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SCIENTIFIC OPINION

Guidance for risk assessment of food and feed from genetically modified plants[1]

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ABSTRACT

This document provides updated guidance for the risk assessment of food and feed containing, consisting or produced from genetically modified (GM) plants, submitted within the framework of Regulation (EC) No 1829/2003 on GM food and feed. The risk assessment strategy for GM plants and derived food and feed proposed seeks to deploy appropriate approaches to compare GM plants and derived food and feed with their respective comparators. The underlying assumption of this comparative approach is that traditionally cultivated crops have gained a history of safe use for consumers and/or domesticated animals. The document provides guidance on how to perform the comparative analysis of the relevant characteristics of the GM plant. The document addresses the details of the different components of the risk assessment: the molecular characterisation, which provides information on the structure and expression of the insert(s) and on the stability of the intended trait(s); the toxicological assessment, which addresses the impact of biologically relevant change(s) in the GM plant and/or derived food and feed resulting from the genetic modification; the assessment of potential allergenicity, of the novel protein(s) as well as of the whole food derived from the GM plant; the nutritional assessment to evaluate whether food and feed derived from a GM plant is not nutritionally disadvantageous to humans and/or animals. In addition every section of the document addresses specifically the requirements for GM plants containing a combination of transformation events, providing guidance on how to establish that the combination is stable and that no interactions occurs between the events that may raise safety concerns. The document does not cover the environmental risk assessment of GM plants which is addressed in a stand-alone environmental risk assessment (ERA) guidance document developed by the EFSA GMO Panel.

KEY WORDS

GMOs, GM plants, GM food, GM feed, guidance, applications, Regulation (EC) No 1829/2003, food safety, feed safety, risk assessment, comparative approach, stacked events, comparator, conventional counterpart

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SUMMARY

The European Food Safety Authority (EFSA) asked the Panel on Genetically Modified Organisms (EFSA GMO Panel) to update its document providing guidance for the risk assessment of food and feed containing, consisting or produced from genetically modified (GM) plants, submitted within the framework of Regulation (EC) No 1829/2003 on GM food and feed. The present version of this guidance document, which was adopted on 14 April 2011, was prepared expanding and completing the previous version (EFSA, 2006a) taking into account the experience gained during the evaluation of past applications, and the outcomes of EFSA GMO Panel Working Groups relevant for the risk assessment of GM plants and derived food and feed. The document does not cover the environmental risk assessment of GM plants which is addressed in a stand-alone environmental risk assessment (ERA) guidance document developed by the EFSA GMO Panel (EFSA, 2010a).

The risk assessment of GM plants and derived food and feed involves generating, collecting and assessing information on a GM plant and its derived products in order to determine their impact on human and animal health. The strategy proposed in this document seeks to deploy appropriate approaches to compare GM plants and derived food and feed with their respective comparators. The underlying assumption of this comparative approach is that traditionally cultivated crops have gained a history of safe use for consumers and/or domesticated animals.

The document outlines the principles of the risk assessment of GM plants and derived food and feed, providing an overview of the comparative approach and definitions of the different steps and objectives of the risk assessment process. The document provides a detailed overview of the different components of the risk assessment: the molecular characterisation, which provides information on the structure and expression of the insert(s) and on the stability of the intended trait(s); the toxicological assessment, which addresses the impact on human and animal health of biologically relevant change(s) in the GM plant and/or derived food and feed resulting from the genetic modification; the assessment of the allergenic potential of the novel protein(s) as well as of the whole food derived from the GM plant; and the nutritional assessment, which aims to demonstrate that the food and feed derived from a GM plant is not nutritionally disadvantageous to humans and/or animals.

As compared to the previous versions, this document also provides up-to-date guidance on the following issues:

- the requirements for the risk assessment of GM plants containing stacked events illustrated and addressed in each section, replacing previous EFSA guidance on this topic (EFSA, 2007);
- the design of the field trials for protein expression analysis;
- the design of the field trials for compositional, agronomic and phenotypic characteristics ensuring sufficient statistical power (see EFSA, 2010b);
- the statistical analysis of field trials data, allowing for an objective quantification of observed differences and equivalences between the GM plant and its comparator (see EFSA, 2010b);
- the selection of appropriate comparator(s) under different possible scenarios (see EFSA, 2011a);
- reference to internationally agreed protocols for toxicological assessment which may be selectively applied for GMO risk assessment;
- specific guidance updating and complementing the allergenicity assessment of newly expressed protein(s) and whole GM food and feed (see EFSA, 2010c);
- animal feeding studies with whole food and/or feed from the GM plant when considered necessary (EFSA, 2008, 2011b).

The present document provides guidance for assessing potential effects of GM plants and derived food and feed on human and animal health, and the rational for data requirements in order to complete a comprehensive risk assessment and to draw conclusions.

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BACKGROUND AS PROVIDED BY EFSA

The scientific Panel on Genetically Modified Organisms of the European Food Safety Authority (EFSA GMO Panel) regularly reviews its guidance document in the light of experience gained, technological progress and scientific developments. The guidance document of the EFSA GMO Panel for the "*Risk assessment of genetically modified plants and derived food and feed*", adopted on 24 September 2004, was updated in 2005 and published in May 2006. In 2008, EFSA requested the EFSA GMO Panel to revise the guidance in view of the scientific developments and in order to support the European Commission to prepare a legal framework for the safety assessment of the GM food and feed.

In May 2008 the EFSA GMO Panel endorsed for public consultation a draft updated guidance document for the Risk Assessment of GM plant and derived food and feed, which was published on the EFSA website from 21 July 2008 until 21 September 2008. This updated guidance document together with the comments received and EFSA's technical support was used by the Commission and the Member States as a basis for the preparation of the draft Regulation on "*Implementing rules concerning applications for authorization of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and the Council"*.

During the process of finalising the update of its guidance document, the EFSA GMO Panel took into account comments which enhanced both scientific quality and clarity and incorporated other scientific outputs of EFSA GMO Panel Working Groups relevant for the risk assessment of GM plants and derived food and feed:

• Safety and nutritional assessment of GM plants and derived food and feed: role of animal feeding trials (EFSA, 2008);
• Statistical considerations for the safety evaluation of GMOs (EFSA, 2010b);
• Assessment of allergenicity of GM plants and microorganisms and derived food and feed (EFSA, 2010c);
• Selection of comparators for the risk assessment of GM plants and derived food and feed (EFSA, 2011a).

The present document, adopted on 14 April 2011, was prepared taking into account the above mentioned scientific outputs and experience gained during the evaluation of the risk assessment of past applications, expanding and elaborating most sections of the previous GMO Panel guidance document on the "*Risk assessment of genetically modified plants and derived food and feed* (EFSA, 2006a), in accordance with current legislation.
It should be noted that guidance for environmental risk assessment of applications with scope cultivation of GM plants has been updated and published in November 2010 in the document "*Guidance on the environmental risk assessment of genetically modified plants"* (EFSA, 2010a).

TERMS OF REFERENCE AS PROVIDED BY EFSA

On 29 February 2008 and in view to support the development by the European Commission of a legal framework for the safety assessment of GM food and feed, EFSA requested the EFSA GMO Panel to revise the guidance document on the "Risk assessment of genetically modified plants and derived food and feed" taking into account the new developments on: 1) the role of animal feeding trials in the safety and nutritional assessment of GM plants and derived food and feed; 2) the risk assessment of stacked events; and 3) the statistical approach for compositional, agronomical and phenotypic characteristics of GM plants. In its request, EFSA also recommended to incorporate further developments made through discussion in EFSA GMO Panel Working Groups' meetings. The outcome of this exercise is the present revised guidance document, which provides up-to-date guidance on several issues, as described in the Background and Summary sections.

1. SCOPE OF THE DOCUMENT

This document provides guidance for the risk assessment of genetically modified (GM) plants[4] and derived food and feed, submitted within the framework of Regulation (EC) No 1829/2003 (EC, 2003a). This document does not cover the environmental risk assessment of GM plants which is addressed in a stand-alone environmental risk assessment (ERA) guidance document developed by the EFSA GMO Panel (EFSA, 2010a). The guidance also applies to feed intended for animals which are not destined for food production. When a product is likely to be used for food or feed purposes, the application should fulfil the requirements for both food and feed (EC, 2003a).

This guidance document is an updated replacement of the '*Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants and derived food and feed*' of May 2006 (EFSA, 2006a, 2009). This guidance document also replaces the '*Guidance document of the scientific panel on genetically modified organisms for the risk assessment of genetically modified plants containing stacked transformation events*' of July 2007 (EFSA, 2007).

This document provides detailed guidance to assist the applicant in the preparation and the presentation of the application, according to Articles 5(8) and 17(8) of Regulation (EC) No 1829/2003. Specific guidance on the presentation of the application is currently under development within EFSA[5].

Food additives (EC, 1989), flavourings (EC, 1988) and feed additives (EC, 2003b) containing, consisting of, or produced from GM plants fall within the scope of this guidance document.

This guidance does not consider issues related to risk management (traceability, labelling, and co-existence). Socio-economic and ethical issues are also outside the scope of this guidance.

2. PRINCIPLES OF RISK ASSESSMENT OF GM PLANTS AND DERIVED FOOD AND FEED

2.1. The comparative approach

The risk assessment strategy for genetically modified (GM) plants and derived food and feed seeks to deploy appropriate methods and approaches to compare GM plants and derived food and feed with their appropriate comparators. The underlying assumption of this comparative approach is that traditionally cultivated crops have a history of safe use for consumers and/or domesticated animals. These traditionally cultivated crops can thus serve as comparators when assessing the safety of GM plants and derived food and feed. The application of this comparative risk assessment in the area of plant composition (Kok and Kuiper, 2003), also denoted as the concept of substantial equivalence (FAO/WHO, 2000; OECD, 1993), serves the purpose of identifying intended and unintended differences and/or lack of equivalences between GM plants and derived food and feed and their comparator(s), taking into account the range of natural variation.

The risk assessment starts with the comprehensive molecular characterisation (MC) of the GM plant in question, followed by the comparative analysis of the relevant characteristics of the GM plant and its comparator(s). In particular, the comparative compositional, phenotypic and agronomic assessment requires the simultaneous application of two complementary tests: the test of difference and the test of equivalence (EFSA, 2010b). The test of difference is used to verify whether the GM plant, apart from the introduced genetic modification(s), is different from its comparator(s) and has therefore the potential to cause adverse effects. The test of equivalence is used to verify whether the agronomic, phenotypic and compositional characteristics of the GM plant fall within the range of natural variation. The range of natural variation is estimated from a set of non-GM reference varieties with a

[4] In the context of this document "genetically modified plants" are defined as genetically modified higher plants, (Gymnospermae and Angiospermae) in line with Directive 2001/18/EC.

[5] http://registerofquestions.efsa.europa.eu/roqFrontend/questionLoader?question=EFSA-Q-2009-900

history of safe use (EFSA, 2010b). The outcome of this comparative analysis will further structure the risk assessment.

Where no comparator can be identified, a comparative risk assessment cannot be made and a comprehensive safety and nutritional assessment of the GM plant and derived food and feed itself should be carried out. This would, for instance, be the case where the food and/or feed derived from a GM plant is not closely related to a food and/or a feed with a history of safe use, or where a specific trait or specific traits are introduced with the intention of changing significantly the composition of the plant.

