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OPINION
of the French Food Safety Agency (Afssa) on the report drawn up by Professor Yvon Le Maho and presented to the European Commission in June 2008

On 5 September 2008, the Directorate-General for Public Health of the French Ministry of Health and Sport asked the French Food Safety Agency (Afssa) to deliver an opinion on the report drawn up by Professor Yvon Le Maho and presented to the European Commission in June 2008.

Background to the referral

Following the delivery of an opinion by the Provisional Committee of the High Authority on GMOs, the French authorities invoked a safeguard clause for the cultivation of MON 810 maize on the grounds that new evidence suggested that varieties obtained from the MON 810 event were liable to pose a serious environmental risk.

In response to the opinion of the Provisional Committee, the Monsanto company, in a report dated 30 January 2008 (Monsanto, 2008), put forward arguments in support of its claims that the variant carried no risks. The Ministry of Ecology, Energy, Sustainable Development and Town and Country Planning (MEEDDAT) asked Professor Le Maho, who is a member of the Provisional Committee, to prepare a reply.

Professor Le Maho's report, which was communicated to the European Commission, sets out health arguments that do not accord with the favourable report delivered by Afssa on 30 April 2008 (Afssa, 2008 (a)), based on its collective expertise. The Le Maho report concludes by stating that the precautionary principle should be applied.

Afssa was asked by the Directorate-General for Public Health in September 2008 to draw up a specialists' report providing a second opinion on the purely health-related aspects of Professor Le Maho's report.

Summary of the conclusions in the Afssa opinion of 30 April 2008 (Annex I):
Opinion delivered by Afssa on an application under Regulation (EC) No 1829/2003 for renewal of a marketing authorisation for the importing, processing and use in food or feed of products derived from the lepidopteran-resistant genetically modified maize variant MON 810.

In its opinion, the French Food Safety Agency considered that :

- the transformation event was characterised by molecular analysis of the MON 810 event,
- the compositional analysis did not reveal any significant difference that compromised the substantial equivalence of MON 810 maize to the control varieties or to conventional varieties of maize,
- the subchronic toxicity study conducted on rats for 90 days did not reveal any adverse effects related to the consumption of maize obtained from the MON 810 event, and
- the nutritional study conducted on chickens did not reveal any nutritional differences

between MON 810 maize grain and the control maize grain.

Accordingly, the French Food Safety Agency believed that, with regard to the data presented in the file, some of which had been updated and a great deal of which had been published in scientific peer reviews, maize obtained from the MON 810 event and its derivative poducts offered the same degree of safety as conventional maize varieties and their derivatives.

Processing of the referral

At its meeting on 20 November 2008, the Scientific Panel on Biotechnology[1] analysed the points in the report concerning the safety of food and animal feed.

Safety of the Cry1Ab protein expressed in MON 810 maize

Extracts from Professor Le Maho's report:

'The Bt protein produced naturally by the bacillus and that produced by MON810 maize do not have the same primary sequences. The protein produced by MON 810 maize may also be modified through the addition of n-acetylglucosamine phosphates or hexose, which may modify spatial conformation as well as its functional characteristics and therefore its hence its possible pathogenicity.'

'The protein produced by the transgene is not identical to that produced by Bacillus thuringiensis*, and its properties in terms of folding, post-translational modification, biodegradability, remanence or specificity, and therefore potential human and environmental toxicity, may be different from those of the natural Cry1Ab toxin.'*

Bt toxins comprise two domains – a toxic N-terminal domain and a crystal C-terminal domain. The whole Cry1Ab protein encoded by the endogenous Cry1Ab gene of *Bacillus thuringiensis*, subspecies *kurtstaki*, strain HD-1, comprises 1 156 amino acids. It is a protoxin that loses its C-terminal part when digested in the gut of an insect. The N-terminal domain is sufficient to provide its toxic activity (Fischhoff *et al.*,1987).

The gene that is present in MON 810 encodes a protein comprising the first 816 N-terminal amino acids of the Cry1Ab protein, including the toxic domain. The polypeptide of 816 amino acids undergoes the same cleavage as the native protein in the gut of the insect. The gene comes from *Bacillus thuringiensis*, subspecies *kurtstaki*, strain HD-1 (Höfte and Whiteley, 1989). The primary structure of the Cry1Ab protein expressed in maize obtained from the MON 810 event is described ion the technical dossier accompanying the marketing application.

As described in the technical dossier, the 3' insertion junction adds nine nucleotides to the mRNA, which leads to the addition of two amino acids in the C-terminal position, namely phenylalanine and arginine).

The publication of an article written by Rosati *et al.* (2008) revealed MRNA variants generated by the alternative splicing of the untranslated transcribed region. These variants have the potential to modify the C-terminal extremity of the Cry1Ab protein by adding to it a polypeptide of 18 amino acids. The article does not inform us about the abundance and stability of these transcriptions or about their translation. Even if the expression of this form of protein cannot be ruled out, it has never been detected.

Neither the comparisons of the sequences of the Cry1Ab protein containing the 18 additional amino acids nor a comparison of the sequences of the 18 amino acids themselves are conducive to the establishment of a significant match with any of the whole catalogue of protein sequences listed in public databases or with the peptides and proteins in the Allergen Database for Food Safety (ADFS).

Lastly, the protein that is present in the maize preserves an accessible cleavage site which results in the elimination of the C-terminal part and hence of the two or, potentially, 18 additional residues. The protein retains the same insect-toxicity properties.

[1] The Scientific Panel on Biotechnology comprises 21 scientists specialising in the following fields: molecular biology, plant physiology, transgenesis and genetic engineering, agricultural science, toxicology, animal nutrition and livestock breeding, food chemistry, microbiology, enzymology and biochemistry.

The second type of difference to which Professor Le Maho refers relates to possible post-translational modifications.
Several factors serve to exclude the possibility that post-translational modifications of the protein expressed in the plant will distinguish it from natural protein. For example:

- a study conducted by the applicant demonstrates that the protein expressed in MON 810 maize is not glycosylated, and
- the molecular weight of the fraction hydrolysed with trypsin (Western-blot technique) is identical, regardless of whether the protein comes from MON 810 maize or from *Bacillus thuringiensis*.

