43)	0.36 ± 0.018 (0.30 – 0.42)	-0.00003 ± 0.0088 (-0.056 – 0.041)	-0.019, 0.019	0.997	0.32 – 0.45 (0.23, 0.51)
Leucine	1.31 ± 0.077 (1.09 – 1.57)	1.32 ± 0.077 (1.08 – 1.55)	-0.014 ± 0.036 (-0.21 – 0.16)	-0.089, 0.062	0.700	1.14 – 1.68 (0.77, 1.92)
Lysine	0.33 ± 0.0097 (0.26 – 0.36)	0.32 ± 0.0097 (0.29 – 0.36)	0.0088 ± 0.0078 (-0.056 – 0.033)	-0.0077, 0.025	0.273	0.24 – 0.34 (0.20, 0.40)
Methionine	0.23 ± 0.0064 (0.20 – 0.27)	0.22 ± 0.0064 (0.20 – 0.24)	0.0038 ± 0.0047 (-0.017 – 0.028)	-0.0061, 0.014	0.427	0.17 – 0.22 (0.14, 0.25)
Phenylalanine	0.51 ± 0.028 (0.43 – 0.61)	0.52 ± 0.028 (0.43 – 0.60)	-0.0012 ± 0.013 (-0.080 – 0.067)	-0.029, 0.026	0.925	0.45 – 0.65 (0.32, 0.73)
Proline	0.93 ± 0.030 (0.79 – 1.05)	0.93 ± 0.030 (0.83 – 1.01)	0.0034 ± 0.019 (-0.15 – 0.10)	-0.037, 0.044	0.861	0.83 – 1.11 (0.68, 1.21)
Serine	0.52 ± 0.022 (0.44 – 0.61)	0.52 ± 0.022 (0.46 – 0.60)	-0.0046 ± 0.012 (-0.087 – 0.058)	-0.030, 0.021	0.703	0.45 – 0.62 (0.34, 0.71)
Threonine	0.056 ± 0.0018 (0.048 – 0.064)	0.056 ± 0.0018 (0.045 – 0.063)	0.00031 ± 0.0013 (-0.0055 – 0.0072)	-0.0025, 0.0031	0.817	0.043 – 0.059 (0.032, 0.072)
Tryptophan	0.37 ± 0.015 (0.22 – 0.43)	0.36 ± 0.015 (0.24 – 0.42)	0.0088 ± 0.016 (-0.21 – 0.14)	-0.026, 0.043	0.596	0.25 – 0.40 (0.17, 0.52)
Valine	0.49 ± 0.020 (0.40 – 0.55)	0.49 ± 0.020 (0.43 – 0.55)	0.0034 ± 0.010 (-0.084 – 0.055)	-0.019, 0.026	0.748	0.42 – 0.55 (0.35, 0.62)

1 % dry weight
2 Abbreviations: S.E. = Standard Error; CI = Confidence Interval

Table III.10. Combined site fatty acid analysis of grain from MON89034 corn compared to a non-transgenic counterpart (USDA, 2006; FSANZ, 2008).

Fatty Acid[1]	MON89034	Control	Difference (MON89034 minus Control)			
Component	**Mean ± S.E.[2]** (Range)	**Mean ± S.E.** (Range)	**Mean ± S.E.** (Range)	**95% CI Lower, Upper**	**p Value**	**Reference Range** (99% Tolerance Interval)
16:0 Palmitic	9.19 ± 0.060 (8.98 – 9.46)	9.12 ± 0.060 (8.91 – 9.34)	0.071 ± 0.049 (-0.14 – 0.33)	-0.034, 0.18	0.171	9.10 – 12.55 (6.12, 15.67)
16:1 Palmitoleic	0.13 ± 0.0058 (0.11 – 0.14)	0.12 ± 0.0058 (0.048 – 0.14)	0.0022 ± 0.0054 (-0.012 – 0.079)	-0.0093, 0.014	0.696	0.050 – 0.19 (0, 0.28)
18:0 Stearic	1.89 ± 0.021 (1.79 – 2.03)	1.82 ± 0.021 (1.76 – 1.87)	0.072 ± 0.021 (-0.055 – 0.18)	0.028, 0.12	0.002	1.57 – 2.45 (0.86, 2.98)
18:1 Oleic	24.96 ± 0.34 (23.38 – 25.75)	24.84 ± 0.34 (23.62 – 26.66)	0.12 ± 0.20 (-1.48, 1.15)	-0.32 – 0.55	0.574	21.17 – 35.33 (7.51, 46.46)
18:2 Linoleic	61.82 ± 0.40 (60.85 – 63.61)	62.07 ± 0.40 (60.51 – 63.41)	-0.25 ± 0.23 (-1.62 – 1.24)	-0.73, 0.24	0.292	50.33 – 63.59 (39.41, 76.74)
20:0 Arachidic	0.39 ± 0.0062 (0.36 – 0.42)	0.38 ± 0.0062 (0.36 – 0.40)	0.013 ± 0.0031 (-0.019, 0.032)	0.0063, 0.019	<0.001	0.32 – 0.47 (0.23, 0.54)
20:1 Eicosenoic	0.28 ± 0.0040 (0.26 – 0.29)	0.28 ± 0.0040 (0.25 – 0.29)	0 ± 0.0024 (-0.014 – 0.011)	-0.0051, 0.0051	0.999	0.23 – 0.32 (0.15, 0.39)
22:0 Behenic	0.16 ± 0.0050 (0.13 – 0.20)	0.15 ± 0.0050 (0.13 – 0.18)	0.0027 ± 0.0062 (-0.019 – 0.029)	-0.010, 0.016	0.665	0.12 – 0.19 (0.081, 0.23)