Intended and unintended effects

Intended effects are those that fulfil the original objectives of the genetic modification. Intended alterations in the phenotype may be identified through a comparative analysis of growth performance, yield, disease resistance, etc. Intended alterations in the composition of a GM plant compared to its comparator, may be identified by measurements of single compounds e.g. newly expressed proteins, macro- and micro-nutrients, anti-nutrients and natural toxins (targeted approach).

Introduction of gene(s) in a plant may result in unintended effects in the modified plant. The risk assessment of GM plants and derived food and feed seeks to identify and characterize both intended and unintended effects with respect to their possible impact on human/animal health.

Unintended effects are consistent differences between the GM plant and its comparator, which go beyond the intended effect(s) of the genetic modification. Unintended effect(s) could potentially be linked to genetic rearrangements or metabolic perturbations and may be predicted or explained in terms of our current knowledge of plant biology and metabolic networks. Unintended effects may be detected through the comparison of the agronomic, phenotypic and compositional characteristics of the GM plant with its comparator cultivated under the same conditions. A starting point in the identification of potential unintended effects is the analysis of the flanking regions of the introduced DNA to establish whether the insertion is likely to impact the function of any endogenous gene of known or predictable function. Furthermore, a comparative analysis on specific compounds, representing components of important metabolic pathways should be carried out. These include macronutrients, micronutrients and specific metabolites as well as known anti-nutrients toxins. Intended and unintended differences and/or lack of equivalences between the GM plant and its comparator(s), taking into account natural variation, should be assessed with respect to their safety, allergenicity and nutritional impact.

2.2. Objectives of the steps of the risk assessment

2.2.1. Hazard identification

Hazard identification is the identification of biological, chemical, and physical agents capable of causing adverse health effects and which may be present in a particular food and feed or group of foods and feeds (Codex Alimentarius, 2007). Hazard identification is the first step in the risk assessment and focuses on the identification of differences and/or lack of equivalences between the GM plant and its comparator, taking into account natural variation, through the comparative analyses of compositional, agronomic and phenotypic characteristics. Identification of differences and/or lack of equivalences will determine the additional studies required to assess the possible impact on human and animal health.

2.2.2. Hazard characterisation

Hazard characterisation is the qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents which may be present in food

and feed. For chemical agents, a dose response assessment should be performed. For biological or physical agents, a dose-response assessment should be performed if the data are obtainable (Codex Alimentarius, 2007).

This step focuses on the possible quantification of the potential toxicological and/or nutritional effects of the identified differences between the GM plant and derived food and feed and its comparator. Studies on laboratory animals and/or target animals may provide useful information for the hazard characterisation. An appropriate test model and suitable test material should be used in order to generate data identifying the onset of adverse effects, and possible dose-response relationships.

2.2.3. Exposure assessment

The aim of the exposure assessment is the quantitative estimation of the likely exposure of humans and animals to the food and feed derived from GM plants (e.g. exposure to food, feed, pollen, new other plant constituents). With regard to humans and animals, an exposure assessment characterises the nature and size of the populations exposed to the food and feed derived from GM plants, and the magnitude, frequency and duration of such exposure. It is necessary that every significant source of exposure is identified. In particular, it is of interest to establish whether the intake of the food and feed derived from GM plants and its constituents are expected to differ from those derived from the conventional product. In this respect particular attention should be paid to GM plants and derived food and feed with modified nutritional properties. This category of GM plants and derived food and feed may require post-market monitoring to confirm the conclusion of the exposure assessment.

2.2.4. Risk characterisation

Risk characterisation is defined as the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterization and exposure assessment (Codex Alimentarius, 2007). The risk characterisation should demonstrate whether the hazard identification and subsequent characterisation is complete or not. Integration and evaluation of data from hazard characterisation and exposure assessment allow evaluating whether an appropriate risk characterisation may be finalised, or whether further data is needed to complete the risk characterisation. For instance if an increased intake of food and feed derived from GM plants by humans or animals is expected, further data on toxicity at extended dose ranges may be needed.

2.3. Elements of the risk assessment of GM plants and derived food and feed

The following elements should be considered for the risk assessment of GM plants and derived products:

a) characteristics of the donor organisms and recipient plant;

b) genetic modification and its functional consequences;

c) agronomic and phenotypic characteristics of the GM plant;

d) compositional characteristics of GM plants and derived food and feed;

e) potential toxicity and allergenicity of gene products (proteins, metabolites) and the whole GM plant and its derived products;

f) dietary intake and potential for nutritional impact;

g) influence of processing and storage on the characteristics of the derived products.

2.3.1. Risk assessment of GM plants containing stacked events

The risk assessment of GM plants containing stacked events requires the risk assessment of the GM plants containing these events independently (i.e. GM plants containing single events).

For GM plants containing a combination of transformation events (stacked events) the primary concern for risk assessment is to establish that the combination of events is stable and that no interactions between the stacked events, that may raise safety concerns compared to the single events, occur.

The risk assessment of GM plants containing stacked events focuses on issues related to:

a) stability of the inserts,

b) expression of the introduced genes and their products and

c) potential synergistic or antagonistic effects resulting from the combination of the events.

Depending on the outcome of this analysis further toxicological and nutritional information may be required.

The risk assessment of a GM plant containing stacked events should address all sub-combinations occurring by natural segregation from the GM plant. Whenever relevant, sub-combinations produced by targeted breeding approaches, which can combine any of the events in all possible permutations, should also be assessed. The risk assessment of these sub-combinations should take into account the different exposure levels covered by the scope of the application. Applicants should provide appropriate data to enable the risk assessment of the sub-combinations.

3. INFORMATION REQUIRED FOR RISK ASSESSMENT OF GM PLANTS AND DERIVED FOOD AND FEED

3.1. Hazard identification and characterisation

3.1.1. Information relating to the recipient and the parental plants

The applicant should provide comprehensive information relating to the recipient or (where appropriate) the parental plants to evaluate all issues of potential concern, such as the presence of natural toxins or allergens, and to identify the need for specific analyses.

For these purposes, the applicant should provide the following:

a) complete name; (i) family, (ii) genus, (iii) species, (iv) subspecies, (v) cultivar/breeding line or strain, (vi) common name;

b) geographical distribution and cultivation of the plant, including its distribution in the European Union;

c) information on the recipient or parental plants relevant to their safety, including any known toxicity and/or allergenicity of constituents and the plant;

d) data on the past and present use of the recipient organism. This information should include: the history of safe use for consumption as food and/or feed; information on how the plant is typically cultivated, transported and stored; whether special processing is required to make the plant safe to eat; and the description of the normal role of the plant in the diet (e.g. which part of the plant is used as a food and feed source, whether its consumption is important in particular subgroups of the population, what important macro- or micro-nutrients it contributes to the diet).

3.1.2. **Molecular Characterisation**

3.1.2.1. Information relating to the genetic modification

The applicant should provide sufficient information on the genetic modification to identify the nucleic acid intended for transformation and related vector sequences potentially delivered to the recipient plant, and to characterise the DNA actually inserted in the plant.

Description of the methods used for the genetic modification

The applicant should provide information on the following elements:

a) method of genetic transformation including relevant bibliographic references;

b) recipient plant material;

c) species and strain of *Agrobacterium* (also known as *Rhizobium*) or other microbes, if used, for the genetic transformation process;

d) helper plasmids, if used, during the genetic transformation process;

e) source of carrier DNA, if used, during the genetic transformation process.

Source and characterization of nucleic acid used for transformation

The applicant should provide information on the donor organism(s) and on the nucleic acid sequence(s) intended to be inserted in order to determine whether the nature of the donor organism(s) or the nucleic acid sequence(s) may trigger any safety issue. Information regarding the function of the nucleic acid region(s) intended for insertion should comprise the following elements:

a) complete sequence of the nucleic acid intended to be inserted, including information on any deliberate alteration(s) to the corresponding sequence(s) in the donor organism(s);

b) history of safe use of the gene product(s) arising from the regions intended for insertion;

c) data on the possible relationship of the gene products with known toxins, anti-nutrients and allergens.

Information regarding each donor organism should comprise its taxonomic classification and its history of use regarding food and feed safety.

Nature and source of vector(s) used including nucleotide sequences intended for insertion

The applicant should provide the following information:

a) a physical map of the functional elements and other plasmid/vector components together with the relevant information needed for the interpretation of the molecular analyses (e.g. restriction sites, the position of primers used in PCR, location of probes used in Southern analysis). The region intended for insertion should be clearly indicated;

b) a table identifying each component of the plasmid/vector (including the region intended for insertion), its size, its origin and its intended function.

3.1.2.2. Information relating to the GM plant

General description of the trait(s) and characteristics which have been introduced or modified

Information provided should include a general description of the introduced trait(s) and its mode of action, of the resulting changes on the phenotype and the metabolism of the plant, and of its intended use (e.g. as a selectable marker or agricultural trait).

Information on the sequences actually inserted/deleted or altered

The applicant should provide the following information:

a) size and copy number of all detectable inserts, both complete and partial. This is typically determined by Southern analysis. Probe/restriction enzyme combinations used for this purpose should provide complete coverage of sequences that could be inserted into the host plant, such as any part of the plasmid/vector, or any remaining carrier or foreign nucleic acid introduced in the GM plant. The Southern analysis should span the entire transgenic locus/loci as well as the flanking sequences and include all appropriate controls;

b) organisation and sequence of the inserted genetic material at each insertion site;

c) in the case of deletion(s), size and function of the deleted region(s), whenever possible;

d) sub-cellular location(s) of insert(s) (in nuclear, plastid, or mitochondrial chromosomes, or maintained in a non-integrated form) and methods for its determination;

e) sequence information for both 5' and 3' flanking regions at each insertion site, with the aim of identifying interruptions of known genes. Bioinformatic analyses should be conducted using up-to-date databases[6] with the aim of performing both intra-species and inter-species similarity searches. In the case of GM plants containing stacked events, applicants should assess the safety of potential interactions between any unintended modifications at each insertion site;

Open Reading Frames (ORFs)[7] present within the insert and spanning the junction sites. The ORFs should be analysed between stop codons, not limiting their lengths. Bioinformatic analyses should be conducted to investigate possible similarities with known toxins or allergens using up-to-date databases, as outlined in more detail in the scientific opinion of EFSA on the assessment of allergenicity of GM plants and microorganisms and derived food and feed (EFSA, 2010c). Depending on the information gathered, further analyses may be needed to complete the risk assessment.

Information on the expression of the inserted/modified sequence

The applicant should provide information to demonstrate whether the inserted/modified sequence results in intended changes at the protein, RNA and/or metabolite levels. In many cases the intended genetic modification will lead to the expression of new protein(s), therefore protein expression data will be the most relevant. In other cases (e.g. silencing approaches or where biochemical pathways have been intentionally modified) the analysis of specific RNA(s) or metabolite(s) may be the most informative.

Data should be derived from plants grown under conditions representative of typical cultivation practices. When commercial production occurs in the field, datasets should be provided from field-grown plants, although expression data from contained environments (e.g. glasshouse) may add value to the risk assessment. Data should be obtained from those parts of the plant relevant to the scope of the application. Where inducible promoters have been used, additional data may be requested on a case-by-case basis. The need for data on developmental expression should be considered on a case-by-case basis, taking into account the promoter used, the intended effect(s) of the modification and scope of the application.

The minimum requirement is data provided from three growing sites or from one site over three seasons. Permutations of the sites and seasons are acceptable as long as the minimum requirement is met.