The guidelines established by EFSA (EFSA, 2006), to which the applicant responds by citing relevant studies, serve to assess the potential toxicity of MON 810 maize. The subchronic toxicity assay in particular is conducted with the genetically modified plant. The animals, which are fed directly with MON 810 maize, are exposed to the Bt protein in the form in which it is present in that maize. The study therefore takes account of effects which, if they occurred in the plant, could perhaps trigger modifications. The findings of this study do not indicate that the protein or the maize is in any way toxic to humans or animals (see 'Toxicological evaluation of GMOs' on page 6 below).

Extract from Professor Le Maho's report:
'Moreover, that produced by MON 810 may be modified by the addition of phosphates, N-acetylglucosamine or hexose, which may lead to a change in the spatial conformation of the protein (Ahmad et al*., 2006), its functional characteristics or, indeed, its possible pathogenic potential (Wang* et al*., 2007, Pang* et al*., 2007, Chen* et al*., 2006, Wells* et al*., 2004, and Lüdemann* et al*., 2005).'*

The examples of post-translational modification of proteins cited in the preceding paragraphs relate to proteins of animal origin with a known molecular-signalling function. The publications describe only the scope for post-translational modifications and refer to their potential consequences but do not demonstrate any actual functional modification.
The Cry1Ab protein has no cellular-signalling function and acts by forming pores in the intestinal cells of certain lepidopterans.
Lastly, the whole plant is subjected to toxicological evaluation in order to reveal any adverse effects of such protein modifications.

Safety assessment of the Cry1Ab protein

The Cry1Ab protein is considered not to pose any risk. This has been the view of the Scientific Committee on Plants (SCP) since 1998, a view that has also been expressed on several occasions by EFSA and Afssa since 2005 (see Annex II). None of the many studies of laboratory animals and target species has identified any adverse effect associated with the consumption of GMOs (see Annex III).

The Cry1Ab protein comes from *Bacillus thuringiensis,* a widespread soil-dwelling bacterium. The class of proteins known as Cry proteins was first isolated in 1901. These are delta endotoxins that act as an insecticide on very specific kinds of pest. Microbiological insecticides containing δ-endotoxins derived from *Bacillus thuringiensis* have been used in farming as an alternative to chemical pesticides since 1960 (McClintock *et al.,* 1995). Today, more than 150 different cry proteins have been described, and the nomenclature of δ-endotoxins comprises 51 classes, from Cry1 to Cry51 (Höfte and Whiteley, 1989). Since 1987, some of these proteins have been used to make crops insect-resistant (Fischhoff *et al.*, 1987).
The presence and agricultural use of this bacterium in the human environment have a history of more than 60 years and have never been a source of risk (Romeis *et al.,* 2006). It has been demonstrated that toxins from *Bacillus thuringiensis* (Bt) used in farming are not toxic to humans or fauna other than the targeted insects (Extoxnet PIPs, 1996). No toxicity has been observed in rats, mice, birds, dogs or guinea pigs to which protein crystals extracted from *Bacillus thuringiensis* serovar israelensis (Bti) have been administered orally (Mayes *et al.,* 1989); this is because of the absence of receptors to these toxins in the intestinal epithelial cells of mammals, birds and fish.

Toxicological assays based on cry proteins have been conducted on mammals (for a review of these, see Betz *et al.,* 2000). After chronic oral administration of 8.4g per kg per day of Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa to rats over a period of 13 weeks, no toxic effects were detected. Similarly, no toxicity of the Cry1Ab protein was observed in an *in vitro* model using mammalian intestinal cells in culture (Bondzio *et al.,* 2008).

When the application for renewal of the marketing licence for MON 810 was examined by Afssa, the applicant supplied the following studies and analyses:

- an updated comparative structural analysis between the Cry1Ab protein and proteins known for their toxic or immunotoxic properties or for their biological or pharmacological activity; this analysis revealed no similarities;
- a study of the degradation of the protein *in vitro* in the presence of simulated gastric fluid; this shows that 90% degradation of Cry1Ab occurs within two minutes (Sanders *et al.,* 1998);
- an acute toxicity study involving the oral administration of the toxic domain of the Cry1Ab protein, synthesised by *E. coli,* which shows that the maximum administrable daily dose of 4 000mg per kg of body weight does not have any observable adverse effect on laboratory mice; the safety margin for humans extrapolated from this dose is very conservative (of the order of 10^7) in relation to the estimated daily food intake of adults and adolescents.

The applicant also provided a subchronic toxicity study conducted over 90 days. This study was published in an international peer-review journal (Hammond *et al.,* 2006). It was conducted with rats on the basis of a protocol conforming to the applicable international guidelines, with a view to studying the effect of the consumption of MON 810 maize grain comprising 11% or 33% of the animals' feed ration compared with that of a control maize from the same genetic background. These doses are equivalent to the consumption of 2.5kg of maize per day by a man weighing 60kg. The findings, which were the subject of an Afssa toxicological report, do not show any adverse effects associated with the consumption of maize obtained from the MON 810 transformation event.

Characterisation of the 3' transgene insertion site

Extract from Professor Le Maho's report:

'Compared with the Cry1Ab protein produced naturally by Bacillus thuringiensis*, its integration into the maize genome has involved complex recombining (Rosati* et al.*, 2008).'*

The title of the publication cited in this sentence is reproduced with a misprint that could mislead readers. The title is given in the list of publications as
'Characterisation of 30 transgene insertion site and derived mRNAs in MON 810 YieldGard maize', whereas the correct title is
'*Characterisation of **3'** transgene insertion site and derived mRNAs in MON 810 YieldGard maize*'.
Cf. *Plant Molecular Biology*, Vol. 67, No 3, pp. 271-281.

