Scientific Institute of Public Health

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BIOSAFETY COUNCIL

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GUIDANCE NOTES FOR

THE SAFETY ASSESSMENT OF GENETICALLY MODIFIED CROPS FOR FOOD AND FEED USE

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Edited by

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Introduction

Ellen Van Haver

1 Scope of the document

This document describes the elements needed for the safety assessment of foods and feeds derived from genetically modified crops. This document does not address environmental, ethical or socio-economical aspects of the marketing of these foods and feeds, but considers the aspects with relevance to human or animal health.

This document provides guidance to notifiers and biosafety assessors as to which extent studies have to be carried out following a case-by-case approach for the evaluation of genetically modified food and feed crops. This guidance document is not a static data package, but should be considered along with newly evolving scientific knowledge and technology.

The scientific aspects and the presentation of information in the dossier should fulfil the requirements of the ongoing legislations and recommendations as specified in section 4 of this chapter. This document complements these requirements by providing more detailed guidelines for the safety assessment of foods and feeds derived from genetically modified crops.

2 Genetically modified food and feed crops

The first transgenic crops destined for human or animal consumption which are currently in use or close to commercialisation, have been modified for improved agronomic properties. These so-called first-generation crops have been altered to confer resistance to common pests or to introduce tolerance to selected herbicides for better weed control. To date only a few crops have been modified for improved quality traits. These traits will continue to be targets of next-generation products to obtain foods and feeds with enhanced or altered nutritional properties. Although these products have not entered the market yet, the safety assessors should be aware of their possible rapid arrival and the possible implications for their safety assessment approach.

This document addresses in first instance the safety evaluation of first-generation crops, but also takes into consideration the information that could be needed for the safety assessment of next-generation products. The need for additional tests could however be extended if, once these products are submitted for authorisation, experience shows that the available tests are not enough for a thorough safety assessment.

3 Food versus feed safety assessment

Many crops (or by-products of these crops) are used for human consumption as well as for livestock feeding. The food and feed safety assessment of such crops will consequently follow the same strategy, starting with a thorough description of the genetic modification, followed by the assessment of the potential toxicity and allergenicity of the newly expressed gene products and metabolites, and finally, the evaluation of the nutritional aspects of the genetically modified crop.

On the implementation level there can however be some differences between the food and feed safety assessment. The necessary parameters to be measured can be different, as well as the methods of analysis to be used. Furthermore, the safety assessment of animal feeds should consider, besides any risk to the animals consuming the feed, any indirect risk to the consumer of animal products.

4 Food and feed safety regulations

Directive 90/220/EEC put in place a step-by-step approval process on a case-by-case assessment of the risks to human health and the environment of any genetically modified organism or product consisting of or containing genetically modified organisms before it can be released into the environment or placed on the market (EU, 1990). Since 17th October 2002 Directive 90/220/EEC is superseded by Directive 2001/18/EC (EU, 2001a). The principles for the environmental risk assessment are laid down in Annex II of Directive 2001/18/EC and guidance notes to this annex are provided by the Decision 2002/623/EC (EU, 2002).

Risk assessments carried out under Directive 2001/18/EC address risks to human and animal health after exposure to the genetically modified organism concerned, including incidental consumption; it does not address the use in food of genetically modified organisms or their products. Genetically modified food destined for human consumption is currently regulated by Regulation (EC) 258/97 on Novel Foods and Novel Food Ingredients (EU, 1997a). The scientific aspects and the presentation of information necessary to support applications for the placing on the market of genetically modified foods are provided by the recommendations 97/618/EC (EU, 1997b). There is currently no specific regulation governing the use of genetically modified feed and the authorisation occurs in accordance with Directive 2001/18/EC. In July 2001, the European Commission adopted a proposal for a Regulation of the European Parliament and of the Council on genetically modified food and feed (EU, 2001b). This draft regulation provides authorisation on genetically modified organisms for food and/or feed use, food and/or feed containing or consisting of genetically modified organisms, as well as food and/or feed produced from or by genetically modified organisms. From the date of application of the genetically modified food and feed regulation, the provisions laid down in this regulation will repeal the provisions in the Novel Food regulation on genetically modified foods.

5 Introduction to food and feed safety assessment

New varieties obtained through traditional breeding methods are generally recognised as safe and continue to enter the market. These varieties are evaluated by breeders for agronomic and phenotypic characteristics, but the genetic and

metabolic changes associated with traits such as disease and pest-resistance, introduced by conventional techniques from wild species, are rarely characterised in detail. Despite of the possible occurrence of undesirable new combinations of genes by conventional breeding, a formal food safety assessment is not required for conventionally bred crops.

The genetic material in crops obtained through genetic modification can also be altered in a way that does not occur naturally by mating or natural recombination. Genetic modification also allows selected individual genes to be transferred from one organism into another, also between non-related species. Crops obtained through genetic modification have, in contrast to conventional obtained crops, to be subjected to rigorous food and feed safety testing procedures.