The applicant should present the following information:

[6] The characteristics and versions of the databases should be provided.
[7] An ORF is defined as any nucleotide sequence that consists of a string of codons that is uninterrupted by the presence of a stop codon in the same reading frame.

a) methods used and the raw datasets. The specificity of the protein analysis method should be demonstrated;

b) if the insert encodes new protein(s), the range and mean values for the levels of the newly produced protein(s). In specific cases, levels of the relevant RNA(s), protein(s) or metabolite(s) should be provided.

For GM plants containing single events for food, feed, import and processing purposes the requirements for the selection of comparators vary depending on the intention of the genetic modification (EFSA, 2011a). If the genetic modification results in newly expressed protein(s) and where the analytical method has been shown to be specific, no comparator may be necessary. In other cases, such as silencing approaches or where the modification is intended to modify the levels of specific metabolites, the experimental design should include a non-GM comparator in order to compare the levels of relevant endogenous RNA(s), protein(s) and/or specific metabolite(s). Where appropriate, the impact of specific treatments linked to the trait (e.g. use of herbicides) should also be assessed.

For GM plants containing stacked events for food, feed, import and processing purposes, the main objective of the analysis is to assess the potential for any interactions between the events which may raise safety concern. If newly expressed proteins are present in the GM plant, their levels should be compared, in the same field trials, with the levels in any set of GM plants that have all been risk assessed and that include between them all of the events stacked in the GM plant under assessment, and no others. This set of GM plants may include either parental GM lines, if previously risk assessed, or GM plants containing the single events in case the parental GM line(s) has not been risk assessed. In other cases, e.g. where silenced traits are stacked, or traits which modify the levels of metabolites, the same principles should be followed. Expression data for specific treatments linked to the trait(s) (e.g. use of herbicides) are only necessary if data obtained from the GM plants containing the respective single events indicate a potential safety concern.

For applications on GM plants containing single or stacked events, which include cultivation in the scope, the above requirements for food, feed, import and processing should be met (including field trial design). Depending on the trait and scope of the application, information may also be required for the assessment of impacts on target and non-target organisms. In such cases, information on expression in various parts of the plant over the growing season is required (EFSA, 2010a). Data should be derived from plants grown under conditions representative of typical cultivation practices in Europe.

The analysis of risk associated with a change of protein and metabolite level(s) is covered in Section 3.1.4.

Historical data from previous applications can complement the risk assessment.

Genetic stability of the inserted/modified sequence and phenotypic stability of the GM plant

The applicant should provide information to demonstrate the genetic stability of the transgenic locus (loci) and the phenotypic stability and inheritance pattern(s) of the introduced trait(s). In the case of GM plants containing stacked events, the applicant should establish that the integrity of the events is retained in the GM plant.

For GM plants containing single events, applicants should provide data from usually five generations or vegetative cycles. The source of the material, the sampling design and the number of plants used for the analysis should be specified. When analysing the inheritance pattern(s), appropriate statistical methods should be applied.

For GM plants containing stacked events, the structure of the inserts should be compared to the structure of the inserts as present in the corresponding single events. Such comparison should be

carried out using plant materials representative of those aimed for commercial production. The applicant should provide adequate justification for the plant material used.

The applicant should also consider the safety implications of the loss of function of specific genes from multi-gene expression cassettes after their insertion into the plant.

3.1.2.3. Conclusions

The molecular characterisation should provide data on the structure and expression of the insert(s), and on the stability of the intended trait(s).

It should be specifically indicated whether the molecular characterisation of the genetic modification(s) raises safety concerns with regard to the interruption of endogenous genes.

The molecular characterisation should specifically aim to identify whether the genetic modification(s) raise(s) any issues regarding the potential for producing new toxins or allergens.

The potential unintended changes identified in this section should be addressed in the relevant complementary part(s) of the risk assessment.

3.1.3. **Comparative assessment**

The risk assessment strategy for GM plants seeks to deploy appropriate methods and approaches to assess the safety of GM plants and derived food and feed. Among them, the comparison of the GM plant and derived food and feed with appropriate comparators is a major, but not unique tool used throughout the risk assessment.

The comparative assessment of compositional, agronomic as well as phenotypic characteristics constitutes, together with the molecular characterisation, the starting point for the risk assessment of GM plants and derived food and feed. It aims to identify differences in composition, agronomic performance and phenotypic characteristics between the GM plants and derived food and feed and its comparator. If found these differences should be further assessed with respect to potential impact on human and animal health. The comparative assessment requires the simultaneous application of two complementary tests: the test of difference and the test of equivalence (EFSA, 2010b). The test of difference is used to verify whether the GM plant, apart from the introduced genetic modification(s), is different from its comparator and might therefore be considered a hazard (potential risk) which, depending on the type of the identified difference, in combination with extent and pattern of exposure, may require further safety evaluation. The test of equivalence is used to verify whether the agronomic, phenotypic and compositional characteristics of the GM plant fall within the normal range of natural variation. Such a range of natural variation is estimated from a set of non-GM reference varieties with a history of safe use (EFSA, 2010b) and therefore allows comparisons of the GM plant with a similar food or feed produced without the help of genetic modification and for which there is a well-established history of safe use.

In case an appropriate comparator is not available, a comparative assessment cannot be made and, therefore a safety and nutritional assessment of the GM plant and derived products should be carried out as for other novel foods. In such cases, the elements to be considered for the risk assessment are the same as those listed in Section 2.3.

Depending on the quality of the available data provided for the risk assessment, animal feeding trials with whole food and/or feed using laboratory animal species (rodents) and/or target animals may be considered, on a case-by-case basis (EFSA, 2008). Test protocols for animal feeding trials with food and feed derived from GM plants are described in the opinion of the EFSA Scientific Committee on 90-day feeding trial protocol (EFSA, 2011b).

GM plants carrying specific traits, e.g. herbicide tolerance and insect resistance, require appropriate treatment comparisons to evaluate safety. Such GM plants will also include those in which the traits are stacked to provide tolerance to multiple herbicides and resistance to multiple insect pests.

For the risk assessment of herbicide-tolerant GM plants, containing single or stacked events, the experimental design should include a comparison of three test materials: the GM plants exposed to the intended herbicide, the comparator treated with conventional herbicide management regimes and the GM plants treated with the same conventional herbicide management regimes.

3.1.3.1. Criteria for the selection of comparator(s)

Regulation (EC) No 1829/2003 defines a conventional counterpart as '*a similar food or feed produced without the help of genetic modification and for which there is a well-established history of safe use*' (Art. 2.12). In line with this legal requirement the EFSA GMO Panel provides details on the criteria for the selection of appropriate comparators, under different scenarios, in the EFSA Guidance for the Selection of comparators for the risk assessment of GM plants (EFSA, 2011a).

The EFSA GMO Panel recommends the use of the term "conventional counterpart" only when referring to: i) the non-GM isogenic variety, in the case of vegetatively propagated crops; ii) a genotype with a genetic background as close as possible to the GM plant, in the case of crops that are propagated sexually. The term "comparator" should be used in all other cases, i.e. cases in which the comparative assessment includes genotypes which do not fit with the definition of conventional counterpart as provided above.

The risk assessment of GM plants containing single events should include the conventional counterpart, as defined above. Additional comparators, e.g. a negative segregant, may be included if deemed useful to support the risk assessment.

The risk assessment of GM plants containing events stacked by conventional breeding should follow the same principles outlined for the risk assessment of GM plants containing single events and the first choice of comparator should be the conventional counterpart, as defined above. However, where applicants can demonstrate that a conventional counterpart for the GM plant cannot be made available, then applicants could use as comparators either a negative segregant(s), but only where such segregant is derived from crosses between GM plants containing events which have been risk assessed and which are all stacked in the GM plant under assessment; or any set of GM plants that have all been risk assessed on the basis of experimental data collected according to the principles of EFSA MC and FF risk assessment. This set of GM plants must include between them all of the events stacked in the GM plant under assessment, and no others. Additional comparators may be included if deemed useful to support the risk assessment.

In cases the stacking of events is performed applying stacking methods other than conventional breeding - such as co-transformation, re-transformation, and multiple gene cassettes - similar principles as described above for stacking by conventional breeding apply.

In all cases, the applicant should provide information on the breeding scheme (pedigree) in relation to the GM plant, the conventional counterpart and/or other comparator(s) used in the risk assessment together with a clear justification for their selection.

3.1.3.2. Field trials: experimental design and statistical analysis

Experimental design

Field trials used for production of material for the comparative assessment should be performed in order to assess differences and equivalences between three test materials: the GM plant, its comparator (selected in accordance to Section 3.1.3.1) and non-GM reference varieties: the objective is to determine whether the GM plant and/or derived food and feed is different from its comparator and/or equivalent to non-GM reference varieties with a history of safe use.

Detailed guidance on the experimental design for the safety evaluation of GM plants is provided in the EFSA guidance on "*Statistical considerations for the safety evaluation of GMOs*" (EFSA, 2010b; van der Voet *et al.*, 2011).

The field trials should be adequately described, giving information on important parameters such as management of the field before sowing, date of sowing, soil type, herbicide use, climatic and other cultivation/environmental conditions during growth and time of harvest, as well as the conditions during storage of the harvested material.

Natural variation may have several sources: variation within a variety arises due to environmental factors and variation between varieties arises due to a combination of both genetic and environmental factors. In order to identify and estimate differences attributable only to genotypes it is essential to control environmental variability. Therefore non-GM reference varieties should be included in the experimental design of the field trials and in sufficient number to ensure an adequate estimate of the variability required to set the equivalence limits.

All test materials (GM plant, comparator, reference varieties and additional comparator(s) where appropriate) should be randomised to plots within a single field at each site, usually in a completely randomised or randomised block experimental design. The different sites selected for the trials should be representative of the range of receiving environments where the crop will be grown, thereby reflecting relevant meteorological, soil and agronomic conditions; the choice should be explicitly justified. The choice of non-GM reference varieties should be appropriate for the chosen sites and should be justified explicitly. In the case that sites cover a very restricted geographic range, the applicant should replicate the trials over more than one year.

Within each site the GM plant, the comparator (and additional comparators, where appropriate) should be identical for all replicates. In addition, unless there is explicit justification for not doing so, at each site there should be at least three appropriate non-GM reference varieties of the crop that have a known history of safe use, which must also be identical between all replicates. The replication at each site is the number of results obtained for each test material; the replication should never be less than four at any site. However, if only two appropriate non-GM reference varieties are available at a particular site then the replication shall be six at that site; if only one is available then the replication shall be eight.

Each field trial should be replicated at a minimum of eight sites, chosen to be representative of the range of likely receiving environments where the plant will be grown. The trials may be conducted in a single year, or spread over multiple years. The non-GM reference varieties may vary between sites and at least six different reference varieties should be used over the entire set of trials.

When the GM plant is tested together with other GM plants of the same species (e.g. *Zea mays*) the production of material for the comparative assessment of these different GM plants may be obtained simultaneously from the same site and within the same field trial, by placing the different GM plants and their comparator(s) in the same randomised block, as illustrated in Table 1. This should be subject to two conditions:

i. each of the comparator(s) should always occur together with its particular GM plant in the same block;

ii. all the different GM plants and their comparator(s) and all the non-GM reference varieties used to test equivalence with those GM plants should be fully randomized within each block.

If the number of plots per block required for such a trial were to exceed 16, then a partially balanced incomplete block design may be used, to reduce the number of plots per block, by excluding some of the GM plants and their comparator(s) from each block. This should be subject to two conditions:

i. each of the comparator(s) should always occur together with its respective GM plant in the same block;

ii. all of the non-GM reference varieties should appear in each of the incomplete blocks and be fully randomised with the GM plants and their comparator(s).