The studies conducted by Rosati *et al.* (2008) identify the precise transgene insertion site and confirm the findings of Hernandez *et al.* (2003), who had not managed to amplify the region corresponding to the insertion site on the basis of DNA from untransformed lines. Sequencing an additional 345 bp in the 3' region, Rosati *et al.* identified a precise locus on chromosome 5, the sequences of which are homologous with those of a gene of rice *(Oryza sativa)* that encodes a HECT E3 ubiquitin ligase. This gene, which the applicant has already identified, is described in the technical dossier. It has not been demonstrated whether the gene is functional in maize (*Zea mays*).
The sequences obtained in the 5' region target another locus, probably a retrotransposon that is present in a locus encoding 22-kDa zeins, and, more precisely, long terminal repeat (LTR) sequences (Holck *et al.,* 2002). Rosati *et al.* (2008), who also sequenced 2 kb of the said region in untransformed plants, did not find a locus encoding 22-kDa zeins. The authors supposed that the insertion would have led to a significant deletion of a genomic region. That deletion was not characterised. The authors confirmed that there was indeed one insert in a single copy, that the

Cry1Ab encoding sequence was truncated and that the NOS terminator had been deleted. The authors themselves mention that their findings are partially consistent with those described by the applicant (US Patent 2004/0180373 A1, pub. date Sep.16, 2004).
Lastly, Rosati *et al.* (2008) specify that the transgene insertion and the deletion of part of the genetic structure and of a genomic region have no impact on either the activity of the protein or the vigour and yield of the maize plants.

To sum up, the molecular characterisation of the event carried out by the applicant and examined by Afssa on receipt of the application for renewal of the marketing licence (Afssa, 2008(a)) contains experimental findings demonstrating the presence in the MON 810 maize genome of one transgenic insert in a single copy. The characterisation includes an analysis of the regions flanking the insert in 5' and 3', which involves a search for open reading frames (ORFs) of the fusion gene, their translation and a comparison of the sequences with those in the databases. None of these displayed homology with the sequences of any protein described as toxic, allergenic or pharmacologically relevant.
We must therefore conclude that the findings of Rosati *et al.* (2008) do not cast any doubt on the safety of MON 810 maize.

Immunogenicity of the Cry1Ab protein

Extract from Professor Le Maho's report:
'Account must be taken of emerging allergy problems linked to new foods or to industrial procedures in food (Wassenberg et al*., 2007). It is known in particular that Cry1Ab produces an immune response in the rat model (Kroghsbo* et al*., 2008).'*

The safety assessment of GMOs certainly does take account of the allergenicity risk of the new protein. Since potential allergenicity cannot be assessed on the basis of a single test, the recommended approach is that of the 'weight of evidence' based on a cumulative body of facts (EFSA, 2006). The following factors are taken into account:

- the source of the gene or genes from which the proteins in question originated,
- the absence of sequential homology of the protein or proteins with known toxic or allergenic proteins,
- the degree of glycosylation of those proteins,
- the digestion time of these proteins *in vitro* in a simulated gastric or intestinal environment, and
- the protein content per gram of dry weight of the maize grains (grains of MON 180 maize have 0.5 µg of Cry1Ab per gram, i.e. 0.0004% of the protein content).

These items of information are provided in the technical dossier accompanying the application for a renewal of the marketing licence for MON 810 maize. The Scientific Panel on Biotechnology concluded that none of these data suggested that MON 810 maize was in any way allergenic. It should be noted that no case has yet been reported of any allergy said to be linked to the consumption of products containing the Cry1Ab protein.

Kroghsbo *et al.* (2008) demonstrate that pure Bt protein used in an experiment induces a specific immune response through inhalation. This observation is not new; back in 1999, it was demonstrated that the Cry1Ac protein, from the same family, induces a mucosal and systemic immune response in mice when administered intraperitoneally or orally (Vazquez *et al.,* 1999).
The production of IgG antibodies that was observed after the mice had inhaled pure Bt protein (1% of their feed ration) is not indicative of an adverse physiological effect on humans and animals. Kroghsbo *et al.* (2008) do not interpret it that way and conclude that, compared with the positive control substance (PHA-E lectin), the Bt protein does not induce an immunotoxic effect. Other proteins may induce the production of IgG antibodies *in vivo* in animal assays (Chen *et al.*, 2001, and Dearman *et al.,* 2003), but this does not make them toxic or allergenic proteins. Kroghsbo *et al.* did not research the IgE level, which would have been a more suitable gauge of allergenicity.

Toxicological evaluation of GMOs

Professor Le Maho criticises the toxicological evaluation of GMOs at several points in his report and questions the choice and duration of the animal assays recommended by EFSA in its guidance document. In his conclusion, for example, he writes the following:

- *'The duration of toxicological tests is inadequate, and they should be conducted on various animal models*.
- *The toxicology tests undertaken to assess transgenic plants do not cover the new health fields (prion diseases, oncology).*

In the absence of long-term tests of the protein in the obtained configuration actually produced by MON810, the precautionary principle should prevail.'

Since it was created, Afssa has advocated the establishment of guidelines defining a strategy for assessing the safety of GMOs (Afssa, 2000).

For this reason, our safety assessments based on the EFSA guidance document for the risk assessment of GMOs involve a number of supplementary studies designed to reveal any potential adverse side-effects resulting from the consumption of a food or feed product or of its derivatives. The critical points of the assessment are the analysis of the transgene insertion into the genome, the nature of the transgenic product, non-equivalence of chemical composition, observation of deleterious effects through acute toxicity and subchronic studies and nutritional non-equivalence for the target animal in the course of the study (EFSA, 2006).

Three types of study may be conducted as part of the toxicological evaluation:

- an acute toxicity study involving the administration of a single dose with a view to identifying any intrinsic toxicity in the protein,
- a subchronic toxicological study of 90 days' duration on rats, in accordance with OECD standards, in order to assess whether the entire plant, as consumed by humans or animals, could produce toxic effects, and
- a study of the nutritional value and tolerance of one or more target species with a view to obtaining information to supplement the findings of the subchronic toxicological study.