In conventional breeding, as well as in genetic engineering, rearrangements or transfer of genes can result in the expression of one or more new constituents in the crop, or change the expression of existing constituents, either positively or negatively. It should therefore be more appropriate if the safety assessment has to be carried out regardless the method of modification used. The use of a particular method of breeding or genetic engineering does not give the resulting plant a particular property. Its properties will depend on what genes are transferred or modified.

This document is in first instance designed for foods and feeds derived from genetically modified crops.

6 Safety assessment strategy

The elements to be considered in the safety assessment strategy concern the characterisation of the genetic modification, the evaluation of the possible toxicity and allergenicity of gene products and metabolites, as well as the consideration of the nutritional aspects of the food or feed in question. These aspects are dealt within separate chapters further on in the document.

Contrary to the risk evaluation of food additives, residues of pesticides and medicinal products, for which the substance to be tested is well characterised, of known purity and of no particular nutritional value, foods and feeds do contain complex mixtures of compounds. The safety evaluation of a single, well-defined chemical is virtually impossible and due to their effect on nutritional imbalances, the application of traditional toxicological testing and risk assessment procedures to whole foods and feeds is not possible. A more focused approach is required for the safety assessment of foods and feeds derived from plants, including genetically modified crops.

The food or feed derived from the genetically modified crop is in first instance compared with its conventional counterpart to establish the extent of equivalence[1]. Following a holistic approach this comparison implies for instance the characterisation of the genetic modification and the analysis of the composition. Based on the identified differences, the genetically modified food or feed will be

[1] Establishing the extent of equivalence is known as the concept of *substantial equivalence*. This concept has attracted some criticism, partly due to the misperception that substantial equivalence is the endpoint of a safety assessment, rather than the starting point (WHO/FAO, 2000; OECD, 2001).

subject to further safety assessment. Different outcomes of the comparative approach can be envisaged (EU, 1997b):

- o substantial equivalence can be established to its conventional counterpart; the need for further testing should be investigated on a case-by-case basis.
- o substantial equivalence can be established except for a single or few specific traits of the genetically modified crop, in which case any further assessment of safety should focus specifically on these traits; safety tests include for instance toxicity and allergenicity testing and analysis of the nutritional impact of the genetically modified food or feed in the diet.
- o neither partial nor total substantial equivalence can be established; on a case-by-case basis, the wholesomeness of the whole food or feed has to be assessed using an appropriate combined nutritional-toxicological approach.

7 References

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Molecular characterisation

Working Group "Molecular characterisation of transgenic plants": Geert Angenon, Marc Boutry, Marc De Loose, Jacques Dommes, Godelieve Gheysen, Pierre Van Cutsem, Nathalie Verbruggen; Secretariat: Adinda De Schrijver

1 Introduction

Following the comparative approach the characterisation of the genetic modification allows to identify at molecular level the differences between the genetically modified crop and its conventional counterpart. In terms of the safety assessment these data are important to characterise the introduced or modified trait and to check if unintended effects of the genetic modification have taken place, for instance as a consequence of possible rearrangements of the DNA at the site of insertion. The (extent of) expressions of (new) proteins in the genetically modified crop should also be assessed to identify the possible occurrence of toxicants and allergens, which have to be evaluated along with the information described in chapters III and IV.

2 Information on the presentation of data

Any relevant scientific articles and reports referred to should accompany the dossier. The quality of the experimental data should also be sufficient to verify clearly any statements made by the applicant and the notifier should submit all sequences in electronic format.

3 Description of the genetic material used for the transformation

For each of the vectors used for transformation, the notifier has to provide a detailed map including the genetic elements listed hereafter, indicating their location, order and orientation in the vector, and the position of relevant restriction sites.

For each of the vectors used for transformation, a list (and a table summarizing name, position and brief description) should be provided of all genetic elements, including coding and non-coding sequences (e.g. origins of replication, T-DNA borders of ***Agrobacterium***, bacterial transposable elements, promoters). For each of these elements the notifier should include:

- A **description** of the genetic element, or a **citation** where the genetic element was isolated and characterized (completed with an accession number in a publicly available database).
- The **portion** and **size** of the genetic element that was inserted in the vector, and its **location** in the vector.
- Information about its **source**. The scientific and the common or trade name of the donor organism should be given. The history of use of the

donor organism (or that of relevant elements thereof) and its relevance to risk assessment should be described, indicating whether the donor organism is responsible for any disease or injury to plants or other organisms (e.g. produces toxicants, allergens, pathogenicity factors or irritants).

- Information on whether the genetic element itself is coding for or involved in the production of proteins responsible for **disease** or **injury** to plants or other organisms (e.g. a toxicant, allergen, pathogenicity factor or irritant).

- Information about the molecular, biochemical and physiological **properties of its products**, as known in the donor organism and aimed at in the transgenic plant.

For direct transformation methods, data should be provided on how the part(s) of the vector(s) used for the transformation was purified and indicate how purity was assessed.

4 Description of the transformation method

The transformation protocol should be **described** and relevant **references** should be provided for the transformation method. In case of direct transformation pure DNA has to be used, implying the absence of carrier DNA.

