ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

REQUEST FOR AN ARTICLE 5 OPINION ON THE SUBSTANTIAL EQUIVALENCE OF COTTONSEED OIL AND FOOD INGREDIENTS DERIVED FROM INSECT PROTECTED COTTONSEED

Company	**Monsanto Europe S.A.**
Product	**Cottonseed oil**
Application	**Substantial equivalence**
Modification	**IPC line 531 – Insect protected** (Bt)
EC guidelines category	**3.1** (the host plant used for the genetic modification has a history of use as a source of food ingredients)

BACKGROUND

1. In June 1997, the UK Competent Authority received a request from Monsanto Europe SA for a scientific opinion on the substantial equivalence as regard their composition, nutritional value, metabolism, intended use and the level of undesirable substances contained therein of food and food products derived from Insect Protected Cottonseed, under article 5 of the Novel Foods Regulation (EC 258/97).

2. Monsanto originally requested an opinion on two products derived from insect protected cottonseed: oil and linters. In November 1997, the Company provided information that linters are processed to the food additives carboxymethyl cellulose (E466) and methylcellulose (E461). Since additives are exempt from the Novel Foods Regulation, the Committee confirmed that consideration need only be given to the oil derived from this line. None of the aspects of the dossier that apply directly to cottonseed linters have been considered in this opinion.

3. The approach taken by the Company in their supporting dossier, was to fully describe the genetic modification event, to demonstrate that the composition of the cottonseed from the modified line was comparable to seed from conventional line and to provide further evidence on the composition of the oil derived from the modified seed, including evidence to demonstrate the absence of DNA and proteins in the refined oil. The premise of this approach was that if the seed was shown to be comparable, then derived oil would also be comparable.

DESCRIPTION OF THE GM LINE

The Host Plant

4. Genetic material was stably inserted into the genome of the host Coker line 312 cultivar of cotton (*G. hirsutum* L.). The host is a commercial breeding line that has been grown in the United States for over 10 years and has a history of safe use in foods for human consumption, including oil derived from cottonseed.

5. The genetically modified line is comparable to the parental variety in morphology and agronomic performance, except for the genes and proteins introduced to the plant for protection against damage from lepidopteran insects, conferred by the introduced CryIA(c) protein.

The Introduced trait

6. Insect-Protected line 531 was modified by the addition of the *cryIA(c)* gene from the common soil bacterium *Bacillus thuringiensis* subsp. *Kurstaki* (*B.t.k.*). The modified plants produce the CryIA(c) protein, which is insecticidal to specific lepidopteran target pests. The genetically modified line also contains two antibiotic resistance marker genes: *nptII* and *aad.*

The Transformation System

7. The T-DNA, which includes the *cryIA(c), nptII* and *aad* genes, was stably transferred into the genome of cotton using *Agrobacterium tumefaciens* mediated transformation. The use of A*grobacteria* in transformation ensures that only T-DNA is integrated in the plant genome and the border sequence, (which contains the necessary genetic elements for transfer) is not. Therefore, once integrated, the insert is no longer functional as a T-DNA, and cannot be remobilised into the genome of another plant.

8. After the plant transformation, residual Agrobacterium cells were killed using specific antibiotics.

Plasmid Vector

9. IPC line 531 was transformed using the single border binary transformation vector, PV-GHBK04. The vector contains well-characterised DNA segments required for selection and replication of the plasmid, as well as a right border for initiating the region of T-DNA, integrated into the plant genome.

10. The Company has provided a list of all genetic elements contained in this vector, together with their sizes and functions. These are described below.

Nature, Function and Expression of Inserted Genes

<u>The modified *cryIA(c)* gene</u>

11. The *cryIA(c)* gene used in this transformation was constructed by combining the first 1398 nucleotides of the *cryIA(b)* gene with nucleotides number 1399 – 3534 of the naturally occurring *cryIA(c)* gene. With the exception of 6 amino acids, the region derived from the CryIA(b) protein is identical to the analogous region of the CryIA(c) protein. The modified *cryIA(c)* gene encodes for a protein that is identical to the CryIA(c) protein found in nature, with the exception of one amino acid at position 766. This discrepancy was unintentional. However, the amino acid at this position is degraded upon exposure to trypsin, or the proteases found within the insect gut, and so will not affect the host range or the active portion of the protein.