Table 1: Example of randomized block design for simultaneous testing of multiple GM plants.

Block	Plot									
	1	2	3	4	5	6	7	8	9	10
1	GM2	CV2	CV1	GM3	NIC3	NIC1	CV3	GM1	NIC2	CV4
2	CV2	GM2	CV3	NIC3	NIC2	GM1	NIC1	CV4	CV1	GM3
3	NIC1	NIC3	GM1	CV1	GM3	NIC2	CV2	CV4	CV3	GM2
4	GM3	GM2	CV1	NIC1	CV2	NIC2	NIC3	CV3	CV4	GM1

GM1, GM2, GM3 = three different GM plants of the same species. NIC1, NIC2, NIC3 = the three conventional counterparts (near-isogenic lines) of the three GM plants under assessment, respectively. CV1, CV2, CV3, CV4 = four non-GM reference varieties (commercial varieties).

Statistical analysis

Analysis of data should be presented in a clear format, using standardised scientific units. The raw data and the programming code used for the statistical analysis should be submitted as part of the application dossier and should be given in an editable form.

Detailed guidance on the statistical analysis for the safety evaluation of GM plants is provided in the EFSA guidance on "Statistical considerations for the safety evaluation of GMOs" (EFSA, 2010b).

For each endpoint, the statistical analysis for the comparative assessment involves two approaches:

i. a test of difference, to verify whether the GM plant is different from its comparator and might therefore be considered a hazard (potential risk) depending on the type of the identified difference, in combination with extent and pattern of exposure;

ii. a test of equivalence to verify whether the GM plant is equivalent or not to non-GM reference varieties with a history of safe use, apart from the introduced trait(s). In testing for difference the null hypothesis is that there is no difference between the GM plant and its comparator against the alternative hypothesis that a difference exists. In testing for equivalence the null hypothesis is that the difference between the GM plant and the set of non-GM reference varieties is at least as great as a specified minimum size (see below) against the alternative hypothesis that there is no difference or a smaller difference than the specified minimum between the GM plant and the set of non-GM reference varieties. Rejection of the null hypothesis is required in order to conclude that the GM plant and the set of non-GM reference varieties are equivalent for the endpoint considered. The equivalence limits used for the test of equivalence should represent appropriately the range of natural variation expected for reference varieties with a history of safe use.

Data transformation may be necessary to ensure normality and to provide an appropriate scale on which statistical effects are additive. For many endpoint response variables, a logarithmic transformation may be appropriate. In such cases, any difference between the GM plant and any other test material is interpreted as a ratio on the natural scale. However, for other endpoints the logarithmic transformation may not be optimal and the natural scale or another scale may be more suitable.

The total variability of each endpoint observed in the field trials should be estimated and partitioned using appropriate statistical models in order to derive two sets of confidence limits and to set a lower and upper equivalence limit (EFSA, 2010b) based on the variability observed among the non-GM reference varieties. One set of confidence limits is used in the test of difference; the other set and the equivalence limits (upper and lower) are used in the test of equivalence.

A linear mixed statistical model (denoted model 1) is used for calculation of the confidence limits for both tests (difference and equivalence); a slightly different mixed model (model 2) is used to estimate the equivalence limits to be used in the equivalence test.

Denote by *I* an indicator variable (uncentered in the mixed model) such that *I*=1 for a field plot having any of the non-GM reference varieties, and *I*=0 otherwise. Then the random factors for model 1 should be, but not necessarily be restricted to, those representing the variation: (i) between the test materials (a set that includes the GM plant, its comparator, each of the non-GM reference varieties and any additional comparators); (ii) in the interaction between the test materials and *I;* (iii) between sites; and (iv) between blocks within sites. Model 2 should be identical to model 1 except that the random factor representing the interaction between the test materials and *I* is omitted.

The fixed factor for both models should have as many levels as there are test materials and represent the contrasts between the means of the test materials. The test materials are as defined above: the GM plant; its comparator; the set of non-GM reference varieties; and any additional test materials. The set of non-GM reference varieties is considered as a single level of the fixed factor. For the difference and equivalence tests, the component of the fixed factor of interest is the single degree-of-freedom contrast between, respectively, the GM plant and its comparator, and the GM plant and the set of non-GM reference varieties.

Both the difference test and the equivalence test are implemented using the correspondence between hypothesis testing and the construction of confidence limits. In the case of equivalence testing the approach used follows the two one-sided tests (TOST) methodology (e.g. Schuirmann, 1987) by rejecting the null hypothesis of non-equivalence when both confidence limits fall between the equivalence limits. The choice of 90% confidence limits corresponds to the customary 95% level for statistical testing of equivalence.

For each endpoint, calculation of the confidence limits, estimation of equivalence limits and associated statistical tests is performed as described below, using the following notation. Sample means are denoted by *m*, with subscripts G, C and R for the GM plant, its comparator and the set of non-GM reference varieties, respectively. The variability encompassed in the standard error of the difference between the means of any two test materials, X and Y, calculated using model *i* (i = 1,2), is denoted *sed*(XY;*i*). The 100*a*% point of Student's *t* distribution is denoted as *t*(*df*;*i*;*a*), where *a* defines the width of the tail of the distribution considered, *i* denotes the model used and *df* is the appropriate number of degrees of freedom, which is recommended to be calculated by the Kenward-Roger method. The least significant difference between the means of any two test materials, X and Y, using model *i,* is calculated as the product of *t*(*df*;*i*;*a*) and *sed*(XY;*i*), and is denoted *lsd*(XY;*i*;*a*).

For the difference test, the two-sided 90% confidence limits are calculated about m_G, as $m_G \pm lsd(GC;1;95)$; the null hypothesis of equality between m_G and m_C is rejected and the test deemed statistically significant if m_C falls outside these limits. For the equivalence test, the two-sided 95% equivalence limits are estimated as $m_R \pm lsd(GR;2;97.5)$ and two-sided 90% confidence limits are

calculated about m_G, as $m_G \pm lsd(GR;1;95)$; the null hypothesis of non-equivalence is rejected and the test deemed statistically significant if and only if the confidence limits lie entirely inside the equivalence limits.

It is convenient to assess visually the quantities involved in the above tests for all the endpoints simultaneously, on a single graph or a few graphs. This may be done by shifting all relevant values for each particular endpoint to a scale that has m_C, the mean of the comparator for that endpoint, as its baseline zero value. Therefore, on this new scale, the values of the means of the GM plant, its comparator and the set of non-GM reference varieties, become, respectively: $m_G - m_C$, 0, $m_R - m_C$.

The confidence limits for the difference test become: $m_G - m_C \pm lsd(GC;1;95)$, the equivalence limits $m_R - m_C \pm lsd(GR;2;97.5)$, and the confidence limits for the equivalence test $m_G - m_C \pm lsd(GR;1;95)$. To facilitate visual interpretation, instead of using the two sets of confidence limits in the graphs, only one, that for the difference test, is displayed. Without some adjustment, the confidence limits for the difference test would not give a valid visual representation for the equivalence test on the graph. This problem is overcome by making an adjustment to the displayed equivalence limits. After this adjustment the displayed confidence limits for the difference test may be used as a basis also for the visual representation of the equivalence test. In this way, one confidence limit may serve visually for assessing the outcome of both tests simultaneously. The adjustment of the equivalence limits consists of two steps: (1) scaling the basic equivalence limits, so that the confidence limits required for the difference and equivalence tests have the same width; and (2) an appropriate shift to facilitate display of the adjusted limits, together with m_G, on the scale that has m_C as its baseline zero value. The adjusted equivalence limits for visual display are calculated by the formula:

$$(m_G - m_C) + \{[(m_R - m_G) \pm lsd(GR;2;97.5)]\ lsd(GC;1;95) / lsd(GR;1;95)\}$$

The graph shows the line of zero difference between the GM plant and its comparator and, for each endpoint: the lower and upper adjusted equivalence limits, the mean difference between the GM plant and its comparator, and the confidence limits for this difference (see the set of possible outcomes for a single endpoint in Figure 1). When, in addition to the comparator, another test material is used as additional comparator, the mean difference between the GM plant and that additional comparator, its confidence limits and its adjusted equivalence limits should be displayed on the same graph referred to above, for all such additional comparators, by referring this to the same zero baseline as defined by the comparator. Note that the line of zero difference on the logarithmic scale corresponds to a multiplicative factor of unity on the natural scale. The horizontal axis is labeled with values that specify the change on the natural scale. In the case of logarithmic transformation, changes of 2x and ½x will appear equally spaced on either side of the line of zero difference.

It is a consequence of the simplified graphical display that confidence limits for the difference test were chosen as 90%, yielding a 10% size for the difference test, in which 1 in 10 of such tests is expected to yield a significant result by chance alone. Despite the expected proportion of spurious significant differences, the applicant should report and discuss all significant differences observed between the GM plant, its comparator and, where applicable, any other test material, focusing on their biological relevance (see Section 3.3).

For reporting, full details should be given for each endpoint analysed, listing: (a) the assumptions underlying the analysis, (b) full specification of the mixed models chosen, including fixed and random effects, (c) results of any test of interaction between the test materials and sites, (d) fixed effects, together with the appropriate estimated residual variation with which it is compared, and variance components for the random factors, (e) estimated degrees of freedom, (f) any other relevant statistics. The likely impact of other growing conditions not tested in the trial should be discussed.