For ***Agrobacterium***-mediated transformation, the **strain** designation of the ***Agrobacterium*** used during the transformation process should be provided, and it should be indicated if and described how the Ti/Ri plasmid based vector was **disarmed**.

For transformation methods that involve the use of **helper plasmids**, these plasmids should be described in detail.

5 Description of the transgene loci

Experimental data should be included revealing the **number of sites** where (part of the) DNA used for the transformation is inserted and it should be indicated whether it is located in the **nucleus, mitochondria or chloroplasts**. For allopolyploid plants it should be indicated into **which parental genome** these insertions have occurred. The methods that were used should be described and their **sensitivity** should be assessed.

For each of the insertion sites the notifier should provide:

- The sequence of the entire insert and of both flanking regions (about 500 basepairs, proven to correspond to plant DNA using appropriate methods). The different genetic elements should be delineated and any rearrangements on a schematical representation of the transgene locus should be indicated. A list of all the genetic elements and rearrangements should be provided and, for each of these, its position and origin has to be indicated and its integrity to be evaluated.

- An examination of the **nature of the flanking sequences** using up-to-date bioinformatics tools (database searches, prediction models, etc.).

- An evaluation of the presence and functionality of **novel chimaeric open reading frames** applying up-to-date bioinformatics tools (database searches and application of prediction models designed to reveal the presence of open reading frames, searches for immunologically and toxicologically relevant similarity, etc.) on the complete sequence. If a chimaeric open reading frame is detected that extends beyond the region, more of the flanking region should be sequenced until the putative end of this open reading frame is reached.

6 Transcript and protein characterisation

The expression of all open reading frames identified in section 5 should be analysed. The methods that were used for the expression analysis should be described and their sensitivity assessed.

- For open reading frames intended to be expressed in the transgenic plant, data should be provided on the levels and the spatial and temporal specificity of expression at the protein level. In case the purpose of the transformation is to alter the expression of endogenous genes (e.g. by antisense constructs, ribozymes, or via the mechanism of RNA silencing), data should be included on the expression of the target.

- For all other genes present on the DNA used for the transformation and (partly) inserted in the genome of the transgenic plant, data should be provided on the levels and tissue specificity of expression at the transcript and/or protein level, unless it can be demonstrated that the necessary regulatory sequences for expression are not linked to the open reading frame, or unless the open reading frame is linked to a non-plant promoter for which it can be demonstrated or for which reference can be provided that it is not functional in the plant, or unless it can be otherwise demonstrated that the open reading frame is not expressed. If transcription occurs, it should be determined whether the transcript is translated.

- For novel chimaeric open reading frames identified in section 5, data should be provided on the levels and tissue specificity of expression at the transcript and/or protein level. If transcription occurs, it should be determined whether the transcript is translated.

The **properties** of the **proteins** that are expressed in the plants or the target proteins of which the expression level has been altered should be described.

If there has been a DNA modification that affects the amino acid sequence of the plant expressed protein, the **modified amino acid sequence** must be provided. It should be indicated whether the modifications are known or expected to result in changes in the properties of the protein.

On a case-by-case basis, data on protein stability in the cell and the environment may be required.

7 Inheritance and stability

Statistically significant data should be provided that demonstrate the inheritance pattern and the stability of the sequences inserted, as well as data that demonstrate

the stability of expression of all proteins that are intended to be expressed in the plant or of the target proteins of which the expression level has been altered.

For plants which are either infertile or for which it is difficult to produce seed (e.g. vegetatively propagated male-sterile potatoes, plants with long sexual generation times such as trees), statistically significant data should be provided to demonstrate that the transgene trait is stably maintained and expressed during vegetative propagation.

8 Detection and identification

The sequence of a **primer pair** should be provided which enables the unequivocal identification of the transformation event, as well as a detailed **protocol** for its use for identification, detection and quantification purposes.

Reference transgenic and control **material** should be provided at the time of deposition of the dossier.

Toxicological assessment

Working Group "Toxicological aspects of genetically modified foods and feeds": Marie-Paule Delcour, Martine Duverger, Benoit Nemery, Hadewijch Vanhooren, Jan Willems; Secretariat: Ellen Van Haver

1 Introduction

Genetically modified plants can be altered for agronomic traits, such as virus-, insect- or herbicide tolerance, and for quality traits, such as enhanced or altered nutritional properties. The genes introduced into the plant may result in the synthesis of new substances that are conventional components of plant foods such as proteins, fats, carbohydrates, or vitamins that are novel in the context of the genetically modified crop. The genetic modification can also result in the synthesis of active substances, which are toxic for adverse exogenous organisms (such as pest organisms). Moreover, as a result from the activity of enzymes generated by the expression of the introduced DNA, new substances may include metabolites of endogenous origin or arising from the use of xenobiotics. Finally, because of technical reasons, the inserted genetic material consists often, in addition to the gene(s)-of-interest, of a molecular marker (in many cases an antibiotic[2] or herbicide resistance marker) and border-DNA (non-coding sequences). At present, transformation methods used result in random integration of the sequences in the genome of the plant, potentially leading to a series of unexpected changes. It is, therefore, clear that requirements for the toxicological assessment of genetically plants may vary from one kind of modification to another. The toxicological evaluation of a genetically modified crop expressing a biological biocide should comply with the requirements for the evaluation of the original organism used as biological biocide, whereas the toxicological evaluation of a well known nutrient may pose much less problems.