1 % total fatty acids
2 Abbreviations: S.E. = Standard Error; CI = Confidence Interval

Table III.11. Combined site mineral analysis of grain from MON89034 corn compared to a non-transgenic counterpart (FSANZ, 2008).

	MON89034	Control	Difference (MON89034 minus Control)			
Component	**Mean ± S.E.[1]** (Range)	**Mean ± S.E.** (Range)	**Mean ± S.E.** (Range)	**95% CI Lower, Upper**	**p Value**	**Reference Range** (99% Tolerance Interval)
Calcium (% DW)	0.0050 ± 0.00034 (0.0038 – 0.0066)	0.0049 ± 0.00034 (0.0040 – 0.0059)	0.00016 ± 0.00011 (-0.00027 – 0.00090)	-0.00008, 0.00040	0.180	0.0031 – 0.0049 (0.0016, 0.0059)
Copper (mg/kg DW)	1.74 ± 0.38 (1.33 – 2.38)	2.07 ± 0.37 (1.26 – 4.54)	-0.33 – 0.53 (-2.96 – 0.78)	-1.45, 0.79	0.547	1.15 – 3.56 (0, 4.20)
Iron (mg/kg DW)	21.40 ± 1.00 (19.23 – 25.23)	22.20 ± 0.99 (19.03 – 28.26)	-0.80 ± 0.67 (-6.50 – 5.90)	-2.22, 0.62	0.250	18.04 – 29.22 (8.88, 34.51)
Magnesium (% DW)	0.12 ± 0.0043 (0.10 – 0.14)	0.12 ± 0.0043 (0.11 – 0.14)	-0.00028 ± 0.0021 (-0.018 – 0.011)	-0.0047, 0.0041	0.893	0.099 – 0.14 (0.075, 0.17)
Manganese (mg/kg DW)	6.79 ± 0.29 (5.43 – 9.32)	6.51 ± 0.29 (5.57 – 8.00)	0.28 ± 0.21 (-1.54 – 2.36)	-0.18, 0.73	0.213	5.56 – 8.64 (3.17, 9.99)
Phosphorus (% DW)	0.33 ± 0.0095 (0.27 – 0.36)	0.33 ± 0.0095 (0.29 – 0.36)	0.00039 ± 0.0043 (-0.038 – 0.026)	-0.0087, 0.0095	0.929	0.25 – 0.37 (0.18, 0.45)
Potassium (% DW)	0.36 ± 0.0065 (0.32 – 0.40)	0.36 ± 0.0065 (0.34 – 0.40)	0.0032 ± 0.0042 (-0.030 – 0.035)	-0.0052, 0.012	0.450	0.32 – 0.40 (0.26, 0.46)
Zinc (mg/kg DW)	22.05 ± 1.14 (18.91 – 26.89)	21.91 ± 1.14 (18.81 – 26.04)	0.14 ± 0.51 (-3.37 – 3.19)	-0.94, 1.22	0.788	16.72 – 34.04 (7.16, 38.55)

1 Abbreviations: S.E. = Standard Error; CI = Confidence Interval; DW = Dry Weight

Table III.12. Combined site compositional analysis of forage from MON89034 corn compared to a non-transgenic counterpart (FSANZ, 2008).