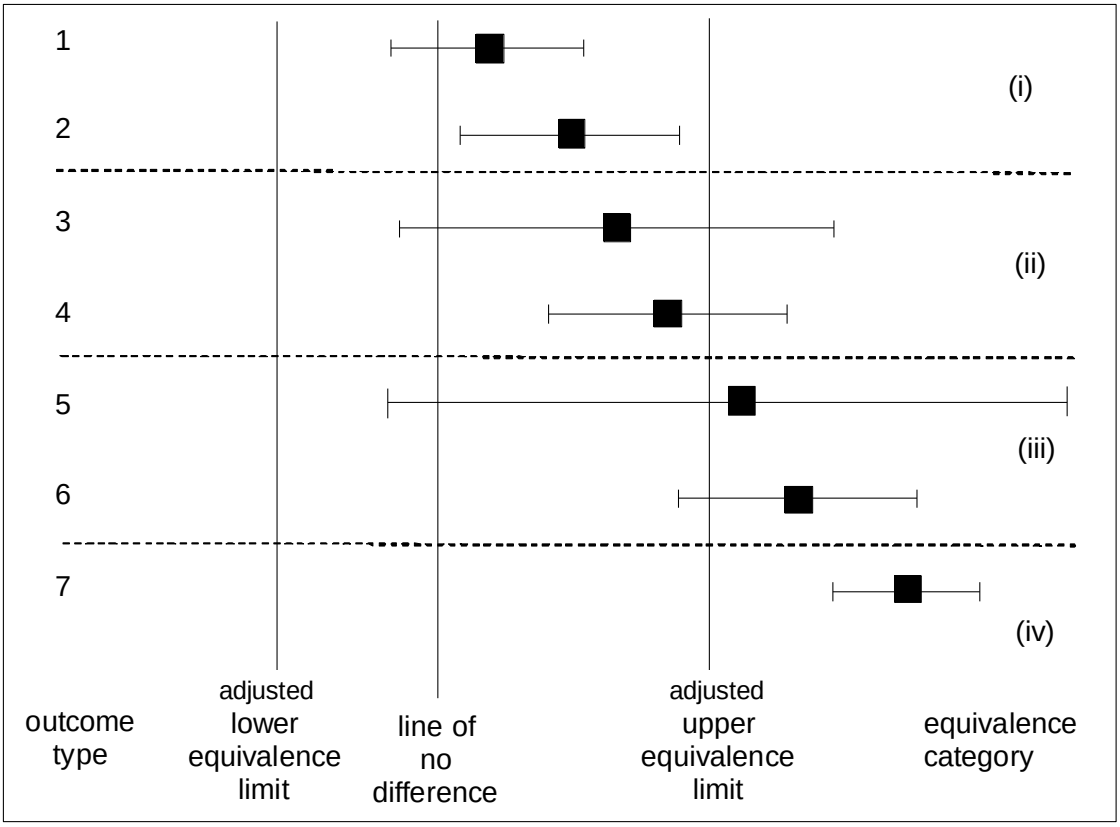

Figure 1. Simplified version of a graph for comparative assessment showing the 7 outcome types possible for a single endpoint. After adjustment of the equivalence limits, a single confidence limit (for the difference) serves visually for assessing the outcome of both tests (difference and equivalence). Here, only the upper adjusted equivalence limit is considered. Shown are: the mean of the GM plant on an appropriate scale (square), the confidence limits (whiskers) for the difference between the GM plant and its comparator (bar shows confidence interval), a vertical line indicating zero difference (for test of difference), and vertical lines indicating adjusted equivalence limits (for test of equivalence). For outcome types 1, 3 and 5 the null hypothesis of no difference cannot be rejected; for outcomes 2, 4, 6 and 7 the GM plant is different from its comparator. Regarding interpretation of equivalence, four categories (i) - (iv) are identified: in category (i) the null hypothesis of non-equivalence is rejected in favour of equivalence; in categories (ii), (iii) and (iv) non-equivalence cannot be rejected. See text for what appropriate conclusions may be drawn.

Regarding the test of difference, each outcome from the graph should be categorised as follows and the respective appropriate conclusion should be drawn.

- Outcome types 1, 3 and 5: the confidence interval bar overlaps with the line of no-difference. The null hypothesis of no difference cannot be rejected and the appropriate conclusion is that there is insufficient evidence that the GM plant and its comparator differ.
- Outcome types 2, 4, 6 and 7: the confidence interval bar does not overlap with the line of no-difference. The null hypothesis of no difference must be rejected and the appropriate conclusion is that the GM plant is different from its comparator.

Regarding the test of equivalence, each outcome from the graph should be categorised as follows, and the respective appropriate conclusion should be drawn.

- Outcome types 1 and 2 (category (i), Figure 1): both confidence limits lie between the adjusted equivalence limits and the null hypothesis of non-equivalence is rejected. The appropriate conclusion is that the GM plant is equivalent to the set of non-GM reference varieties.
- Outcome types 3 and 4 (category (ii), Figure 1): the mean of the GM plant lies between the adjusted equivalence limits, but the confidence interval bar overlaps at least one of the adjusted

equivalence limits on the graph. Non-equivalence cannot be rejected, but the appropriate conclusion is that equivalence between the GM plant and the set of non-GM reference varieties is more likely to be the case than lack of equivalence. Further evaluation may be required.

- Outcome types 5 and 6 (category (iii), Figure 1): the mean of the GM plant lies outside the adjusted equivalence limits, but the confidence interval bar overlaps with at least one of the adjusted equivalence limits. Non-equivalence cannot be rejected and the appropriate conclusion is that equivalence between the GM plant and the set of non-GM reference varieties is less likely to be the case than lack of equivalence. Further evaluation is required.
- Outcome type 7 (category (iv), Figure 1): both confidence limits lie outside the adjusted equivalence limits. The appropriate conclusion is that the evidence analysed here demonstrates non-equivalence between the GM plant and the set of non-GM reference varieties. Further evaluation is required.

In case of significant difference and/or lack of equivalence for any particular endpoint, further analysis should be done to assess whether there are interactions between any of the test materials and site, possibly using a simple standard ANOVA approach. Whatever approach is adopted, details should be given, for each endpoint analysed, listing: (a) the assumptions underlying the analysis, and, when appropriate: (b) degrees of freedom, (c) the estimated residual variation for each source of variation, and variance components, (d) any other relevant statistics. These additional analyses are intended to aid the interpretation of any significant differences found and to study potential interactions between test materials and other factors.

3.1.3.3. Compositional analysis

Analysis of the composition is crucial when comparing the GM plant and derived food and feed with its comparator. The material to be used for the comparative assessment should be selected taking into account the uses of the GM plant and the nature of the genetic modification. Unless duly justified, analysis should be carried out on the raw agricultural commodity, as this usually represents the main point of entry of the material into the food and feed chain. Additional analysis of processed products, should be conducted where appropriate on a case-by-case basis. The sampling, the analysis and the preparation of the material tested should be carried out according to appropriate quality standards.

Besides the analysis on the level of the newly expressed proteins (see Section 3.1.2.2), the compositional analysis should be carried out on an appropriate range of compounds. The compounds for analysis should be selected in accordance with the OECD consensus documents on compositional considerations for new plant varieties (OECD, a) which include proximates (including moisture and total ash), key macro- and micro-nutrients, anti-nutritional compounds, natural toxins, and allergens, as well as other plant metabolites characteristic for the plant species. The vitamins and minerals selected for analysis should be those present at levels which are nutritionally significant and/or those with a nutritional significant contribution to the diet at the levels at which the plant is consumed. Depending on the intended effect of the genetic modification and the nutritional value and use of the plant, specific analyses may be required for an appropriate assessment. For example, a fatty acid profile should be included for oil-rich plants (main saturated, mono-unsaturated and poly-unsaturated fatty acids) and an amino acid profile (individual protein amino acids and main non-protein amino acids) for plants used as an important protein source.

The characteristics of the introduced trait may trigger further analysis of specific compounds including metabolites of potentially modified metabolic pathways. The applicant should consider, when appropriate, the inclusion of compounds other than the nutrients, toxins, and anti-nutrients and allergens identified by the OECD consensus documents and justify the selection of these compounds.

The same conditions apply for GM plants containing stacked events. Additional compounds may be selected for analysis depending upon the introduced traits, as appropriate.

3.1.3.4. Agronomic and phenotypic characteristics

The comparative assessment of the GM plant, containing single or stacked events, with its comparator should address also aspects of the biology of the plant, in the form of agronomic and phenotypic traits (e.g. yield, plant morphology, flowering time, day degrees to maturity, duration of pollen viability, response to plant pathogens and insect pests, sensitivity to abiotic stress). The protocols for field trials to study these characteristics should follow the specifications made under Section 3.1.3.2. Additional information on agronomic traits of the GM plant should be provided from additional field trials, where appropriate.

3.1.3.5. Effects of processing

The applicant should assess whether or not the processing and/or preserving technologies applied are likely to modify the characteristics of the GM end-products compared with their comparators. The applicant should provide a detailed description of the different processing technologies used on the plant, paying special attention to the steps which may lead to significant changes in composition, both with respect to quality and quantity.

Toxicological tests with the processed products are not required if the GM plant (or relevant parts of it) is demonstrated to be safe and there are no indications that the processed products would be any different from their non-GM counterparts. The applicant should provide adequate justification in this regard.

A genetic modification targeting metabolic pathways may result in changes in the concentration of plant constituents and lead to the production of new metabolites (e.g. nutritionally enhanced foods/feeds, functional foods). Processed products derived from such GM plants may require specific approaches for their risk assessment. The applicant should provide a scientific rationale for the selected approach. On a case-by-case basis, experimental data may be required. This may include information on the composition, on the level of undesirable substances, or on the nutritional value.

In the case the GM plant contains new compounds, the effects of processing on these compounds should be evaluated. Other aspects to be taken into account are: the possible impact of processing on the increased or reduced levels of specific compounds; the need for specific processing measures of the food and feed with respect to these altered levels; whether the processing results in the formation of new compounds.

Depending on the nature of the newly expressed protein(s), it may be necessary to assess the extent to which the processing steps lead to the concentration or to the elimination, denaturation and/or degradation of these protein(s) in the final product.

It may also be required to evaluate whether intact and functional transgenic DNA remain after processing.

3.1.3.6. Conclusions

The conclusion of the comparative assessment should clearly state whether:

a) compositional characteristics of the GM plant and derived food and feed are, except for the introduced trait(s), different to those of its comparator and/or equivalent to the non-GM reference varieties, taking into account natural variation;

b) agronomic and phenotypic characteristics of the GM plant are, except for the introduced trait(s), different to those of its comparator and/or equivalent to the non-GM reference varieties, taking into account natural variation;

c) further assessment is needed for those characteristics showing differences between the GM plants and derived food and feed and the comparator, or showing lack of equivalence with respect to non-GM reference varieties, taking into account natural variation;

d) there are indications of interactions between the combined events in the case of GM plants containing stacked events.

3.1.4. **Toxicological assessment**

The purpose of performing toxicological studies of compounds, using either experimental animals and/or *in vitro* systems, is to characterise any hazard linked to their presence and to determine exposure levels that do not result in adverse effects to humans and animals, using uncertainty or safety factors. These factors take into account differences between test animal species and humans, and inter-individual variations among humans. This internationally accepted approach is similar to the one applied for testing chemicals in foods described elsewhere (Renwick *et al.*, 2003; Smith, 2002). The information requirements and testing strategies for whole foods like GM plants and derived food and feed are outlined in the following sections.

The toxicological impact of any biologically relevant change in the GM plant and/or derived food and feed resulting from the genetic modification (e.g. expression of introduced genes, gene silencing or over-expression of an endogenous gene) should be assessed.

Toxicological assessment should demonstrate that the intended effect(s) of the genetic modification has no adverse effects on human and animal health, and that the unintended effect(s), which have been identified or assumed to have occurred based on the preceding comparative molecular, compositional or phenotypic analyses, have no adverse effects on human and animal health.

The applicant should consider the needs for toxicological testing based on the outcomes of the molecular and comparative analyses referred to in Sections 3.1.2 and 3.1.3, *i.e.* the intended and unintended differences and/or lack of equivalences identified between the GM plant and its comparator taking into account natural variation.

Toxicological assessment must consider:

a) presence and levels of newly expressed proteins;

b) potential presence of other new constituents;

c) possible changes in the levels of endogenous constituents beyond normal variation;

d) impact of other changes in composition due to the genetic modification.

In addition to the exposure of consumers and animals through food and feed intake, the applicant should also report any adverse effect(s) of exposure due to individuals' professional activities (e.g. farming, seed processing). Appropriate studies should be performed to further characterise these adverse effects.

In case the applicant considers that a conclusion on safety can be reached without conducting some of the tests recommended in this chapter and/or that other tests are more appropriate, the applicant should state the reasons for not submitting the recommended studies and/or for carrying out studies other than those mentioned in the following section.

3.1.4.1. Standardised Guidelines for Toxicity Tests

Internationally agreed test methods described by the OECD (OECD, b) or by the European Commission (EC, 2002) should be used for toxicity testing. Test protocols may need to be adapted for the toxicological testing of GM plants and derived food and feed. Any adaptation of these protocols, or use of any methods that differ from these protocols, should be explained and justified. Furthermore, new methods (e.g gene expression, profiling, metabolomics, etc.) may complement standard methods to address specific issues.