The purpose of subchronic toxicological studies is to assess the potential effects of repeated consumption of a product by humans or animals and the unexpected or unintentional toxic effects that would not be revealed by a chemical analysis or by acute toxicity studies.
The duration of a subchronic toxicity study (90 days in the case of rats) results from a choice between the minimum (14 days) and maximum periods (six months) that apply to the testing of medicinal products on rodents. A longer study is only warranted for research into carcinogenic effects, which may last for two years in the case of rats.
The value of studies on laboratory animals in the context of safety assessments of foodstuffs comprising or derived from GMOs has already been the subject of an Afssa opinion, which sheds some light on the ideal duration of animal assays (Afssa, 2002, and Afssa, 2008(b)). The same subject has also been discussed and documented in a recent report from the EFSA GMO Panel (EFSA, 2008(b)).
The capacity of 90-day subchronic studies to detect potential toxic effects has been confirmed. That duration is considered sufficient to permit the identification of effects produced by compounds which would induce toxicological disorders following chronic exposure (Munro *et al.,* 1999). A subchronic toxicity study of 90 days' duration serves as a beacon or sentinel study (EFSA, 2008(b), Knudsen and Poulsen*,* 2007, Knudsen *et al.*, 2008) that can generate a demand for additional experimentation if it produces favourable findings or raises doubts based on the customary principles governing the risk assessment of GMOs.
In other words, it is certainly on the basis of all the evidence that EFSA and Afssa assess the safety of GMOs and their derivative products for human and animal consumption and decide, **on a case-by-case basis**, whether there is a need to supplement the studies produced by industry. Afssa strongly recommends a 90-day subchronic toxicity study on rats for primary genetic transformation events (Afssa, 2008(b)). Toxicity studies *in vivo*, focusing on a protein or foodstuff, are conducted on laboratory animals of the rodent order (rats or mice) in accordance with the OECD protocols.
The use of animals other than rodents, namely rabbits, is recommended where there is a need to demonstrate effects on reproduction and/or development.

The capacity of rodent assays to detect carcinogenic effects

'Toxicological studies must from now on also target oncogenic research. Tests on new-born animals have also long been performed in viral and non-viral oncology. They have thus made it possible to served to discover oncogenes, which cause a great many cancers in human beings (Gelman et al.*, 1993, Bonham* et al.*, 1992, Hassan* et al.*,1990, and Darlix* et al.*, 2007).'*

The assessment of the safety of GMOs takes account of research into any unintentional and unexpected toxic effects produced by regular consumption of a GMO or of products derived from it. Such effects may be linked to products of gene expression or to the metabolites or degradation products of the gene, to overproduction of a toxic substance that exists naturally in plants, such as an alkaloid, or to the presence of new metabolites or residues resulting from the treatment of the plant with a herbicide. Analysis of the GMOs that have been tested to date by Afssa shows that the effects of such compounds, which are present in very low concentrations in GMOs and hence in consumed foodstuffs, are well documented and that none of them has ever been regarded as indicative of carcinogenic potential.

Toxicological studies lasting more than 90 days or focusing on specific functions such as reproduction and development may be requested on the basis of:

- the nature of the expressed protein or proteins under examination,
- specific risks associated with potential exposure, such as gossypol from cotton plants or phyto-oestrogens from soya-bean plants,
- the nature and quantitative significance of the differences in chemical composition observed between the GMO and its non-GM control organism, and
- findings from the other elements of the assessment (nutrition and 90-day subchronic toxicity).

The references cited in support of Professor Le Maho's statements relate to the discovery of viral or non-viral oncogenes involved in controlling the cell cycle. This situation is not comparable with the gene encoding the Cry1Ab toxin, since the latter is not known to play a part in controlling the growth or division of cells.
As far as tests on new-born animals (juveniles) are concerned, these were first proposed only recently for medicinal products intended for use in paediatrics. They are not systematically practised.

The capacity of assays to encompass new fields of research

'Toxicology tests performed on the Cry1Ab protein in no way cover the new research fields that emerged in recent studies on prion diseases (CJD, mad-cow disease, scrapie; contaminations and transplantations), which have had a significant global impact with harmful effects on human and animal health, due to new procedures used in agriculture, and which are linked to conformation modifications of proteins.
The Cry1Ab recombining protein has not been tested in accordance with current methods in the field of prion research (new-born rats and i.c. or i.p. injections, followed by studies with a minimum duration of 120 to 300 days) (Liberski and Brown, 2007; Unterberger and Voigtländer, 2007). It must be emphasised that such studies would have made it possible to avoid the 'mad cow' crisis and, more recently, the crisis involving the growth hormone affecting young children (Lewis et al., 2006; Pauli, 2005).'

This paragraph posits a link between the Cry1Ab protein and prion diseases such as scrapie and CJD and cites publications dealing with these diseases. The current state of knowledge about prion diseases in terms of biochemistry and the behaviour of PrP proteins does not permit such linkage. In the case of prion diseases, a protein homologous with the protein responsible for the disease is present in the cells of the host. The susceptibility of the host even seems to be conditioned by the degree of sequential or conformational homology of the host's PrP protein with that of the infected donor organism. There is no link between the structure of the recombinant protein Cry1Ab, which is of bacterial origin, and the PrP protein of mammals. Besides, maize-eaters do not express the Cry1Ab gene or any other protein displaying homology with Cry proteins.

The epizootic disease BSE – bovine spongiform encephalopathy – is associated with the recycling of an infectious agent within the same species through the use of contaminated meat and bone meal in animal feed; as for the cases of Creutzfeldt-Jakob disease, which is linked to the growth hormone, batches of hormones were contaminated by the CJD agent, which has already adapted to humans, because the hormones were taken from the bodies of persons infected by that pathogen.

Injections of laboratory rodents with samples taken from experimentally infected animals are used for research into prion diseases. They do not by any means constitute a diagnostic tool that could be used as a matter of routine on the basis of some sort of matrix which could have predicted the appearance of prion diseases.
Moreover, in the example of the cases of iatrogenic CJD resulting from the contaminated growth hormone, success in eliminating the risk of CDJ transmission was due to the use of a recombinant growth hormone.

Independence of toxicological studies

'In this context, such tests [toxological tests] should be undertaken entirely independently of the company and double blind. Once the results have been obtained, they should be made public.'
'Moreover, scientists should have access to the original data for the toxicological tests used. Blocking their dissemination, as has happened in previous years with regard to the results of tests on rats fed or not fed on MON 863 maize, prevents the advance of scientific knowledge and is, moreover, contrary to European legislation (notably Directive EEC/2001/18) and to French legislation.'

The toxicological studies provided by applicants to support applications for marketing licences are conducted by specialised firms which are external to the commissioning companies, possess GLP (good laboratory practice) status and apply the protocols enacted by international bodies (the OECD and EMEA). The procedures governing the conduct of these studies are those that apply to safety assessment of medicinal products. The principles and requirements of GLP serve to ensure the traceability of all data from the studies and to guarantee their authenticity. The laboratories in question are regularly checked by inspectors acting on behalf of a national authority. Up to the present day, non-clinical safety studies of medicinal products have not been conducted in the form of double-blind tests.
The assessors have access to all the raw data in the files. The content of these files is confidential, not secret. The applicant's intellectual property rights must be respected, and such data cannot be publicly disseminated unless that right is guaranteed.