Substances expressed by the insertion of the defined DNA sequences should be subjected to the toxicological evaluation as described in this document. In case additional components or altered levels of existing components are present as an unintended result of the genetic modification (e.g. by the disruption, modification or silencing of active genes or the activation of silent genes), the assessment of these substances should follow the same criteria as those intended by the genetic modification. To date, toxicity assessment is relying on the traditional validated methods starting from some required tests, followed by others requested on a case-by-case basis. Moreover, testing remains accessible to new supporting developing methods. Profiling technologies such as metabolomics, proteomics and transcriptomics are considered as emerging technologies to extend the breadth of comparative analyses and to identify the need for further risk assessment. Should new technologies be applied, the expectation is then that all approaches are properly validated and that statistical analyses have been performed to the highest standard.

[2] Antibiotic resistance marker genes in genetically modified organisms which may have adverse effects on human health and the environment, will have to be phased out according to Directive 2001/18/EC (EU, 2001).

If the new substance modifies the fate of xenobiotic substances, e.g., biocides applied to the plant, the advice of other scientific committees is requested competent within the framework of Directive 91/414/EEC concerning the placing of plant protection products on the market (EU, 1991). Similarly, if the new substance claims therapeutic properties, the advice of the scientific committee competent within the framework of Regulation (EEC) 2309/93 for the authorisation of medicinal products for human and veterinary use (EU, 1993), should be requested.

The assessment of toxicological effects on non-target organisms other than humans and animals (farm or pet) is beyond the scope of this document.

2 Comparative analysis (molecular characterisation and compositional analysis)

Following the comparative approach as mentioned in Chapter I, section 6, the degree of substantial equivalence of the genetically modified food or feed with its conventional counterpart will determine the extent of further toxicological analyses.

2.1 Information on expressed DNA-sequences

Information should be provided as described in Chapter II on the new traits expressed in the plant, as well as on the possible occurrence of additional expressed products as a consequence of unintended effects of the genetic modification.

2.2 Marker-DNA

The safety of marker genes should be assessed, as would be the case for any other expressed gene product. If evaluation of the information as mentioned in Chapter II suggests that the presence of the marker gene or gene product presents a risk to human or animal health, the marker gene or gene product should not be present in the genetically modified crop. Alternative transformation technologies that do not result in clinically relevant antibiotic resistance marker genes should be encouraged in the future development of genetically modified organisms.

2.3 Allergenicity

All newly expressed proteins should be assessed for potential allergenicity as mentioned in Chapter IV. It is recommended to consider the outcome of the allergenicity assessment along with the toxicological evaluation.

3 Toxicological assessment

3.1 Study of literature

The notifier should perform a comprehensive literature review, discussing the absence of toxicity to humans and animals of the new substances (proteins and non-proteins). This literature search has to be clearly referenced (e.g. search methods used).

A comprehensive literature review has also to be performed concerning the toxic potential of the donor organisms used.

3.2 Screening for structure-activity relationship

The homology between the new substance (proteins and non-proteins) and known toxic components has to be screened by e.g. comparing the sequence of a protein with known protein toxins, using databases, predicted 3-D-structure, and amino acids sequence in regions of the protein that are critical to toxicological properties. If the newly expressed substance is an enzyme, the characteristics and biological effects of that enzyme should be described and considered. Database consultation and the use of computer-based amino acid search programs should be clearly documented and verifiable.

3.3 Exposure assessment

An estimation of the intake of the new substance (protein and non-protein) has to be carried out:

- per unit plant
- per unit food compound

in order to derive a daily intake (DI).
This predicted exposure to the substance of interest has to be compared with the potential exposure to the same substance, produced by the donor organism.

3.4 Toxicological tests

3.4.1 Requirements

Protein and non-protein new substances
Toxicological studies should be conducted using internationally agreed state of the art protocols, such as OECD/EU protocols, and be carried out according to the principles of Good Laboratory Practice (GLP).

If the substance of interest to be used in the toxicology tests is produced through molecular biology techniques in another organism (e.g. bacteria) than the plant, it has to be proven that the test substance is structurally, biochemically and functionally equivalent to the substance produced in the plant. Factors that should be examined to establish equivalence are: post-translational modification, full length amino acid sequence, amino acid composition/sequence, molecular weight (using the most appropriate methods), functional characteristics (immunorecognition in a Western blot assay and similar bioactivity).

3.4.2 Metabolic / toxicokinetic studies

Protein new substances
An ***in vitro*** digestibility assay in simulated gastric and/or intestinal fluids is required. It is important to note that resistance to ***in vitro*** digestion is not a toxicity endpoint by itself, but simply an indication that the protein warrants closer examination and perhaps different types of testing. On a case-by-case basis, also an ***ex vivo*** gastric fluid test (e.g. pig, cattle, dog) or ***in vivo*** models may be required.

