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 ROUNDUP® READY COTTON

Company	**Monsanto Europe S.A.**
Product	**Cottonseed oil**
Application	**Substantial equivalence**
Modification	**RRC 1445 – Herbicide tolerant** (herbicide Roundup ®- active ingredient glyphosate)
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 Roundup Ready Cottonseed, under article 5 of the Novel Foods Regulation (EC 258/97).

2. Monsanto originally requested an opinion on two products derived from cottonseed: oil and linters. In November 1997, the Company provided information that linters are processed to the food additives carboxy methylcellulose (E 466) and methylcellulose (E461). Since additives are exempt from the Novel Food 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 modified line is comparable to the parental variety in morphology, disease- and pest-resistance and agronomic performance, except for the genes and proteins that were introduced to the plant and the tolerance to Round-up® herbicide, conferred by the introduced CP4 EPSPS protein.

The Introduced trait

6. RRC line 1445 was modified by the addition of the *cp4 epsps* gene from the common soil bacterium *Agrobacterium* subsp. *CP4*. The modified plants produce the 5-enolpyruvylshikimate-3-phosphate synthase protein (CP4 EPSPS), the presence of which confers resistance to Roundup® herbicide. The genetically modified line also contains two antibiotic resistance marker genes: *nptII and aad.*

The Transformation System

7. The T-DNA, which includes the *cp4 epsps, nptII* and *aad* genes, was stably transferred into the genome of cotton using *Agrobacterium tumefaciens* mediated transformation. The use of A*grobacterium* 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. RRC line 1445 was transformed using the single border binary transformation vector, PV-GHGT07. 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 in to the plant genome. The plasmid also contains a *gox* gene, which encodes for glyphosate oxidoreductase. This was not transferred into RRC line 1445.

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

The *cp4 epsps* gene

11. This gene confers resistance of the herbicide Roundup® to the modified cotton plants and is under the control of the constitutive promoter CmoVb (isolated from the cauliflower family of viruses, specifically the figwort mosaic virus). The gene is derived from *Agrobacterium* subsp. *CP4*. A synthetic version of this gene, with plant-preferred sequences, was used in the production of RRC line 1445. The activity of the synthetic CP4 EPSPS protein was compared with that from the native *cp4 epsps* gene and was found to be identical.

12. The region that codes for the chloroplast transit peptide from the native CP4 EPSPS is fused to the region of DNA that codes for the synthetic protein. This enables the protein to be targeted to the chloroplast where aromatic amino acid biosynthesis takes place.

Antibiotic selection

13. RRC line 1445 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

14. 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.

15. Analyses of RRC line 1445 were carried out in parallel with another, non-commercial RRC line, 1698, which was transformed with the plasmid PV-GHGT06, a derivative of PV-GHGT07. PV-GHGT06 lacks the *gox gene cassette,* which is contained in PV-GHGT07. In some of the Southern blot experiments, PV-GHGT06 was used as a probe or a control, rather than plasmid PV-GHGT07. This is not likely to have any bearing on the outcome of these experiments.

Insert Number

16. One T-DNA insert was transferred into the genome of RRC line 1445, which is not present in the genome of the parental line. Data on this section indicates that RRC line 1445 has a single locus containing DNA from PV-GHGT07. Segregation data provided also supports this conclusion.

Insert Integrity and Copy Number

17. Southern blot analysis confirms the presence of the *cp4 epsps* gene and the CMo Vb promoter in the PV-GHGT07 plasmid and digested genomic DNA from RRC line 1445.

18. The presence of the *nptII* and *aad* genes in the genomic DNA of RRC line 1445 was also demonstrated, with no hybridising bands being seen in the genomic DNA from the parental, demonstrating the absence of these two genes in the control line.

19. Southern analysis indicates that the *ori-V* sequence was partially truncated before or during the insertion event.

20. A defined band of the expected size of the *gox* gene was seen in the digested plasmid PV-GHGT07. The absence of this band in the genomic RRC line 1445 DNA demonstrates that this gene was not integrated into the genome of the cotton plant when it was transformed to produce the RRC line 1445. It was also absent from the DNA of Coker 312, control line.