Contesting statistical analyses

'Moreover, the capacity of the statistical methods used is questionable, since they appear to show very little sensitivity to differences, even though some of these are significant'.
'Re-examining these results, Séralini et al. *(2007) highlighted differences in weight variation between male and female rats as well as signs of hepatorenal toxicity. A study supported by the company (Doull* et al.*, 2007) then challenged this interpretation, arguing that a dose-effect relationship had not been shown and that the results differed according to sex.'*

Professor Le Maho is referring here to the re-examination of the toxicological data on MON 863 maize. That re-examination, which was undertaken by Séralini *et al.* (2007), involved the use of various statistical methods, culminating in the same results. Where Séralini *et al.* (2007) differ is in the way they interpreted the differences.
EFSA, the Biomolecular Engineering Committee (*Commission du génie biomoléculaire – CGB*) and Afssa have each issued an opinion on the Séralini *et al.* publication, and all of them conclude that the new analysis does not refute the previous opinions on MON 863, namely Afssa, 2007, AESA, 2007, and CGB, 2007). Professor Le Maho's report does not cite these three opinions.

When formulating their guidelines, Afssa and EFSA produced detailed documents on the conduct of statistical analyses of data (EFSA, 2008(a) and Afssa, 2002). If an assessment reveals a shortage or absence of data, the dossier is rejected (cf. Afssa's published opinions on

GMOs).

CONCLUSION of the French Food Safety Agency

Afssa considers that the parts of Professor Le Maho's report on risk assessment which relate to Afssa's sphere of responsibility do not contain any new elements that might cast doubt on the safety of maize obtained from the MON 810 event.
The technical dossier accompanying the marketing application satisfies all the requirements of the European guidelines in that:

- their substantial equivalence has been demonstrated,
- toxicology tests, including a subchronic toxicity study on rats, have not identified any adverse effects linked to the consumption of such maize, and
- numerous supplementary studies on target species (Annex II) have been conducted, and none of them has indicated any toxicity.

The data on consumption of MON 810 maize and exposure to it over a period of more than ten years are informative, even though there has been no formal system for reporting potential undesirable effects in livestock. Favourable reassessments of MON 810 maize have only recently been made by Afssa (Afssa, 2008(a)) and EFSA.
After analysing Professor Le Maho's report, EFSA's Scientific Panel on Genetically Modified Organisms concluded that, in terms of risk to human and animal health and the environment, the information package provided by the report did not present new scientific evidence that would invalidate the previous risk assessments of maize MON 810 (EFSA, 2008(c)).

The criticisms expressed in Professor Le Maho's report do not constitute grounds for reappraisal of the method established by Afssa for the toxicological risk assessment of GMOs in foodstuffs.

Key words: GMOs, MON 810, Professor le Maho.

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ANNEX I

Maisons-Alfort, 30 April 2008

OPINION

of the French Food Safety Agency (Afssa) on an application under Regulation (EC) No 1829/2003 for renewal of a marketing licence for the importing, processing and use in food or feed of products derived from the lepidopteran-resistant genetically modified maize variant MON 810

On 27 February 2008, the Directorate-General for Competition, Consumer Affairs and Anti-Fraud Policy of the French Ministry of the Economy, Industry and Employment asked the French Food Safety Agency (Afssa) to deliver an opinion on an application under Regulation (EC) No 1829/2003 for renewal of a marketing licence for the importing, processing and use in food or feed of products derived from the lepidopteran-resistant genetically modified maize variant MON 810.

Under Regulation (EC) No 1829/2003, and particularly its Articles 6 and 16, the European Food Safety Authority (EFSA) has an obligation to assess dossiers relating to genetically modified food and feed and to deliver an opinion to the European Commission. EFSA, however, has decided to allow Member States to make observations on the initial dossier. It was in this framework that the Directorate-General for Competition, Consumer Affairs and Anti-Fraud Policy requested the opinion of Afssa.

After consulting the Scientific Panel on Biotechnology at the Panel's meeting of 17 April 2008, the French Food Safety Agency delivers the following opinion:

(A) General information

The request is for the examination of a dossier with a view to the renewal of a marketing licence for the importing, processing and use in food or feed of products derived from the lepidopteran-resistant genetically modified maize variant MON 810. Maize obtained from the MON 810 event was licensed for cultivation and use as animal feed in February 1998 by virtue of Directive 90/220/EEC, subsequently replaced by Directive 2001/18/EC. Derivative products of maize obtained from the MON 810 event were authorised for use in food and feed in February 1998 by virtue of Regulation (EC) No 258/97, later replaced by Regulation (EC) No 1829/2003. In July 2004, maize obtained from the MON 810 event and its derivative products intended for use in food and feed were notified in accordance with Articles 8 and 20 of Regulation (EC) No 1829/2003 and were entered in the Community Register in April 2005.

The purpose of the present application is the renewal of all the existing licences that have expired after ten years under the terms of Articles 8 and 20 of Regulation (EC) No 1829/2003. The assessment of the environmental risk arising from the cultivation of GMOs does not fall within Afssa's sphere of competence, and the present opinion relates to the food safety of maize obtained from the MON 810 event.

Information regarding maize has already been examined by Afssa in the context of its assessment of applications for marketing licences in respect of hybrids obtained from the MON 810 event, namely the T25 x MON 810, NK603 x MON 810, LY038 x MON 810, MON 88017 x MON 810 and MON 810 x MON 863 maize varieties.

MON 810 maizes contain the gene that encodes the toxic domain of the Cry1Ab protein, which comes from *Bacillus thuringiensis*, subsp. kurstaki. That protein is able to create pores in the intestinal cells of some lepidopteran species, which results in the disturbance

of the intestinal absorption system. The expressed domain of Cry1Ab is toxic to the European corn borer (*Ostrinia nubilalis*) and the Mediterranean corn borer *(Sesamia nonagroides*).
Maize varieties obtained from the MON 810 event have been marketed in the United States since 1997. They have subsequently been grown in many other countries (Uruguay, the Philippines, Spain, France, Germany, the Czech Republic, Portugal and Slovakia).