It is essential that facilities in which toxicological tests are performed, apply appropriate quality assurance systems in order to ensure that the results are of high quality. Such principles are laid down by Directive 2004/10/EC of the European Parliament and Council of 11 February 2004 on the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances (EC, 2004) . If such tests are carried out outside the Union, they should follow "the OECD Principles of Good Laboratory Practice" (GLP). With regard to studies other than toxicological studies, they should be conducted under ISO or GLP standards or other appropriate quality assurance.

Table 2: Non-exhaustive lists of OECD guidelines for testing of chemicals which may be selectively applied for (geno)toxicological testing relevant for GMO risk assessment (OECD, b).

No. OECD	Title
402	Acute Dermal Toxicity
406	Skin Sensitisation
407	Repeated Dose 28-day Oral Toxicity Study in Rodents
408	Repeated Dose 90-Day Oral Toxicity Study in Rodents
410	Repeated Dose Dermal Toxicity:21/28-Day
415	One-Generation Reproduction Toxicity
416	Two-Generation Reproduction Toxicity Study
417	Toxicokinetics
421	Reproduction/Developmental Toxicity Screening Test
471	Bacterial reverse mutation test
473	*In vitro* mammalian chromosome aberration test
474	Mammalian erythrocyte micronucleus test
475	Mammalian bone marrow chromosome aberration test
476	*In vitro* mammalian cell gene mutation test
479	*In vitro* sister chromatid exchange (SCE) assay in mammalian cells
482	DNA damage and repair, unscheduled DNA synthesis in mammalian cells *in vitro*
487	Draft guideline on: *In vitro* mammalian cell micronucleus test

Selection of test protocols depends upon the type of GM plant and derived food and feed, on the genetic modification and intended and unintended alterations, on the intended use and exposure/intake, and on the available knowledge. Most studies recommended for the risk assessment of food derived from GM plants are relevant also for the assessment of feed derived from GM plants.

3.1.4.2. Assessment of newly expressed proteins

All newly expressed proteins should be assessed. The studies required to investigate the potential toxicity of a newly expressed protein should be selected on a case-by-case basis, depending upon the knowledge available with respect to the protein's source, its function/activity and its history of human/animal consumption. Specific toxicity testing is not required in case a proper use and safe consumption as food and feed of both the plant and the newly expressed proteins are duly documented.

If specific testing is required, the tested protein should be equivalent to the newly expressed protein as it is expressed in the GM plant. If, due to the lack of sufficient amount of test materials, a protein produced by microorganisms is used, the structural, biochemical and functional equivalence of this microbial substitute to the newly expressed plant protein should be demonstrated. Comparisons of the molecular weight, amino acid sequence, post-translational modification, immunological reactivity and, in the case of enzymes, the enzymatic activity, are needed to provide evidence of equivalence. In case of differences between the GM plant expressed protein and its microbial substitute, the significance of these differences should be evaluated with respect to the potential safety impact.

To demonstrate the safety of newly expressed proteins, the applicant should provide:

a) molecular and biochemical characterisation of the newly expressed protein, including the amino acid sequence, molecular weight, post-translational modifications and a description of the function. In the case of newly expressed enzymes, information on the enzyme activities including the temperature and pH range for optimum activity, substrate specificity and possible reaction products should be provided. Potential interactions between the newly expressed proteins and other plant constituents should be evaluated with respect to safety impact;

b) up-to-date search for homology to proteins known to cause adverse effects, e.g. toxic proteins. A search for homology to proteins exerting a normal metabolic or structural function may also contribute valuable information. The database(s) and the methodology used to carry out the search should be specified;

c) information on the stability of the protein under the relevant processing and storage conditions for the food and feed derived from the GM plant. The influences of temperature and pH changes should be examined. Potential modification(s) of the proteins (e.g. denaturation) and/or production of stable protein fragments generated through processing and storage should be characterised;

d) data concerning the resistance of the newly expressed protein to proteolytic enzymes (e.g. pepsin), e.g. by *in vitro* investigations using appropriate and standardised tests (see also Section 3.1.5). Stable breakdown products should be characterised and evaluated with regard to the potential risks associated with their biological activity;

e) repeated dose toxicity studies using laboratory animals, unless reliable information demonstrating the safety of the newly expressed protein (including its mode of action) can be provided, and it is demonstrated that the protein is not structurally and functionally related to proteins adversely affecting human or animal health. The repeated dose 28-day oral toxicity study in rodents with the newly expressed protein should be performed according to OECD guideline 407 (Table 2). It is recommended to use a sufficient number of animals per group e.g. 10/sex in order to obtain an adequate statistical power. Depending on the outcome of the 28-day toxicity study, further targeted investigations may be required.

If there is a possibility for synergistic or antagonistic interactions between two or more newly expressed proteins that may impact on safety, the applicant should perform additional studies with combined administration of these proteins.

Acute toxicity testing of the newly expressed proteins is of little additional value to the risk assessment of the repeated human and animal consumption of food and feed derived from GM plants and, therefore, is discouraged.

3.1.4.3. Assessment of new constituents other than proteins

The applicant should provide a safety assessment of identified new constituents. This assessment should include an evaluation of their toxic potency, which may also require toxicological testing as described in the Guidance on submissions for food additive evaluations by the Scientific Committee

on Foods (SCF, 2001) and Commission Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 (EC, 2008) for the preparation and the presentation of applications and the assessment and the authorisation of feed additives. If there is a documented history of safe use and consumption as food and/or feed for the new constituents, then toxicological testing is not needed.

3.1.4.4. Assessment of altered levels of food and feed constituents

This section applies if the genetic modification intentionally or unintentionally altered the content of food and feed constituents beyond the levels expected for the non-GM counterpart. To demonstrate the safety of the altered content of food and feed constituents, such as macro- and micronutrients, anti-nutrients, natural toxins as well as other plant metabolites, the applicant should carry out a risk assessment based on the knowledge of the physiological function and/or toxic properties of these constituents, as well as the anticipated changes in intake levels. The result of this assessment would determine if, and to what extent, toxicological tests are required.

3.1.4.5. Assessment of the whole food and/or feed derived from GM plants

If the composition of the food and/or feed derived from GM plant is substantially modified, or if there are any indications for the potential occurrence of unintended effects based on the preceding molecular, compositional or phenotypic analyses, not only new constituents but also the whole food and feed derived from the GM plant should be tested. In such case the testing program should include a 90-day toxicity study in rodents (EFSA, 2008).

In the case of GM plants containing stacked events, toxicological testing of the whole food and/or feed derived from the GM plant should be considered when there are indications of possible interactions between the events stacked within the GM plant. Such indications may be obtained from the outcome of the molecular characterization, and knowledge of the mode of action of the newly expressed proteins, and possibly from the compositional characterization of the GM plant containing stacked events.

Design and performance of 90-day feeding study in rodents

The design of the toxicity study with whole food and feed derived from a GM plant should be performed according to the principles of OECD guideline 408 (Table 2) following an adapted protocol. Normally a minimum of two test doses and a negative control are used. The highest dose should be the maximum achievable without causing nutritional imbalance; the lowest dose should contain the tested food and/or feed in an amount at least equivalent to the one consumed by humans or animals. It is recommended that, whenever possible, information on natural variation of test parameters is derived from historical background data rather than from the inclusion of reference varieties, consisting of commercially available food and feed derived from non-GM plants with a history of safe use, in the experiments. The statistical analysis should focus on the detection of possible differences between the test material and its control. Detailed discussion is available in the opinion of the EFSA Scientific Committee on 90-day feeding trial protocol (EFSA, 2011b).

Depending on the outcome of the 90-day feeding study, further toxicity studies may be needed (e.g. studies on reproductive/developmental effects, chronic toxicity).

Supplemental information to 90-day feeding studies in rodents on the possible occurrence of unintended effects may be obtained from comparative nutritional studies conducted with young rapidly growing animal species (broiler chicks as animal model for non-ruminants; lambs for ruminants; or other rapidly growing species; see also Sections 3.1.6.1 and 3.1.6.2). Also for these

studies it is recommended that, whenever possible, information on natural variation of test parameters is derived from historical background data rather than from the inclusion of reference varieties, consisting of commercially available food and feed derived from non-GM plants with a history of safe use, in the experiments. The statistical analysis should focus on the detection of possible differences between the test material and its control.

In cases where molecular, compositional, phenotypic, agronomic and other analyses have demonstrated equivalence between the GM plant and derived food and feed and its comparator, except for the inserted trait(s), and have not indicated unintended effects, the performance of animal feeding trials with rodents or other (target) animal species (e.g. broilers) is of little additional value if any, and is therefore not deemed necessary on a routine basis.

Interpretation of animal studies

Changes in test parameters must be evaluated with respect to: (i) relationship with the applied doses, (ii) possible correlations with changes in other biologically related parameters (iii) incidental occurrence, (iv) gender specificity, and (v) normal biological variation. When a difference is noted at only the highest dose applied, other factors should be considered to determine whether there is a relationship with treatment. Information on the background variability in a given parameter may be obtained from data from other animals of the same species/strain tested in the same or other experiments, or from internationally harmonised databases.

Attention should be paid to the fact that certain effects may be specific for the test animal, but not for humans due to interspecies differences.

3.1.4.6. Conclusions

The conclusion of the toxicological assessment should indicate whether:

a) the available information on the newly expressed protein(s) and other new constituents resulting from the genetic modification gives indications of adverse effects in particular, whether and at which dose levels adverse effects were identified in specific studies;

b) the information on natural constituents, with levels different from those in the comparator, provides indications of adverse effects, in particular, whether and at which dose levels adverse effects were identified in specific studies;

c) toxicologically relevant effects have been identified from the animal studies made on the whole food and feed derived from the GM plant, compared to its comparators;

The applicant should evaluate the result of the toxicological assessment in the light of anticipated intake of the food and feed derived from GM plants.

3.1.5. **Allergenicity assessment**

Food allergy is an adverse reaction to food and represents an important public health problem. Food allergy is different from toxic reactions and intolerance. Allergy is a pathological deviation of the immune response to a particular substance, which affects only some individuals where a combined effect of variations in the environment and genetic predisposition has resulted in allergic sensitisation. In allergic individuals, sometimes minute amounts of a food that is well tolerated by the vast majority of the population can cause serious symptoms and death. It is not the allergen *per se*, but the allergic person's abnormal reaction to the allergen that causes the adverse health effect. Food allergy can be caused by various immune mechanisms. However, IgE-mediated food allergy represents the main form of food allergy, that causes the most severe reactions and the only form causing life-threatening

reactions. This IgE-mediated food allergy has been the focus in the risk assessment of allergenicity of GMOs. Importantly, food allergy consists of two separate phases: first *sensitisation* where no symptoms occur while the capacity of the immune system to react increases dramatically, and later *elicitation (provocation)* with clinical manifestations. When ingested, the allergen(s), i.e. the sensitising food or food component is to some extent degraded by digestive enzymes, absorbed by the gut mucosa (small amounts even by the oral mucosa), processed in specialised cells of the immune system and then presented to the reactive immune cells that produce an immune response. Sensitisation can also occur if the food allergen comes into contact with the skin or is inhaled.

The majority of the constituents that are responsible for allergenicity of foods as well as of pollens are proteins. Some protein breakdown products, i.e. peptide fragments, may conserve part of the allergenicity of the native protein and thus can also be considered as allergens. The specific allergy risk of GMOs is associated i) with exposure to newly expressed protein(s) that can be present in edible parts of the plants or in the pollen. This point is related to the biological source of the transgene and ii) with alterations to the allergenicity of the whole plant and derived products e.g. due to over-expression of natural endogenous allergens as an unintended effect of the genetic modification. This point is related to the biology of the recipient organism itself.