	MON89034	Control	Difference (MON89034 minus Control)			
Component[1]	**Mean ± S.E.[2]** (Range)	**Mean ± S.E.** (Range)	**Mean ± S.E.** (Range)	**95% CI Lower, Upper**	**p Value**	**Reference Range (99% Tolerance Interval)**
Acid Detergent Fibre	5.48 ± 0.19 (3.82 – 7.24)	5.27 ± 0.19 (4.17 – 7.00)	0.21 ± 0.25 (-3.18 – 3.07)	-0.30, 0.72	0.410	4.11 – 6.33 (2.77, 7.56)
Neutral Detergent Fibre	10.06 ± 0.37 (8.59 – 12.08)	9.75 ± 0.37 (8.48 – 11.75)	0.31 ± 0.34 (-2.26 – 2.05)	-0.41, 1.03	0.370	8.20 – 11.30 (5.93, 13.63)
Total Dietary Fibre	15.17 ± 0.47 (13.39 – 17.02)	14.67 ± 0.47 (12.82 – 17.62)	0.50 ± 0.54 (-3.61 – 4.20)	-0.66, 1.65	0.375	12.99 – 18.03 (9.20, 20.27)
Ash	1.41 ± 0.036 (1.25 – 1.56)	1.39 ± 0.036 (1.28 – 1.51)	0.014 ± 0.041 (-0.11 – 0.13)	-0.072, 0.10	0.734	1.12 – 1.62 (0.74, 1.96)
Carbohydrates	84.85 ± 0.42 (83.29 – 86.52)	84.96 ± 0.42 (83.58 – 86.22)	-0.11 ± 0.18 (-1.42 – 0.84)	-0.50, 0.28	0.562	82.91 – 86.78 (81.08, 88.80)
Moisture	9.52 ± 0.77 (7.89 – 12.80)	9.50 ± 0.77 (7.86 – 13.10)	0.021 ± 0.22 (-1.00 – 0.87)	-0.44, 0.48	0.923	7.60 – 15.30 (0.45, 19.52)
Protein	10.43 ± 0.42 (8.54 – 11.98)	10.36 ± 0.42 (9.22 – 11.52)	0.070 ± 0.19 (-1.26 – 1.28)	-0.34, 0.48	0.725	9.33 – 11.82 (7.54, 13.13)
Total Fat	3.32 ± 0.069 (3.05 – 3.89)	3.29 ± 0.069 (3.05 – 3.75)	0.025 ± 0.089 (-0.50 – 0.29)	-0.16, 0.21	0.784	2.66 – 3.71 (2.20, 4.55)

1 % dry weight, except for moisture
2 Abbreviations: S.E. = Standard Error; CI = Confidence Interval

Table III.13. Combined site vitamin analysis of grain from MON89034 corn compared to a non-transgenic counterpart (FSANZ, 2008).

	MON89034	Control	Difference (MON89034 minus Control)			
Component[1]	**Mean ± S.E.[2]** (Range)	**Mean ± S.E.** (Range)	**Mean ± S.E.** (Range)	**95% CI Lower, Upper**	**p Value**	**Reference Range** (99% Tolerance Interval)
Folic Acid	0.35 ± 0.037 (0.26 – 0.48)	0.36 ± 0.037 (0.23 – 0.53)	-0.0080 ± 0.022 (-0.11 – 0.11)	-0.054, 0.038	0.717	0.13 – 0.45 (0.012, 0.69)
Niacin	30.08 ± 1.11 (25.72 – 34.84)	29.59 ± 1.11 (24.93 – 35.75)	0.48 ± 0.65 (-4.44 – 5.64)	-0.82, 1.79	0.461	16.17 – 29.19 (6.97, 37.83)
Vitamin B1	3.07 ± 0.13 (2.39 – 3.44)	2.94 ± 0.13 (2.39 – 3.36)	0.13 ± 0.17 (-0.66 – 0.68)	-0.24, 0.49	0.474	2.19 – 5.60 (0.37, 6.35)
Vitamin B2	1.42 ± 0.046 (1.24 – 1.65)	1.42 ± 0.046 (1.16 – 1.61)	0.0015 ± 0.050 (-0.30 – 0.45)	-0.099, 0.10	0.976	1.34 – 1.91 (0.91, 2.30)
Vitamin B6	6.22 ± 0.23 (5.28 – 6.99)	6.26 ± 0.23 (5.37 – 6.80)	-0.036 ± 0.18 (-0.72 – 1.10)	-0.41, 0.34	0.838	5.08 – 7.47 (3.12, 9.30)
Vitamin E	6.77 ± 0.42 (5.55 – 8.62)	6.63 ± 0.42 (2.72 – 9.02)	0.14 ± 0.36 (-2.35, -3.83)	-0.64, 0.91	0.714	2.71 – 13.94 (0, 20.49)

1 mg/kg dry weight, except for moisture
2 Abbreviations: S.E. = Standard Error; CI = Confidence Interval

Table III.14. Combined site anti-nutrient and secondary metabolite analysis of grain from MON89034 corn compared to a non-transgenic counterpart (FSANZ, 2008).