(C) Information relating to the genetic modification

The MON 810 event results from the regeneration of the whole plant from a callus of the B73 variety of maize, transformed by means of a biolistic method. The DNA strands used for the transformation are plasmids PV-ZMBK07 and PV-ZMGT10.
The PV-ZMBK07 plasmid consists of a plasmidic origin of replication (*ori*), the *nptII* gene, which provides resistance to Kanamycin, and a 4.9-kb chimeric expression cassette containing:

- the promoter from the cauliflower mosaic virus (CaMV) governing the synthesis of the 35S RNA, along with two copies of its enhancer sequence,
- the heat-shock protein 70 intron from the maize gene (*ZmHSP70)*,
- the sequence, optimised for the vegetation, encoding a variant of the Cry1Ab toxin from *Bacillus thuringiensis* subsp. *Kurstaki*, strain HD1, and
- the transcription terminator of the *A. tumefaciens* gene encoding nopaline synthase.

The PV-ZMGT10 plasmid consists of an origin of replication (*ori*) from *E. coli*, the bacterial gene *nptII* and two cassettes designed to regulate the expression of:

- the CP4 EPSPS protein from *Agrobacterium tumefaciens*, and
- the GOX (glyphosate oxidoreductase) protein from the LBAA strain of *Ochrobactrum anthropi*.

The function of these two proteins in the maize is to establish tolerance to glyphosate by means of two mechanisms, namely synthesis of an EPSPS, the enzyme targeted by glyphosate, to render it insensitive to that herbicide and synthesis of a GOX to deactivate the glyphosate molecules.

Be that as it may, the MON 810 event does not amount to the integration of both plasmids, for only a fragment from the PV-ZMBK07 plasmid is incorporated into the host genome (a fraction of the chimeric Cry1Ab gene).

(D) Information relating to the GM plant

(1) The MON 810 event involves only a fragment of the PV-ZMBK07 plasmid containing the Cry1Ab gene that makes maize plants resistant to certain lepidopterans.

(2) Southern-blot analyses, involving the use of a broad spectrum of restriction enzymes and probes corresponding to each of the two plasmids, show that the MON 810 maize line has no *ori* sequences or *nptII* genes or any fragment derived from the PV-ZMGT10 (CP4 EPSPS and GOX genes).
The complete insertion sequence and its 5' and 3' ends were generated, and analyses of these served to establish that the inserted DNA covers less than 3.6 kb, corresponding to:

- 307 bp derived from the CaMV 35S enhancer/promoter, the 5' half of which was not integrated,
- all of the 803 bp corresponding to the intron of the maize HSP70 gene, and
- the first 2 448 bp coding for the first 801 amino acids of the complete sequence of Cry1Ab, which comprises 1 151 amino acids.

No particular termination sequence was found to halt the transcription of the transgene, since the NOS terminator envisaged in the construct was not integrated at the time of the MON 810 transformation event.

The Cry1Ab protein expressed in the chimeric gene is truncated in relation to the initial expression cassette but nevertheless provides the transformed MON 810 maize with the expected resistance to certain insects belonging to the order of *Lepidoptera*.

The findings of the molecular analyses show that MON 810 contains a single copy of the inserted fragment and that this is the only insertion into the nuclear genome of the maize.

The regions upstream and downstream of the insert were sequenced in 2001, and in 2005 and 2007 new sequences were produced and were compared with the sequences registered in the public databases. The findings of these analyses show the following:

- At the upstream junction or 5' region, the DNA sequence contains five potential ORFs (open reading frames) of 15 to 80 amino acids. Comparison of the sequence of each of the five polypeptides with those listed in public databases reveals no homology with any protein that is toxic, allergenic or pharmacologically relevant. Moreover, there is nothing to indicate that this junction region will be transcribed and translated.
- At the downstream junction or 3' region, the DNA sequence contains six potential ORFs, and a comparison of the sequence of each of the six polypeptides with those listed in public databases reveals no homology with any protein that is toxic, allergenic or pharmacologically relevant. The analyses undertaken in 2005 showed that the longest ORF, with 278 amino acids, is similar to a rice protein defined as HECT ubiquitin-protein ligase. The insertion site is in exon 7 of this potential gene.

(3) **Information on the expression of the insert**

Cry1Ab-protein content in the various tissues of the maize plant – from leaves, from the whole plant as used in animal feed and from grains – was measured by enzyme-linked immunosorbent assay (ELISA) at six sites where MON 810 maize was being grown in the United States in 1994 and at sites where MON 810 or MON 810 hybrids were growing in Europe in 1995.

The average content measured in leaves and grains is shown in Table 1 below.

Table 1: Average content of Cry1Ab measured in maize varieties obtained from the MON 810 event, expressed in µg per g of fresh weight. The figures in brackets show the lowest and highest measured values.

	MON 810 United States, 1994	**MON 810 Europe, 1995**	**Hybrid MON 810 Europe, 1995**
Leaf	9.35 (7.93-10.34)	8.60 (7.59-9.39)	9.26 (8.20-10.51)
Grain	0.31 (0.19-0.39)	0.53 (0.42-0.69)	0.46 (0.35-0.60)

(5) **Genetic stability of the insert and phenotypic stability of the GM plant**

The genetic stability of the insert that was present in maize obtained from the MON 810 event was verified by means of Southern blot, and the phenotypical stability of its expression was primarily tested on the basis of the toxicity of the maize obtained from the MON 810 event to the European and Mediterranean corn borers that feed on it.
The findings of these analyses, which were undertaken on seven generations of crosses with the recurrent parent (B73) and six generations of crosses with another line (MO17), confirmed the ancestor-descendant stability of the insert and phenotype and revealed classical Mendelian inheritance of a dominant character.

(7) **Information on any toxic, allergenic or other harmful effects on human and animal health arising from the GM food/feed**

(7.1.3) Several studies have been conducted for the purpose of comparing the chemical composition of maize obtained from the MON 810 event with that of control varieties

and are summarised in Table 2 below.

Table 2: Description of the various assays conducted and parameters measured in studies for the comparison of the chemical composition of maize obtained from the MON 810 event with those of control varieties.