Non-protein new substances
For new non-protein substances (e.g. those exerting biocidal or pharmacological effects), toxicity should be assessed on a case-by-case basis, depending on the identity and biological function of the substance in the plant and dietary exposure, and according to the appropriate guidelines and the conventional toxicological approach (including metabolism studies, studies on toxicokinetics). Also the use of human relevant testing systems for metabolic profiling will be encouraged, although most of these have not yet been validated.

3.4.3 Acute toxicity

Acute oral toxicity of the new substance (protein and non-protein)
Acute oral toxicity testing in laboratory rodents is required to confirm the lack of toxicity suggested by the literature reviews performed. A single dose study may also generate useful data to describe the relationship of dose to systemic and/or local toxicity. Further, these data can be used to select doses for repeated dose toxicity studies.
The maximum hazard dose test is generally adequate to address substance toxicity. It should be performed with a single high dose according to Directive 96/54/EEC (EU, 1996). The animals should be observed for 14 days to ascertain that no adverse sign occurs, and should then be subjected to gross necropsy. In the observation period, incorporation of parameters such as changes in skin and fur, eyes and mucous membranes, but also respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and behaviour pattern should be considered in the design of these studies.
As an alternative approach, mammalian toxicity can be tested with the purified substance. The dose level to be used in the test should be selected from one of the four fixed dose levels, namely 5, 50, 500 or 2000 mg/kg BW according to test method B1 bis of Directive 96/54/EEC (EU, 1996).

As the doses of the substance to be administered may be quite large, it is possible that the quantities required cannot be reasonably purified from the genetically modified crop, necessitating a production in an alternative organism (see 3.4.1).

3.4.4 Irritation tests

Protein and non-protein new substances
Dermal and eye irritation testing should be considered, as workers may be exposed to pollen and crop dust.

Non-protein new substances
Photosafety testing (photoirritation), conducted consistent with the appropriate guideline protocols, should be considered on a case-by-case basis.

3.4.5 Sensitisation

Protein and non-protein new substances
Sensitisation testing (as workers are exposed to pollen and crop dust) should be considered along with the allergenicity assessment as described in Chapter IV.
To assess the potential for immunological sensitisation, tests of interest are the Guinea Pig Maximisation test or newer tests such as the Local Lymph Node Assay according to the OECD guideline No. 429 (OECD, 2002). Promising developing methods, such as the Mouse Intra-Nasal Test (Robinson et al, 1996; Robinson et al,

1998) and the Brown Norway rat model (Penninks and Knippels, 2001), although not yet validated, should be considered as complementary information.

The use of the i.p. route of administration of the substance may provide a more direct way to assess systemic toxicity and is also an alternative approach to evaluate the sensitizing potential of the test substance (Dearman and Kimber, 2001).

There may be special instances where an inhalation exposure (worker exposure) should be considered, for example when a protein related to an aeroallergen is found to be expressed in an anemophilous plant, with copious wind-born pollen, or when a significant exposure to grain dust is anticipated.

Non-protein new substances
Photosafety testing (photoallergy), conducted consistent with the appropriate guideline protocols, should be considered on a case-by-case basis.

3.4.6 Genotoxicity

In general, genotoxicity testing, whether it is performed or not, should always be well motivated.

Protein new substances
In vitro mutagenicity tests (bacterial mutagenicity tests, chromosome aberration tests including cytogenicity tests in cultured mammalian cells) should be performed on a case-by-case basis depending on the identity and biological function of the substance in the plant and dietary exposure.

Non-protein new substances
In vitro mutagenicity testing (bacterial mutagenicity tests, chromosome aberration tests including cytogenicity tests in cultured mammalian cells) is obligatory, unless convincing evidence can be provided to deviate from standard procedures.

The use of in vitro toxicological profiling, such as general cyto- and genotoxicity testing and the use of eukaryotic and bacterial stress gene assays, may become an important part in evolving strategies for a tiered approach (Noteborn et al., 2000).

3.4.7 Repeated dose toxicity - Oral route

Protein new substances
The 28-day oral toxicity test should be performed as a minimum requirement with a diet that properly nourishes the test animal (rodent), yet contains sufficient amounts of the new protein. The highest dose level should be the maximal achievable without causing nutritional imbalance, while the lowest level used should be comparable to the anticipated human intake.
The repeated dose study should include a tier I immunotoxicity screen according to the modified OECD guideline No. 407 (OECD, 1995) to establish dose-response characteristics and provide an indication for a Tier II screen. In other words, additional targeted investigations should be conducted if the new protein is suspected to act on specific organs or tissues including the endocrine, reproduction, or nervous system.

Non-protein new substances
Non-protein new substances, biological available metabolites, stable degradation products, should be evaluated according to the traditional toxicological approach on a case-by-case basis as provided by Directive 91/414/EEC (EU, 1991) or Directive 89/107/EEC (EU, 1988). This implies the submission of information on a core set of studies and the consideration of whether any other type of study might also be appropriate.