	MON89034	**Control**	**Difference (MON89034 minus Control)**			
Component	**Mean ± S.E.**[1] (Range)	**Mean ± S.E.** (Range)	**Mean ± S.E.** (Range)	**95% CI Lower, Upper**	**p Value**	**Reference Range** (99% Tolerance Interval)
Phytic Acid % DW	0.75 ± 0.050 (0.53 – 0.87)	0.73 ± 0.050 (0.56 – 0.88)	0.016 ± 0.027 (-0.15 – 0.18)	-0.037, 0.069	0.537	0.50 – 0.94 (0.21, 1.22)
Ferulic Acid µg/g DW	2131.38 ± 108.09 (1790.25 – 2525.31)	2148.05 ± 108.09 (1878.66 – 2669.85)	-16.67 ± 50.08 (-330.17 – 264.79)	-116.98, 83.65	0.740	1412.68 – 2297.36 (1136.69, 2806.24)
p-Coumaric Acid µg/g DW	194.25 ± 7.12 (166.11 – 253.04)	183.96 ± 7.12 (167.76 – 210.13)	10.28 ± 7.08 (-24.37 – 70.84)	-4.73, 25.30	0.165	99.30 – 285.75 (0, 378.57)

1 Abbreviations: S.E. = Standard Error; CI = Confidence Interval; DW = Dry Weight

Table III.15. Summary of the statistically significant differences between MON89034 maize and a non-transgenic counterpart (USDA, 2006; FSANZ, 2008).