Place and date	US, 1994 6 sites	France, 1995 4 sites	France/Italy, 1995 3 sites	France/Italy, 1995 5 sites (2)
Event **Control variety**	MON 810 MON 818	MON 810 MON 820	MON 810 MON 820	MON 810 hybrid, Control hybrid
Tissue source	grain	grain	whole plant (animal feed)	Grain and whole plant (animal feed)
Measured parameters	6 proximal parameters (1) 18 amino acids 5 fatty acids Fibres Phytic acid Calcium Phosphorus	5 proximal parameters (1) 18 amino acids 9 fatty acids 2 fibres (NDF and ADF)	6 proximal parameters (1) 2 fibres (NDF and ADF)	6 proximal parameters (1) 18 amino acids 8 fatty acids 2 fibres (NDF and ADF)

(1) The proximal parameters are ash content, water content, dry matter, carbohydrates, lipids, proteins and calories.
(2) The data were measured by means of infra-red spectrometry.

The statistical analysis (variance analysis) of the values measured for the various parameters sporadically reveals significant differences, but the observed content of each component remains within the range of values previously measured for maize. All of these studies therefore demonstrate substantial equivalence between the maize obtained from the MON 810 event and the control varieties.

With regard to the analyses of chemical composition undertaken in connection with applications for marketing licences in respect of hybrids with MON 810 as one of their parents, some of these applications were examined by the French Scientific Panel on Biotechnology (*CES Biotechnologie*), which concluded that there was substantial equivalence between each hybrid and its control variety, particularly in the cases of NK603 x MON 810 (opinion of 13 September 2005), LY038 x MON 810 (opinion of 5 June 2007) and MON 88017 x MON 810 (opinion of 3 May 2007).

*(7.4) **Agronomic traits***

Numerous analyses of agronomic and phenotypical traits have been undertaken since 1994, and all the findings indicate that there are no differences between genetically modified plants and control plants other than their resistance to lepidopterans.
It has also been shown that the cultivation of maize which is resistant to insect pests helps to reduce mycotoxin (fumonisin) content resulting from contamination and the development of fungus, which are promoted by insect attacks (Afssa, 2004).[2]

*(7.4) **Effect of processing***

The technical dossier provides a general description of the various categories of products derived from maize grains but does not contain any information on the ways in which they are produced or their Cry1Ab-protein content.

[2] Afssa, *OGM et alimentation : peut-on identifier et évaluer des bénéfices pour la santé ?*, 2004

*(7.8) **Toxicology***

(7.8.1) **Safety assessment of newly expressed proteins**

The Cry1Ab protein was deemed risk-free by the SCP in 1998, a view endorsed by EFSA and Afssa on several occasions since 2005. The analysis of the toxicity of Cry or Bt proteins was subjected to review (Betz *et al.,* 2000),[3] which found no evidence of toxicity to humans.

The following points should be taken into consideration:

- √ The Cry1Ab protein comes from *Bacillus thuringiensis,* a widespread natural soil-dwelling bacterium.
- √ There are no receptors for Cry proteins in mammals, birds or fish.
- √ The protein is present in its natural state in the human environment, including foodstuffs.
- √ Cry1Ab exhibits no structural similarity to any of the proteins listed in international databases which are known for their toxic or immunotoxic properties or for their biological or pharmacological activity in humans or animals other than their toxicity to certain lepidopterans for which it was selected. The relevant BLAST analyses were updated in 2004 and 2007.
- √ An acute toxicity study involving the oral ingestion by mice of the toxic domain of the Cry1Ab protein, synthesised by means of *E. coli*, revealed no deleterious effect on the tested mice at the maximum administrable daily dose of 4 000mg per kg of body weight.
- √ The safety margin for humans that has been calculated on the basis of the maximum single dose, taking account of the highest possible content of Cry1Ab protein in maize grain, is very conservative (of the order of 10^7) in relation to the estimated daily food intake of adults and adolescents.

The acute toxicity study was conducted with the toxic domain of the protein, produced in *E. coli*, which comprises 350 more amino acids than the domain of the protein expressed in maize obtained from the MON 810 event.

(7.8.2) **Testing of new constituents other than proteins**

A subchronic toxicity study was conducted over a period of 90 days, on the basis of a protocol conforming to the applicable international guidelines, with rats of both sexes – 20 rats of each sex per treatment – with a view to studying the effect of the consumption of MON 810 maize grain comprising 11% or 33% of the animals' feed ration compared with that of a control maize – amounting to 33% of the feed ration – from the same genetic background.

The chemical composition of the MON 810 maize grain administered to the animals has been determined and is consistent with the findings of the composition analysis presented above (see subsections 7.1 to 7.3).

The general clinical condition of the animals and their weight gain and food consumption as well as haematological, biochemical serum and urinary parameters were measured after 5 and 14 weeks. When the animals were sacrificed at 14 weeks, macroscopic and microscopic observations were made of their organs.

The following points should be noted:

- Weight gain and the nutritional value of feed do not differ in the animals whose diet is based on MON 810 maize from those fed with the control maize.
- Some haematological and biochemical serum parameters vary but display inconsistencies between the sexes; these observations do not provide evidence of any toxicological implications.

[3] F.S. Betz, B.G. Hammond and R.L. Fuchs, 'Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests', in *Regulatory Toxicology and Pharmacology*, Vol. 32, Issue 2, pp. 156-173, 2000.

- Some parameters do, however, differ significantly between the groups. Some of these significant differences that were observed between animals whose diet was based on MON 810 maize and those fed with the control maize were not replicated in a comparison with animals fed with a diet based on conventional maize varieties. These variations cannot therefore be directly linked with the nature of the animals' diet and are therefore of no toxicological significance.
- Histological analysis of the animals' organs does not reveal any alteration or difference between the animals whose diet is based on MON 810 maize and those fed with the control maize.

(7.9) ***Allergenicity***

The allergenicity of Cry1Ab has already been considered on several occasions (by the SCP in 1998 as well as by EFSA and Affsa) in connection with the assessment of other genetically modified maize varieties and plant species expressing that protein.