3.1.5.1. Assessment of allergenicity of the newly expressed protein

Allergenicity is not an intrinsic, fully predictable property of a given protein but is a biological activity requiring an interaction with individuals with a pre-disposed genetic background. Allergenicity therefore depends upon the genetic diversity and variability in atopic humans. Frequency, severity and specificity of allergic reactions also depend upon geographic and environmental factors. Given this lack of complete predictability it is necessary to consider several aspects in the risk assessment process, to obtain a cumulative body of evidence which minimises any uncertainty with regard to the protein(s) in question.

When studying the structural characteristics and the biological and physicochemical properties of a newly expressed protein, it is essential that the tested protein is equivalent with respect to structure and activity to the newly expressed protein in the GM plant. Studies carried out using purified target proteins prepared by expression in organisms such as *Escherichia coli* are acceptable as long as the properties of the microbial substitute protein are identical to those of the protein expressed in the plant, thus taking into account all post-translational modifications that specifically occur in the plant.

It should be verified whether the source of the transgene is allergenic. When the introduced genetic material is obtained from wheat, rye, barley, oats or related cereal grains, the applicant should also assess the newly expressed proteins for a possible role in the elicitation of gluten-sensitive enteropathy or other enteropathies which are not IgE-mediated. Where events have been stacked, the applicant should provide an assessment of any potential for increased allergenicity to humans and animals on a case-by-case approach. These potential effects may arise from additive, synergistic or antagonistic effects of the gene products.

In line with the recommendations of EFSA (EFSA, 2010c) and the Codex *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology (Codex Alimentarius, 2009), an integrated, case-by-case approach, i.e. so called weight-of-evidence approach, should be used in the assessment of possible allergenicity of newly expressed proteins.

- Amino acid sequence homology comparison between the newly expressed protein and known allergens: in every case, a search for sequence homologies and/or structural similarities between the expressed protein and known allergens should be performed to identify potential IgE cross-reactivity between the newly expressed protein and known allergens. The quality and the comprehensiveness of the databases used should be considered. Improvement and harmonisation

of the algorithms that are used should be sought. The alignment-based criterion involving 35 % sequence identity to a known allergen over a window of at least 80 amino acids is considered a minimal requirement (EFSA, 2010c). All sequence alignment parameters used in the analysis should be provided including calculation of percent identity (PID). It is recommended that the calculation of PID is performed on a window of 80 amino acids with gaps so that inserted gaps are treated as mismatches. In some cases, for assessing short peptidic fragments such as ORFs, a search for sequences of contiguous identical or chemically similar amino acid residue can be conducted. However, this search is not recommended routinely for the identification of potential linear IgE binding epitopes because of its poor sensitivity or specificity.

- Specific serum screening: when there is indication of sequence homology or structure similarities, an important procedure for assessing the potential that exposure to the newly expressed proteins might elicit an allergic reaction in individuals already sensitised to cross-reactive proteins, is based on *in vitro* tests that measure the capacity of specific IgE from serum of allergic patients to bind the test protein(s). It is noted that there is inter-individual variability in the specificity and affinity of the human IgE response. In particular the specificity of the IgE antibodies to the different allergens present in a given food/source and/or to the different epitopes present on a given protein may vary amongst allergic individuals. In order to optimize the sensitivity of the test, individual sera from well-characterised allergic individuals should be used rather than pooled sera. Specific serum screening should be performed in the following cases:

  If the source of the introduced gene is considered allergenic even if no sequence homology of the newly expressed protein to a known allergen is demonstrated or if the source is not known to be allergenic but there is any indication of relationship between the newly expressed protein and a known allergen, based on sequence homology or structure similarity.
  Specific serum screening should be undertaken with sera from individuals with a proven allergy to the source or to the potentially cross-reacting allergen using relevant immunochemical tests. IgE-binding assays (such as Radio or Enzyme Allergosorbent Assay (RAST or EAST), Enzyme Linked Immunosorbent Assay (ELISA) and electrophoresis followed by immunoblotting with specific IgE-containing sera) are adequate methods.

- Pepsin resistance and *in vitro* digestibility tests. Stability to digestion by proteolytic enzymes has long been considered a characteristic of allergenic proteins. Although it has been established that no absolute correlation exists (EFSA, 2010c; Fu *et al.*, 2002), resistance of proteins to pepsin digestion is still proposed as an additional criterion to be considered in an overall risk assessment. The pepsin resistance test is generally performed under quite standardized conditions (Thomas *et al.*, 2004), at low pH values and high pepsin:protein ratios. It is recognized that the pepsin resistance test does not reflect the physiological conditions of the digestion. The digestibility of the newly expressed proteins in specific segments of the population such as infants and individuals with impaired digestive functions may be assessed using *in vitro* digestibility tests using different conditions (EFSA, 2010c). Also, since the protein encoded by the newly introduced genes will be present in the product as a complex matrix, the impact of the possible interaction between the protein and other components of the matrix as well as the effects of the processing should be taken into account in additional *in vitro* digestibility tests. Depending on the outcome of the *in vitro* digestibility test, it could also be useful to compare intact, heat-denatured and pepsin-digested proteins for IgE binding, since an altered digestibility may impact on the allergenicity of the newly expressed protein.

- Although additional tests including *in vitro* cell based assays or *in vivo* tests on animal models have not been validated so far for regulatory purposes, they may be considered useful to provide additional information e.g. on the potential of the newly expressed protein for de novo sensitisation.

3.1.5.2. Assessment of the allergenicity of the whole GM plant

When the recipient of the introduced gene is known to be allergenic, the applicant should test any potential change in the allergenicity of the whole food derived from a GM plant by comparison of the allergen repertoire with that of its appropriate comparator(s). This recommendation is based on the possibility that the genetic modification might have induced an unintended effect, e.g. resulting in an over-expression of natural endogeneous allergen(s).

The approach should be applied on a case-by-case basis depending on the available information on the allergenic potential of the recipient organism. It is generally performed by analytical methodologies such as proteomics in association with the use of allergic human sera as probes. Sera from clinically well-characterised allergic individuals that are the reference material for IgE binding studies may be available in limited number and quantity. In order to minimize the use of human sera, preliminary important information on the likelihood of an unintended alteration of the overall allergenicity of the GM plant can be obtained by using sera of animals experimentally sensitised in well-defined conditions and by including relevant identified endogenous allergens in the comparative compositional analysis of the GM plant and its appropriate comparator(s).

The integrated process applies to the assessment of the allergenicity of the edible components and the pollen of GM plants (i.e. covers both food and respiratory allergy risk).

In addition, the applicant should provide, where available, information on the prevalence of occupational allergy in workers or in farmers who have significant exposure to the GM plant, or to the airborne allergens they may contain.

On a case by case post market monitoring programs can also be proposed to confirm the absence of increased allergenic risk in actual conditions of exposure.

3.1.5.3. Adjuvanticity

Adjuvants are substances that, when co-administered with an antigen increase the immune response to the antigen and therefore might increase as well the allergic response. In cases when known functional aspects of the newly expressed protein or structural similarity to known strong adjuvants may indicate possible adjuvant activity, the possible role of these proteins as adjuvants should be considered. As for allergens, interactions with other constituents of the food matrix and/or processing may alter the structure and bioavailability of an adjuvant and thus modify its biological activity (EFSA, 2010c).

3.1.5.4. Conclusions

The conclusion of the allergenicity assessment should clearly indicate whether:

- the novel protein(s) is likely to be allergenic;
- the food derived from GM plant is likely to be more allergenic than the comparator.

When there is a likelihood of allergenicity in one of the above mentioned cases, the food derived from GM plant should be further characterised in the light of anticipated intake and appropriate conditions for placing on the market, including labelling, should be proposed.

3.1.6. **Nutritional assessment**

The applicant should provide a nutritional evaluation to demonstrate that the introduction of food and feed derived from a GM plant into the market is not nutritionally disadvantageous to humans and

animals, respectively. This evaluation should include an assessment of: (i) the nutritional relevance of newly expressed proteins and other new constituents; (ii) the changes in the levels of endogenous constituents in the GM plant and derived food and feed; (iii) the potential alterations in the total diet for the consumers/animals.

If the GM plant and derived food and feed have been assessed as compositionally not different from its comparator except for the introduced trait(s) (see Sections 3.1.2 and 3.1.3), no further studies to demonstrate nutritional equivalence are required. If, on the basis of the comparative assessment it is not possible to conclude on nutritional equivalence, further studies should be carried out (see Sections 3.1.6.1 and 3.1.6.2).

Further information is available in the Report of the EFSA GMO Panel Working Group on Animal Feeding Trials (EFSA, 2008) and in the opinion of the EFSA Scientific Committee on 90-day feeding trial protocol (EFSA, 2011b).

3.1.6.1. Nutritional assessment of food derived from GM plants

The nutritional assessment of food derived from GM plants should consider:

(a) the composition of the food with regard to the levels of nutrients and anti-nutrients (see compositional studies as described in Section 3.1.3.3);

(b) the bioavailability and biological efficacy of nutrients in the food taking into account the potential influences of transport, storage and expected treatment of the foods;

(c) the anticipated dietary intake of the food and resulting nutritional impact.

When the comparative assessment has identified compositional characteristics of the food derived from a GM plant that are different and/or not equivalent to those of its comparator, their nutritional relevance should be assessed further, for instance performing specific studies in rodents, poultry and/or livestock depending upon the GM crop under assessment (ILSI, 2003, 2007).

In cases where an altered bioavailability may raise concern for specific sub-population(s), the level of the nutrient in the food should be determined, taking into account all the different forms of the compound. The methods to test bioavailability should be selected on a case-by-case basis.

3.1.6.2. Nutritional assessment of feed derived from GM plants

When the comparative assessment has identified compositional characteristics of the feed derived from a GM plant that are different and/or not equivalent to those of its comparator, their nutritional relevance should be assessed in target animal species selected depending upon the GM crop under assessment (e.g.: poultry, pigs or ruminants). In the case of GM plants modified for altered content of nutrients, livestock studies with model or target species should be performed in order to determine the bioavailability of individual nutrients in the feed derived from a GM plant compared to its comparator (ILSI, 2003, 2007).

In the case of GM plants modified with traits enhancing animal performance, through increased nutrient density (e.g. increased oil content) or through a higher level of a specific nutrient (e.g. an essential amino acid or a vitamin), an appropriate control diet with similar nutrient profile should be formulated. Such diet should use a non-GM control supplemented with the specific nutrient as present in the GM plant. In case of food derived from animals fed feed with modified nutritional value, it may be necessary to assess their nutritional profile.

Target animal feeding studies should span: (i) from the growing and/or the finishing period to slaughter for chickens, pigs, and cattle; (ii) the major part of the lactation cycle for dairy cows; and

(iii) the laying cycle for laying hens or quails. Growth studies with aquatic species, such as carp or other typical herbivores, are preferable for feedstuff intended only for aquaculture.

As appropriate, other tests demonstrating that the nutritionally altered feed fulfils the expected nutritional value should be provided, on a case-by-case basis. The experimental design and statistical approach of feeding studies will depend upon the choice of animal species, the type of plant trait(s) studied and the magnitude of the expected effect. Endpoints' measurements will vary according to the target species used in the study, but should include animal health and welfare, animal losses, feed intake, body weight, and animal performance (EFSA, 2008). Specific studies may be carried out to measure the digestibility and bioavailability of nutrients, in case these have been targeted by the genetic modification.