3.5 Whole-food toxicology testing

In principle, whole food testing should allow to answer the question whether unintended adverse effects (secondary pleiotropic effects) have been introduced following the genetic modification.

Whole food testing should be performed on a case-by-case basis in the following situations: 1) a completely new gene and/or transgenic organism; 2) organisms extensively changed as a result of biotechnology (metabolic pathway engineering); 3) new substances as anti-nutrients; 4) new substances without a clear threshold (e.g. bacterial toxins); 5) products with predicted high intake levels of the new protein; 6) non-rapidly degradable proteins (e.g. protease inhibitors, lectins) or crop plants with profoundly altered compositions (e.g. low glutelin rice, golden rice); 7) transgenic plants inactivating herbicides producing metabolised degradation products, which might be present in the plant; 8) the presence or altered level of phytotoxins (e.g., alkaloids).

The food product should be tested in the appropriate test animal, over an appropriate time span. For foods, a 90-day feeding study in rodents should be performed. For feeds, it is recommended that the study is conducted with a fast growing livestock species such as broiler chickens. Special attention must be paid to the avoidance of problems of nutritional imbalance (see also Chapter V).
Complete end-points (including biochemical, haematological, histological end-points) according to the OECD-guidelines for toxicity testing in analogy with irradiated foods are requested.

The food product tested should be in a similar form to that which would be consumed by humans or animals (e.g. processed foods).

The plants used should be grown under conditions that represent normal practice for the crop plant (e.g. pesticide use in case of herbicide resistance).

4 Background documents and references

(Draft) Codex Guideline for the conduct of food safety assessment of foods derived from recombinant-DNA plants, March 2002. http://codexalimentarius.net/biotech/en/DNAPlant.htm

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Allergenicity assessment

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1 Introduction[3]

Allergenicity is defined in this chapter as the capacity to elicit an IgE immune response upon animal or human immunisation or exposure. All newly expressed proteins[4] in genetically modified plants that could be present in the final food or feed destined for human or animal consumption should be assessed for their potential to cause allergic reactions. This should include consideration of whether a newly expressed protein is one to which certain individuals may already be sensitive as well as whether a protein new to the food supply carries the risk to cause allergic sensitisation and to induce allergic reactions in some individuals. The necessity to test for allergenicity of genetically modified organisms destined for animal consumption is supported by the possibility to find back the transgenic protein in animal-derived products for human consumption, such as milk or eggs.

At present, there is no single definite test that can be relied upon to predict allergic responses in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case-by-case approach, as described below, be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

The endpoint of the assessment is a conclusion as to the likelihood of the protein for being a food allergen. The decision tree as enclosed in annex 1 to this chapter will be helpful to determine the endpoints but is not to be strictly followed.

2 Assessment Strategy

The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known

[3] The expert consultation group 'Allergenic aspects of genetically modified foods/feeds' of the Biosafety Council formulated the Belgian comments to the Ad Hoc Open-Ended Working Group on Allergenicity which convened in Vancouver in September 2001. This Working Group was established by the Codex Ad Hoc Intergovernmental Task Force on Foods derived from Biotechnology in order to develop detailed guidelines for the assessment of potential allergenicity of genetically modified foods (FAO/WHO, 2002). The text, as described below, is based on these guidelines which have been further elaborated and completed by the expert consultation group of the Biosafety Council.

[4] This assessment strategy is not applicable for assessing whether newly expressed proteins are capable of inducing gluten-sensitive or other enteropathies. In addition, the strategy is not applicable to the evaluation of foods where gene products are down regulated for hypoallergenic purposes.

allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation, heat stability and/or acid treatment.

As there is no single test that can predict the risk of human IgE response to oral exposure, the first step to characterize newly expressed proteins should be the comparison of the amino acid sequence and certain physicochemical characteristics of the newly expressed protein with those of established allergens in a weight of evidence approach. This will require the isolation of any newly expressed proteins from the genetically modified plant, or the synthesis or production of the substance from an alternative source, in which case the material should be shown to be structurally, functionally and/or biochemically equivalent to that produced in the genetically modified plant. Particular attention should be given to the choice of the expression host, since post-translational modifications allowed by different hosts (i.e.: eukaryotic vs. prokaryotic systems) may have an impact on the allergenic potential of the protein.

It is important to establish whether the source is known to cause allergic reactions. Genes derived from known allergenic sources should be assumed to encode an allergen unless scientific evidence demonstrates otherwise.

The level of a protein in a food cannot be taken as a criterion for assessment of allergenicity. The first reason is that there is no scientifically determined minimal level of exposure for an allergic reaction. Moreover, nobody can ascertain that the level of expression of a transgene-encoded protein cannot be increased under certain circumstances (climatic, soil,…) so that it becomes life-threatening for the allergic individuals.