Antibiotic selection

12. IPC line 531 contains *nptII* gene under the control of the constitutive CaMV 35s promoter and the *aad* gene, which is driven by its own bacterial promoter and, as such, is not expressed in the modified cotton line.

Characterisation of the Insertion Event

13. Southern blot analyses were conducted to characterise the inserted T-DNA in terms of insert number (number of integration events), copy number (number of T-DNA copies at a particular genetic locus) and insert integrity (gene size, composition and linkage). The characterisations were carried out on genomic DNA isolated from leaf tissue from both the modified and parental (control) lines.

Insert Number

14. Molecular characterisation of Bollgard cotton event 531 demonstrated there are two T-DNA inserts. The primary functional insert contains single copies of the full-length cry1Ac gene, the nptII gene and the aad antibiotic resistance gene. This T-DNA insert also contains an 892 bp portion of the 3' end of the cry1Ac gene fused to the 7S 3' transcriptional termination sequence. This segment of DNA is at the 5' end of the insert, is contiguous and in the reverse orientation with the full-length cry1Ac gene cassette and does not contain a promoter. The second T-DNA insert contains 242 bp of a portion of the 7S 3' polyadenylation sequence from the terminus of the cry1Ac gene and is not functionally active in the plant genome.

Insert Integrity and Copy Number

15. Restriction digest analysis demonstrated that the larger insert containing an intact fragment containing the *cryIA(c)* and *nptII* genes has been integrated into the cotton genome. This T-DNA copy is no larger than 8.2 Kb, and so maximally contains the *cryIA(c), nptII* and *aad* genes, as well as part of the *oriV* site.

16. Further restriction analysis indicates that this insert also contains a second, but smaller T-DNA derived copy (1.7kb, which hybridised to the *cry1A(c)* gene probe, but not to the *nptII* gene probe. Since the origin of transfer typically initiates from the right border, this fragment contains 0.44kb of 7S 3' termination sequence, and 0.89kb of the 3' end of the *cry1A(c)* gene. This portion of the *cryIA(c)* gene is not insecticidally active and so will have no impact on the action of the introduced trait.

17. The results from these analyses established that the two T-DNA copies are contiguous to each other, and in a tail-to-tail arrangement.

18. The second insert has been fully characterised and has been shown to be 242bp in length. It contains only a portion of the transcriptional termination sequence from the *cry1Ac* gene and is functionally inactive in the plant genome.

Genetic Stability of IPC line 531

Segregation

19. The *cryIA(c)* gene segregated in a manner consistent with a single intact gene insertion that is stably transferred with crossing. The selfed data from crosses further demonstrates the stability of the insert during transfer over subsequent generations, with the structural and local maintenance of the inserted genes being conserved over four back crossed generations of derivatives of IPC line 531 in several elite cultivar lines.

20. Segregation data for the R1 plants (selfed progeny of the initial transformant - referred to as RO) and the progeny of the R1 plants is presented. These data demonstrate that a single active copy of the *cryIA(c)* gene has been inserted in IPC line 531.

Stability of the insert

21. Stability of the T-DNA insertions in Bollgard cotton event 531 was determined by analysing the R5 and R6 generations, as well as two commercial cotton lines containing Bollgard cotton event 531 by Southern blot analysis. The results from these experiments indicated that the functional insert was present in all the generations of Bollgard cotton event 531 that were analysed. However, the 242 bp T-DNA segment containing a portion of the 7S 3' genetic element was detected in the R5 and R6 inbred generations, but was not detected by Southern blot analysis in the two commercial lines containing Bollgard cotton event 531 that were tested. A likely explanation for the absence of the 242 bp insertion in the commercial lines is that it segregates independently of the functional insertion since the commercial lines were derived from the original Bollgard cotton event 531 through traditional breeding methods.