	MON89034p	Control	Difference (MON89034 minus Control)			
Component (Units)[1]	**Mean**	**Mean**	**% of Control**	**p Value**	**MON89034 Range**	**Commercial Tolerance Interval**
Combined Site						
Forage Phosphorus (% DW)	0.25	0.21	19.24	0.010	0.22 – 0.32	0.071, 0.32
Grain 18:0 Stearic (% Total FA)	1.89	1.82	3.97	0.002	1.79 – 2.03	0.86, 2.98
Grain 20:0 Arachidic (% Total FA)	0.39	0.38	3.43	<0.001	0.36 – 0.42	0.23, 0.54
More Than One Site						
Site IA Grain Carbohydrates (% DW)	83.38	84.52	-1.34	0.008	83.29 – 83.55	81.08, 88.80
Site OH Grain Carbohydrates (% DW)	84.26	83.80	0.55	0.009	83.99 – 84.59	81.08, 88.80
Site IL-1 Grain Copper (mg/kg DW)	1.76	1.36	29.35	0.023	1.51 – 2.21	0, 4.20
Site NE Grain Copper (mg/kg DW)	2.15	1.67	28.66	0.023	1.92 – 2.38	0, 4.20
Site IL-1 Grain Iron (mg/kg DW)	20.86	19.48	7.11	0.048	19.23 – 21.79	8.88, 34.51
Site OH Grain Iron (mg/kg DW)	21.37	25.74	-17.00	0.006	20.59 – 21.76	8.88, 34.51
Site IL-1 Grain 18:0 Stearic (% Total FA)	1.96	1.82	7.94	<0.001	1.89 – 2.02	0.86, 2.98
Site IL-2 Grain 18:0 Stearic (% Total FA)	1.98	1.82	9.05	<0.001	1.93 – 2.03	0.86, 2.98
Site IL-1 Grain Arachidic (% Total FA)	0.41	0.39	5.23	0.007	0.40 – 0.42	0.23, 0.54
Site IL-2 Grain 20:0 Arachidic (% Total FA)	0.39	0.37	6.83	0.021	0.38 – 0.40	0.23, 0.54
Site OH Grain 20:0 Arachidic (% Total FA)	0.38	0.37	3.12	0.035	0.38 – 0.39	0.23, 0.54
One Site Only						
Site IA Grain Alanine (% DW)	0.88	0.81	7.83	0.030	0.87 – 0.88	0.48, 1.08
Site IA Grain Arginine (% DW)	0.51	0.46	10.83	0.005	0.50 – 0.52	0.33, 0.56
Site IA Grain Aspartic Acid (% DW)	0.77	0.71	8.66	0.003	0.77 – 0.78	0.43, 0.90
Site IA Grain Cysteine (% DW)	0.25	0.23	7.54	0.014	0.24 – 0.26	0.18, 0.27
Site IA Grain Glutamic acid (% DW)	2.27	2.09	8.66	0.011	2.26 – 2.28	1.25, 2.75
Site IA Grain Glycine (% DW)	0.41	0.38	6.94	0.020	0.40 – 0.41	0.28, 0.46
Site IA Grain Histidine (% DW)	0.34	0.32	7.16	0.022	0.34 – 0.34	0.22, 0.38
Site IA Grain Leucine (% DW)	1.49	1.37	8.96	0.032	1.48 – 1.51	0.77, 1.92
Site IA Grain Lysine (% DW)	0.35	0.32	6.66	0.028	0.33 – 0.36	0.20, 0.40
Site IA Grain Methionine (% DW)	0.25	0.23	11.20	0.003	0.25 – 0.27	0.14, 0.25
Site IA Grain Phenylalanine (% DW)	0.58	0.53	9.45	0.028	0.57 – 0.59	0.32, 0.73
Site IA Grain Proline (% DW)	1.05	0.98	7.29	0.028	1.04 – 1.05	0.68, 1.21
Site IA Grain Serine (% DW)	0.60	0.56	8.28	0.004	0.60 – 0.61	0.34, 0.71
Site IA Grain Threonine (% DW)	0.37	0.34	8.45	0.004	0.37 – 0.37	0.24, 0.41
Site IA Grain Tyrosine (% DW)	0.43	0.36	17.50	0.006	0.42 – 0.43	0.17, 0.52
Site IA Grain Protein (% DW)	11.89	10.85	9.59	0.005	11.73 – 11.98	7.54, 13.3
Site IL-1 Forage Moisture (% FW)	69.03	66.53	3.76	0.031	68.50 – 69.40	57.62, 86.45
Site NE Forage Ash (% DW)	3.20	4.39	-27.12	0.021	2.93 – 3.38	1.93, 6.31
Site NE Forage Carbohydrates (% DW)	88.16	84.98	3.74	0.004	86.86 – 88.84	83.05, 90.74
Site NE Grain Neutral Detergent Fibre (% DW)	10.52	9.05	16.27	0.028	10.43 – 10.69	5.93, 13.63
Site OH Forage Acid Detergent Fibre (% DW)	31.31	23.58	32.78	0.012	26.92 – 46.82	16.76, 43.76
Site OH Forage Neutral Detergent Fibre (% DW)	43.21	37.87	14.11	0.027	40.07 – 46.82	25.94, 55.67
Site IA Grain 18:3 Linolenic (% Total FA)	1.21	1.34	-9.40	0.009	1.20 – 1.23	0.63, 1.77
Site IL-1 Grain 16:1 Palmitoleic (% Total FA)	0.13	0.14	-6.87	0.012	0.12 – 0.13	0, 0.28
Site IL-2 Grain 18:1 Oleic (% Total FA)	24.75	23.82	3.93	0.003	24.14 – 25.25	7.51, 46.46
Site IL-2 Grain 18:2 Linoleic (% Total FA)	61.87	63.17	-2.07	0.001	61.19 – 62.42	39.41, 76.74
Site NE Grain 20:1 Eicosenoic (% Total FA)	0.28	0.29	-1.50	0.030	0.28 – 0.28	0.15, 0.39
Site IA Grain Calcium (% DW)	0.0064	0.0058	10.96	0.012	0.0062 – 0.0066	0.0016, 0.0059
Site IA Grain Manganese (mg/kg DW)	8.34	6.99	19.32	0.017	7.62 – 9.32	3.17, 9.99
Site IA Forage Calcium (% DW)	0.24	0.26	-8.77	0.033	0.24 – 0.24	0.016, 0.38
Site NE Forage Phosphorus (% DW)	0.25	0.17	46.95	0.036	0.23 – 0.28	0.071, 0.32
Site IL-2 Grain Folic Acid (mg/kg DW)	0.37	0.32	13.81	<0.001	0.35 – 0.38	0.012, 0.69
Site OH Grain p-Coumaric Acid (µg/g DW)	218.38	185.63	17.64	0.032	187.79 – 253.04	0, 378.57

1 Abbreviations: DW = Dry Weight; FW = Fresh Weight; FA = Fatty Acids