Genetic Stability of RRC line 1445

Segregation

21. Segregation ratios observed in R1 plants (selfed progeny from the initial transformant) supports the conclusion that the inserted T-DNA segregates as a single Mendelian locus. The Southern blot analysis demonstrates that the T-DNA was stably maintained for three generations.

Stability of the insert

22. For RRC line 1445, Southern blot analysis shows that the *cp4 epsps* gene is stably maintained from the R3 through to the R5 generations. The *nptII* gene is also shown to be stably maintained for three generations.

Stability of expression

Stability of gene expression

23. Data on levels of introduced CP4 EPSPS and NPTII proteins demonstrate that that production in leaves and seed are comparable in 1993 and 1994. The results from these analyses indicate that levels of expression of the introduced *cp4 epsps* and *nptII* genes are consistent from one generation to the next.

Stability of phenotypic expression

24. RRC line 1445 has exhibited consistent levels of Roundup-tolerance in field conditions since it was first tested in trials in 1991. The trait has been stably maintained through subsequent generations of plant propagation

and breeding in different genetic backgrounds and under different environmental conditions. This was confirmed on a commercial scale in 1997 in the US. This demonstrates stable maintenance of the phenotype under different field conditions over 5 years.

EFFECT OF PRODUCTION PROCESS ON NOVEL FOOD

25. A description of the typical production process for cottonseed oil was provided and it is intended that oil from the RRC cotton line 1445 will be processed in the same way. In order to eliminate naturally occurring toxicants in cotton, the oil undergoes extensive processing during production.

26. 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

27. 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 detection of approximately 100pg of DNA (equivalent to 1ng/100ml control oil spiked with genomic cotton DNA). Refined oil from RRC line 1445 was analysed using this method, and no DNA was detected.

28. The refined cottonseed oil from RRC line 1445 and Coker 312 was found to be comparable in quality to commercially processed cottonseed oil from non-GM sources. These data 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 RRC line 1445 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.

29. The insertion of the genes to provide herbicide tolerance did not alter the processing characteristics of the cottonseed or the quality of refined oil.

30. At the further request by the Committee, a report was submitted in August 2001 regarding the detectability of amino acids in refined oil from RRC line 1445. The results demonstrated that there is no detectable protein in refined oil at the limit of detection, which is 0.082µg/ml oil, and that the processing of oil removes protein to non-detectable levels. The Committee was content with these data.

COMPOSITION OF THE COTTONSEED

31. 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.

32. Extensive compositional analyses were performed on cottonseed from Roundup® Ready cotton line 1445 and the parental line, from field trials carried out in 1993 and 1994 at 6 field sites across the US cotton belt.

33. Although there were clear fluctuations between the control and test lines over the two years, observed differences still fell within the limits of previously reported ranges found in the literature for refined cottonseed oil. It was also demonstrated that the introduction of the EPSPS protein, which catalyses a step in the aromatic amino acid biosynthetic pathway, and the application of Roundup® herbicide on the modified line had no effect on the amino acid profile of the seeds.

34. The values obtained for the major toxicants were shown to be statistically equivalent between the two lines throughout the field trials.

CONCLUSION

The Committee was satisfied that

- line RRC 1445 has been well characterised regarding the genetic modification event;
- the T-DNA has been stably inserted and;
- the composition of cottonseed oil derived from line RRC 1445 is comparable in terms of protein, oil, carbohydrate, moisture, ash and calories in composition to the parental line and to commercial varieties, and that there is no novel genetic material present in the refined oil.

35. The Committee is therefore of the opinion that oil derived from herbicide tolerant cottonseed line RRC 1445 is substantially equivalent to oil from conventional cottonseed lines, in terms of composition, nutritional value, metabolism, intended use and level of undesirable substances.