3 Initial Assessment

3.1 Source of the Protein

As part of the data supporting the safety of foods derived from genetically modified plants, information should contain any reports of allergenicity associated with the donor organism. Allergenic sources of genes would be defined as those organisms for which reasonable evidence of IgE mediated allergy is available after inhalation, ingestion or skin contact with any part of the organism. Knowledge of the source of the introduced protein allows the identification of tools and relevant data to be considered in the allergenicity assessment. These include: the availability of sera for screening purposes; documented type, severity and incidence of allergic reactions, prevalence of occupational allergy (inhalation/worker exposure); physicochemical (structural characteristics and amino acid sequence) and immunological properties (when available) of known allergenic proteins from that source.

3.2 Amino Acid Sequence Homology

The purpose of a sequence homology comparison is to assess the extent to which a newly expressed protein is homologous in sequence to a known allergen (food, respiratory or any other type). This information may suggest whether that protein has an allergenic potential. Sequence homology searches of all newly expressed proteins with all known allergens should be done. Searches should be conducted using various algorithms such as FASTA or BLASTP to predict overall structural homologies. Strategies such as stepwise contiguous identical amino acid segment

searches may also be performed for identifying sequences that may represent linear epitopes. The size of the contiguous amino acid search should be based on a scientifically justified rationale in order to minimize the potential for false negative or false positive results[5]. Validated search and evaluation procedures should be used in order to produce biologically meaningful results.

IgE cross-reactivity between the newly expressed protein and a known allergen should be considered a possibility when there is more than 35% homology in a segment of 80 or more amino acids (FAO/WHO 2001) or other scientifically justified criteria. All the information resulting from the sequence homology comparison between the newly expressed protein and known allergens should be reported to allow a case-by-case scientifically based evaluation.

Sequence homology searches have certain limitations. In particular, comparisons are limited to the sequences of known allergens in publicly available databases and the scientific literature. There are also limitations in the ability of such comparisons to detect non-contiguous epitopes capable of binding specifically with IgE antibodies.
Most of B cell epitopes of soluble proteins, and in particular epitopes recognized by IgE antibodies, are made of amino acid residues located at distance on the protein and brought together by the tertiary conformation of the molecule. The only way to determine which amino acids are involved in the epitopes is by elucidating the 3-D structure through crystallography and X-ray structure. The number of allergens that have been crystallized today is very limited and, apart from phospholipase A2, Der p 2 and Bet v1, crucial information is therefore lacking.

A negative sequence homology result based on the findings of a less than 50% homology between sequences of 6 to 8 amino acids of the newly expressed protein and of known allergens, indicates that a newly expressed protein is not a known allergen and is unlikely to be cross-reactive to known allergens. A result indicating absence of significant sequence homology should be considered along with the other data outlined under this strategy in assessing the allergenic potential of newly expressed proteins. Further studies should be conducted as appropriate (see also sections 4 and 5). A positive sequence homology result indicates that the newly expressed protein carries the risk to be an allergen. If the product is to be considered further, it should be submitted to serum screening using serum from individuals sensitized to the homologous allergen.

3.3 Pepsin Resistance

Resistance to pepsin digestion has been observed in several food allergens; thus a correlation exists between resistance to digestion by pepsin and allergenic potential[6]. The establishment of a consistent and well-validated pepsin degradation protocol may enhance the utility of this method. However, it should be taken into account that a lack of resistance to pepsin does not exclude that the newly expressed protein can be a relevant allergen and that pepsin digestion might reveal allergenic epitopes. Moreover, the wide use of proton-pump inhibitors to reduce gastric acidity, and thereby the efficiency of pepsin digestion, further reduces the relevance of pepsin

[5] It is recognized that the 2001 FAO/WHO consultation suggested moving from 8 to 6 identical amino acid segments in searches. The smaller the peptide sequence used in the stepwise comparison, the greater the likelihood of identifying false positives, inversely, the larger the peptide sequence used, the greater the likelihood of false negatives, thereby reducing the utility of the comparison.

[6] The method outlined in the U.S. Pharmacopoeia (1995) was used in the establishment of the correlation (Astwood et al. 1996).

resistance assays in the evaluation of the allergenicity potential of newly expressed proteins.

Although the pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Additional protocols may be used where adequate justification is provided.

4 Serum Screening

For those proteins that originate from a source known to be allergenic, or have sequence homology with a known allergen, testing in immunological assays should be performed.
Sera from individuals with a clinically validated allergy to the source of the protein or to the allergen with sequence homology can be used to test the specific binding to IgE class antibodies of the protein in a specific serum screen test. A critical issue for testing will be the availability of human sera from sufficient numbers of individuals. In addition, the quality of the sera and the assay procedure need to be standardized to produce a valid test result.
The search for the presence of IgE antibodies to the protein of interest can be carried out for instance by dot blotting. This consists in first applying drops of the protein solution onto a membrane and let the protein bind to it. Next, protein dots are incubated with the sera (one dot for one serum to be tested), and bound IgE are detected by using a system of labelled IgE-specific antibodies. Demonstration of IgE binding indicates that the target carries the risk to be an allergen. The assay procedure needs to be set up on a case-by-case basis to produce a valid test result with limited risk of false negative and false positive reactions. Critical parameters are the amount of protein, the volume of serum, and the detecting system of antibodies. Serum screening should be performed on the purified transgene-encoded protein. If the protein cannot be purified from the transgenic plant and the protein is glycosylated in its natural form, a recombinant form obtained in another high-producing system than bacteria, for example yeast, allowing glycosylations, should be considered. The purification procedure should not exclude any glycovariant of the target protein. It will also be required to compare intact, pepsin digested and heat denatured proteins for IgE binding.
As for the number of sera that should be used for testing, twenty-four different well documented sera is statistically enough to detect an allergen with a 99% confidence interval. One positive serum out of at least 24, if available, is enough to declare that the protein carries the risk to be allergenic.
For proteins from sources not known to be allergenic, and which do not exhibit sequence homology to a known allergen, as well as in the case of a negative outcome of a specific serum screening, targeted serum screening (i.e. the assessment of binding to IgE in sera of individuals with clinically validated allergic responses to broadly-related categories of allergens) may be considered.