Stability of expression

Stability of gene expression

22. Data presented on the levels of introduced CryIA(c) and NPTII proteins demonstrate that production in leaves and seed are comparable in 1992 and 1993 (and 1994 for CryIA(c) only). When IPC line 531 was backcrossed, no significant differences, within the limits of the assay, were observed between protein levels. This demonstrates that the levels of expression of the introduced *cryIA(c)* and *nptII* genes are consistent from one generation to the next.

Stability of phenotypic expression

23. IPC line 531 has expressed the introduced trait of insect-resistance in field conditions since it was first planted in trials in 1990. Subsequent generations further demonstrated the stable maintenance of this trait in plant propagation, breeding in field trials under differing environmental conditions and on a commercial scale in 1996, in the US and Australia. This demonstrates stable maintenance of the insect-protected phenotype over 6 years.

EFFECT OF PRODUCTION PROCESS ON NOVEL FOOD

24. A description of the typical production process for cottonseed oil was provided and it is intended that oil from the IPC line 531 will be processed in the same way. In order to eliminate naturally occurring toxicants present in conventional cotton, the oil undergoes extensive processing during production.

25. The Committee sought further information regarding the processing conditions to ensure that they will eliminate protein and DNA from the refined oil. The Company provided a reference with a more detailed description of the production methodology of the oil. This information satisfied the Committee's concerns.

COMPOSITION OF THE COTTONSEED OIL

26. The Committee sought further assurance regarding the sensitivity of the original protocol to detect DNA and protein. At this request, the Company revised the study protocol for DNA extraction from refined cottonseed oil. The new method demonstrated a limit of DNA detection of approximately 100pg (equivalent to 1ng/100ml control oil spiked with genomic cotton DNA). Refined oil from IPC line 531 was analysed using this method, and no DNA was detected. The limit of detection for the protein assay was determined as being 0.082µg/ml of oil.

27. The refined cottonseed oil from IPC line 531 and Coker 312 was found to be comparable in quality to commercially processed cottonseed oil from non-GM sources. These results indicate that the levels of the important fatty acids, as well as the toxic cyclopropenoid fatty acids, are comparable in refined cottonseed oil fractions produced from IPC line 531 and the control line. There was no detectable gossypol in refined oil, and levels of alpha tocopherols were also comparable in both lines and were within the range published for other commercial varieties.

28. The insertion of the genes to provide insect resistance did not alter the processing characteristics of the cottonseed or the quality of refined oil.

29. A further field study carried out in 1998, also demonstrated that oil derived from Bollgard™ (commercial line of insect-protected cotton) was compositionally equivalent to that derived from the non-genetically modified control line, and three other commercial lines.

COMPOSITION OF THE COTTONSEED

30. The Company argues that the composition of the modified seed, in terms of protein, oil, carbohydrate, moisture, ash and calories, is comparable to the parental, non-GM variety, and, as such, the resulting oil will also be comparable.

31. Extensive compositional analyses were performed on cottonseed from Insect-Protected cotton line 531 grown in both 1992 and 1993. Field sites were selected in major cotton growing regions of the US, under a variety of environmental conditions and insect pressures from agronomically important insect pests. Both test and control lines were grown under identical conditions at each location, with the agronomic practices and conditions being monitored and recorded.

32. The level of the major components was shown to be compositionally equivalent between the two lines over the two field trials. All values obtained for these components were within published ranges.

CONCLUSION

32. The Committee was satisfied that

- line IPC 531 has been well characterised regarding the genetic modification event;
- the T-DNA has been stably inserted and;
- the composition of oil derived from line IPC 531 is comparable in terms of protein, oil, carbohydrate, moisture, ash and calories to the parental line and to commercial varieties, and that there was no novel genetic material present in the refined oil.

33. The Committee is therefore of the opinion that oil derived from insect protected cottonseed line IPC 531 is substantially equivalent to oil from conventional cottonseed lines, in terms of composition, nutritional value, metabolism, intended use and level of undesirable substances.