Assessment of the allergenicity of the Cry1Ab protein is based on the following considerations:

√ Cry1Ab is derived from an organism which is not known as a source of allergen.
√ The search for amino-acid sequences of proteins known to be allergens that are identical to the sequence of the Cry1Ab protein, which was updated in 2005 and 2007, involved a comparison with sequences of 80 amino acids and a search for a match with eight contiguous amino acids but did not result in the discovery of any matches.
√ The Cry1Ab is sensitive to proteolysis by pepsin in an acidic environment (simulated gastric fluid); 90% digestion of the protein occurs within two minutes of incubation.
√ Cry1Ab is not N-glycosylated, and the concentration of Cry1Ab in the maize grain is very low (0.5 µg/g, i.e. 0.0004% of the protein content of the grain).

It should, nevertheless, be noted that these data, namely the findings on the degradation of proteins and their digestion *in vitro* and the comparison of their sequences, do not constitute definite proof of the absence of any toxic or allergenic potential but that, given the present state of knowledge, such certainty cannot be obtained for any protein.

(7.10) ***Nutritional assessment of GM food/feed***

A nutritional study was conducted on chickens, comprising eight treatments with 80 males and 80 females per treatment, in which the chickens were fed over a period of 42 days with two diets – one for the initial period, from day 1 to day 21, and one for the growth and finishing period, from day 22 to day 42 – based on MON 810 maize (54% and 60% respectively for the two periods) with a view to comparing them with chickens fed in the same conditions with control maize from the same genetic background and with four commercial varieties of maize grown in the United States in 1999.

The researchers verified that the chemical composition of the MON 810 maize was equivalent to that of the control maize and reference varieties and that the rations contained 19 mycotoxins[4] and four pesticides.

The observations focused on eight parameters relating to livestock production, six relating to meat cuts and three sets of two parameters concerning the composition of thigh and breast muscle. It was established that the mortality rate recorded in the course of the experimentation was not treatment-related.

[4] It should be noted that the content of B1 fumonisins was significantly lower in the MON 810 maize than in the control maize.

Statistical analysis of these findings shows that there were no observable differences caused by treatments between the chickens fed with MON 810 and those fed with the control maize or the reference commercial varieties in terms of the measured parameters described above.

It may be concluded from the analysis of these findings that the nutritional value of MON 810 maize grain is equivalent to that of the non-GM control maize.

Of the numerous studies (see Annex III) conducted with other species – pigs, salmon, dairy cows and young bullocks – none revealed any toxicity or difference in nutritional value between maize varieties obtained from the MON 810 event and control varieties.

In spite of a jumbled presentation of data in the technical dossier, the French Food Safety Agency therefore considers that:

- the molecular analysis of the maize obtained from the MON 810 event characterises the transformation event,
- the compositional analysis does not reveal any significant difference that would compromise the substantial equivalence between MON 810 maize and the control maize or conventional maize varieties,
- the subchronic toxicity study conducted on rats over a 90-day period does not reveal any deleterious effects linked to the consumption of maize obtained from the MON 810 event, and
- the nutritional study conducted on chickens does not reveal any nutritional differences between MON 810 maize grain and the control maize grain.

Accordingly, the French Food Safety Agency considers that, in view of the data presented in the technical dossier, some of which have been updated, and the large volume of data published in scientific peer-review literature (see the bibliography appended to the opinion), maize varieties obtained from the MON 810 transformation event and their derivative products attain the same level of safety to human and animal health as conventional maize varieties and their derivative products.

Key words: GMOs, MON 810 maize, lepidopteran-resistant, renewal

Pascale Briand

Director-General

ANNEX III

Nutritional assessment of MON 810 maize and tolerance in target animals

Studies conducted for the purpose of verifying the non-toxicity of MON 810 maize, tolerance to it and its nutritional equivalence to non-GM control varieties on rats and other target species are summarised in Table 1 below.

These experiments resulted in the necessary sacrifice of 200 rats, 3 792 chickens, 144 pigs, 24 young bullocks, 21 000 salmon larvae and 34 pregnant cows. Permanent ruminal fistulas and bovine duodenum were also used.

Table 1: Summary of animal studies conducted with MON 810 maize.

Animals, duration	Event	% of feed ration	Production parameters	Biology	Reference
Rats *90 days*	MON 810	11-33G (1)	Semi-chronic toxicity Performance/sex Organs	12 Haematology 17 Serum	Hammond, 2006
Chickens *42 days*	MON 810xNK 603	55G	9 Performance (2)/sex Carcass (3)	6 Muscle composition	Taylor, 2003(a)
Chickens *42 days*	MON 810 MON 810xGA 21	55G 54-61G	Performance; survival rate Carcass	6 Muscle composition	Taylor, 2003(b)
Chickens *42 days*	MON 810 x MON 863	53G	Performance/sex: n=5 Carcass	6 Muscle composition	Taylor, 2003(c)
Chickens *42 days*	MON 810 x MON 88017	55G	Performance/sex Carcass: n=9	6 Muscle composition	Taylor, 2005
Chickens *42 days*	MON 810	50 G	Performance Mycotoxins (-)	Absence of DNA Haematology	Rossi, 2005
Chickens *42 days*	MON 810			Absence of DNA Muscle	Jennings, 2003(b)
Chickens *39-42 days*	MON 810		Presence of Cry1Ab (+) in digestive system (-), blood and organs		Deaville, 2005
Pigs *96 days*	MON 810	68-84G	15 Performance 14 Carcass/meat		Weber, 2000
Salmon *240 days*	MON 810	12,1 G	Performance Body composition	Organ weight 5 Haematology	Sanden, 2006
Dairy cows *21/28 days*	MON 810	42S ;34G 60S ;0G	Production/ Milk composition Quality (4)	10 Digestibility *in vitro*	Donkin, 2003
Dairy cows *Young bullocks*	MON 810	60S ; 20G	Absence of Cry DNA residue in milk, kidneys, liver and spleen		Jennings, 2003(b)
Dairy cows	MON 810	18.5 G	Presence of DNA, digestive tract (+) serum (-), milk (-)		Phipps, 2003

(1) % of grain (G) and silage (S) in the feed ration
(2) productivity: weight/weight gain, consumption index, survival rate
(3) Carcass: yield, weight of meat cuts, weight of fatty tissue
(4) Composition of milk: proteins, fats, lactose, total cells

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See the bibliography appended to the opinion (page 10).

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