3.1.6.3. Conclusions

The conclusion of the nutritional assessment of food and feed derived from GM plants should indicate:

- if the food and feed is nutritionally equivalent to its comparator, taking into account natural variation;
- in case of lack of equivalence, if the identified changes have an impact on the anticipated intake of the food and feed;
- if the unintended effects of the genetic modification, either identified during hazard identification or assumed based on the preceding molecular, compositional and phenotypic analyses, have affected the nutritional value of the food and feed;
- in case of GM plants containing stacked events, if there are changes in nutritional value due to additive, synergistic or antagonistic effects of the gene products. This may be particularly relevant where the combined expression of the newly introduced genes affects biochemical pathways. This assessment will clearly require a case-by-case approach.

3.2. Exposure assessment - Anticipated intake/extent of use

An estimate of the expected intake is an essential element in the risk assessment of GM plants and derived food and feed. Information on the intended function, the dietary role, and the expected level of use of the food and feed derived from the GM plant should be provided by the applicant. When the genetic modification targets agronomic traits the intake of the plant species is not expected to be changed.

The applicant should determine the concentrations of the newly expressed proteins, other new constituents and endogenous constituents with levels altered as a result of the genetic modification (e.g. due to changes in metabolic pathways) in those parts of the GM plant intended for food or feed use. Expected intake of these constituents should be estimated taking into account the influences of processing, storage and expected treatment of the food and feed in question, e.g. potential accumulation or reduction. In cases where the genetic modification has resulted in an altered level of an endogenous constituent, or if a new constituent occurs naturally in other food and feed products, the anticipated change in total intake of this constituent should be assessed considering realistic as well as worst intake scenarios.

The applicant should estimate the anticipated average and maximum intake levels of the food and feed based on representative consumption data for products derived from the respective conventional plants. Probabilistic methods may be used to determine ranges of plausible values. The applicant should identify particular groups of the population with an expected high exposure and should consider them within the risk assessment. Any assumption made on the exposure assessment should

be described. Recent developments in methodologies and appropriate consumption data should be used. Data on import and production quantities may provide additional information for the intake assessment.

The applicant should provide information on known or anticipated human/animal intake considering all possible routes of exposure.

3.3. Risk characterisation

3.3.1. Introduction

The risk characterisation of GM plants and derived food and feed is based on data from hazard identification, hazard characterisation, and exposure/intake assessment. A comprehensive risk characterisation should be carried out considering all the available evidence from several analyses including molecular, phenotypic, agronomical and compositional analysis, together with toxicity and allergenicity testing. The risk characterisation may give indications for specific measures during post-market monitoring of food and feed derived from GM plants.

Uncertainties identified at any stage of the risk assessment should be highlighted and quantified, to the extent possible (EFSA, 2006b). Distinction should be made between uncertainties reflecting natural variation in ecological and biological parameters (including variations in susceptibility in populations), and variation reflecting differences in responses between species.

Depending on the issue to be addressed and the available data, risk characterisation may be only qualitative, but may also be quantitative. The estimated risk and associated uncertainties should be as precise as possible.

3.3.2. Issues to be considered for risk characterisation

Risk assessment of GM plants and derived food and feed should be carried out in an integrative manner and, on a case-by-case basis depending on the type of genetic modification, should take into consideration environmental factors including cultivation practice that may influence food and feed quality. The applicant should take into account the different issues considered in the hazard identification and characterisation and in the exposure assessment. The list of issues provided in this section is by no means exhaustive.

Molecular characterisation

Evaluation of the characteristics and previous use of the donor and the recipient organism is a key element to identify the need for specific analyses, e.g. occurrence of specific toxins, or allergens in the unmodified recipient plant, which may be unintentionally increased as result of the genetic modification.

Transformation protocols, molecular characterisation strategies and the specificity and sensitivity of the methods used should be discussed in relation to the intentional and possibly unintentional insertion and expression of gene sequences.

Where sequence analysis has identified a potential hazard, it should be demonstrated how approaches like bioinformatic analysis, compositional/agronomical analysis, and possibly animal feeding trials with the whole food and feed contribute to the safety assessment. The value of the results obtained should be evaluated in the light of the available knowledge on the structure and function of genomic databases of the plant species in question.

In cases of GM plants containing stacked events, the additional risks possibly arising from the combined effects of the stacked genes, e.g. effects on biochemical pathways should be evaluated.

Comparative assessment

An important issue to be evaluated is whether the comparative assessment was carried out appropriately. The choice of comparator is key to such assessment and its selection must be performed according to the principles described in Section 3.1.3.1. The goal of the comparative assessment is to identify possible differences and/or lack of equivalences between the GM plant and its comparator, taking into account natural variation. These differences and/or lack of equivalences should be assessed with respect to their possible impact on food and feed safety and nutritional properties. The estimated risk and associated uncertainties should be as precise as possible and taken into account.

Information on variation of constituents from databases

In principle it could be possible to establish expected constituents' variation for the comparative assessment (i.e. equivalence limits) using data obtained from databases. However, the GMO Panel recommends establishing equivalence limits from the non-GM reference varieties included in the field trials (see Section 3.1.3.2) rather than from databases. It is critical that the databases used contain detailed information on the particular variety concerned and provide a sufficient characterisation of the environments concerned to allow the elimination of confounding effects, linked to environmental differences, in the comparison of GM plants with commercial varieties.

Toxicological assessment

The data generated to estimate possible risks for human and animal health associated with the consumption of food and feed derived from GM plants should be evaluated with respect to the expression of new proteins and/or metabolites, and to the presence of altered levels of endogenous plant proteins and/or metabolites in the food and feed, taking also into account unintended effects of the genetic modification. If specific studies demonstrate that single constituents and/or the whole food and/or feed can induce adverse effects, these should be addressed by applicants (e.g. dose response relationships, threshold levels, delayed onset of adverse effects, risks for certain groups in the population, use of uncertainly factors in extrapolating from animal data to humans).

The relevance of short-term toxicity data to predict possible long-term adverse effects of newly expressed proteins and/or new metabolites in the GM plant and derived food and feed should be discussed. The absence or inclusion of specific data on long-term studies (e.g. on reproductive and developmental toxicity) should also be discussed, when applicable. In the case of feeding studies with the whole food and feed the outcomes should be evaluated taking into account experimental limitations (e.g. dose range, dietary composition, confounding factors).

Data on the characteristics of the new compounds present in the GM plant, which may affect humans and animals, should be considered. If the compounds have known adverse health effects and maximum levels for their presence in plants or derived products are laid down in specific legislations, these maximum levels should be taken into account. If these are not available, reference values for acceptable or tolerable levels of intake, such as the Acceptable Daily Intake (ADI) or Tolerable Upper Intake Level (UL), should be taken into account in relation to the anticipated intake. In cases where the compounds have been safely consumed in foods, the intake levels of consumers from a conventional diet can be implicitly considered as safe.

Exposure Assessment

The methodologies used for intake estimations of food derived from GM plants (in particular those with modified nutritional quality) should be evaluated with respect to their uncertainties associated with predictions on long-term intake. Post-market monitoring requirements for foods with modified nutritional qualities should monitor the occurrence of changes of the overall dietary intake patterns,

the magnitude of such changes, and whether or not the product induces known or unexpected side effects. If a post-market monitoring is deemed necessary, the reliability, sensitivity and specificity of the proposed methods should be discussed.

3.3.3. The result of risk characterisation

The applicant should ensure that the final risk characterisation clearly demonstrates that:

a) consumption of food and feed derived from GM plants is as safe as the respective comparators;

b) the food derived from a GM plant is not nutritionally disadvantageous for the consumer compared to the food which is intended to replace;

c) the feed derived from a GM plant feed is not nutritionally disadvantageous for animals compared to the feed which is intended to replace;

d) the feed derived from a GM plant does not harm or mislead the consumer by impairing distinctive features of the animal products compared to conventionally produced feed.

The applicant should indicate what assumptions have been made during the risk assessment in order to predict the probability of occurrence and severity of adverse effect(s) in a given population, and the nature and magnitude of uncertainties associated with estimating these risks.

The applicant should also include detailed information justifying the inclusion or the non inclusion in the application of a proposal for labelling in accordance with Articles 5(3)(f) and 17(3)(f) of Reg. (EC) No 1829/2003 (EC, 2003a).

3.4. Post Market Monitoring (PMM) of food and feed derived from GM plants

Where appropriate a Post Market Monitoring (PMM) programme should be performed for food and feed derived from GM plants. PMM does not substitute a thorough pre-marketing toxicological testing programme, rather complements it in order to confirm the pre-market risk assessment. It may increase the probability of detecting rare unintended effects. Therefore the PMM for food and feed should be designed to generate reliable and validated flow of information between the different stakeholders which may relate consumption of food and feed derived from GM plants to any (adverse) effect on human and animal health.

As pre-market risk assessment studies cannot fully reproduce the diversity of the populations who will consume the marketed product, the possibility that unpredicted side effects may occur in some individuals, such as those with certain disease states, those with particular genetic/physiological characteristics or those who consume the products at high levels remains. Indeed, risk assessment also relies on an estimate of exposure to the food, which is variable and subject to uncertainty before the food is marketed. A PMM should therefore address the following questions: is the product use as predicted/recommended? Are known effects and side-effects as predicted? Does the product induce unexpected side effects? (Wal *et al.*, 2003)

PMM should be required only in specific cases, such as foods with altered nutritional composition and modified nutritional value and/or with specific health claims. A similar approach can apply to feed with altered nutritional characteristics.

4. RECOMMENDATIONS FOR FUTURE DEVELOPMENTS

Since compositional analysis is one of the cornerstones of the comparative risk assessment of GM plants and derived food and feed, further development, harmonization and validation of databases covering the composition of crops, and providing reliable estimates of their variability due to different environmental conditions, is recommended. Also in the case of animal feeding trials of whole food and feed derived from GM plants further collection and standardisation of data on test parameters with respect to natural variation is recommended. Systematic and standardised collection of data would also allow meta-analysis of safety relevant data. Initiatives in such respect should preferably be taken at the international level.

The EFSA GMO Panel takes into consideration all available scientific evidence in its evaluation of risk assessment. Part of the information provided in GM plant applications has been generated under GLP conditions, in particular with respect to performance of animal toxicity studies. Further development, standardisation and validation of methodologies for molecular characterisation and compositional analysis, including quality assurance and statistical methodologies, is encouraged.

In cases where a comparative assessment is not applicable, a comprehensive food and feed safety and nutritional assessment of the GM plant and derived food and feed should be performed. This should include, among others, a detailed compositional analysis and specific toxicological/nutritional analyses, selected according to the agronomic and compositional properties of the food and feed under assessment. Further development and detailing of this strategy is needed.

To further map the possible effects of the genetic modification in GM plants, profiling technologies such as genomics, transcriptomics, proteomics and metabolomics, should be further explored for the comparative assessment (Cellini *et al.*, 2004; Davies *et al.*, 2010; ILSI, 2004; Kuiper *et al.*, 2003). These non-targeted approaches may be, for instance, of particular relevance for food and feed derived from GM plants with specific metabolic pathways modified, e.g. those leading to enhanced nutritional profiles, obtained through the insertion of single or multiple genes. The potential to apply these technologies to studies with animals fed feed derived from GM plants should be further investigated.

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