In the case of a newly expressed protein derived from a known allergenic source, a negative result in in vitro immunoassays may not be considered sufficient, but should prompt additional testing. The search for the presence of specific IgE antibodies can be completed by a search for the capacity to activate human basophils, using, for instance, whole blood assays. Additionally, a passive cutaneous anaphylaxis test (PCA) can be carried out, in which the serum containing the putative IgE antibodies is injected into the abdominal skin of rats, which are later challenged by i.v. injection of the protein under scrutiny and a dye to visualize the skin extravasation reaction.

Alternatively, a rat basophil leukaemia (RBL) cell line that is transfected with the alpha chain of the human high-affinity receptor for IgE could be used to avoid the necessity to prepare fresh basophils from the peripheral blood of human subjects. In such a system, RBL are passively sensitized by incubation with human serum containing putative IgE antibodies. RBL are then washed and incubated with different concentrations of the protein under scrutiny. Activation of RBL is followed by measuring the production of β-hyaluronidase. A positive result in such tests would indicate a potential allergen.

5 Additional tests

After the first two screening steps which are the essential components of the assessment strategy for possible allergenicity, a number of other analyses characterising the properties of the protein should be recommended which further document and strengthen the status of "non-allergenic" proteins.

T-cell epitope search

It may be important to determine the possible sharing of T-cell epitopes between transgene-encoded proteins and allergens. Recent evidence shows that, on the one hand, the T-cell receptor (TCR) recognizes more a conformation than an actual sequence and, on the other hand, that tightness and duration of contact between the TCR and the peptide are more important than recognition itself. The lack of sequence homology between a new transgene-encoded protein and a known allergen offers no valuable information to determine as to whether a new transgene-encoded protein presents a risk of being allergenic. Recent databases nevertheless offer the possibility to explore in more details the interaction between peptides and MHC class II molecules and are based on virtual matrices in which the contribution of each amino acid with each pocket of the MHC molecule is quantified (see for instance Hammer et al, 1994 and the TEPITOPE database).

Animal models

The use of animal models does not seem to be useful to identify allergenic proteins in IgE-mediated allergy because MHC restrictions of immune responses preclude any conclusion. Nevertheless, if animal models for the identification of protein allergens are further developed and validated, the use of animal models can be considered as an enhancing step in the weight-of-evidence approach.

For the other types of sensitivity, animal tests should be considered along with the information provided in Chapter III, Section 3.4.5.

Testing of the whole genetically modified plant

Another problem that should be considered in the allergenicity assessment of genetically modified foods/feeds is that the insertion of a new gene might also increase the level of expression of proteins naturally present in the conventional plant. Therefore, if the host contains allergenic proteins, the expression level of such allergens might be increased and such plants in general might become more allergenic. In this case the whole genetically modified plant or crop should be assessed for allergenicity.

6 Recommendations

Sequence homology searches should be performed by an independent organisation (possibly designated by the organisation in charge of the safety evaluation).

Serum screening and purification of the transgene-encoded protein should preferentially be carried out by an independent laboratory.

To allow serum screening, steps should be taken and funding should be raised to organise an international serum bank, linked to a facility that also will be able to perform the testing. Banks could possibly be raised on regional scale (South- and North-America, Europe, Africa, Asia, etc.) within a framework. The advantage of an international input of serum samples is the increased likelihood of containing IgE antibodies against a wide variety of proteins, to which people in certain areas of the world are more (or even selectively) exposed.

7 Conclusions

Proteins that are positive in the sequence homology search or serum screen test should be considered as allergenic or at least as carrying the risk to be allergenic. Transgenic crops of which the newly expressed proteins are allergenic or at least carry the risk to be allergenic should not be approved for marketing.

When sequence homology analysis and serum screening tests are negative, the protein can be considered as being probably non-allergenic. However, this can never mean that the protein is definitively considered as such, especially as many of these proteins have never been inhaled or ingested by humans before. Post-marketing surveillance on the occurrence of allergy should therefore be strongly supported.

If a protein shows pepsin resistance, or contains T-cell epitopes cross-reacting with epitopes of known allergens, post-marketing surveillance should be exerted.

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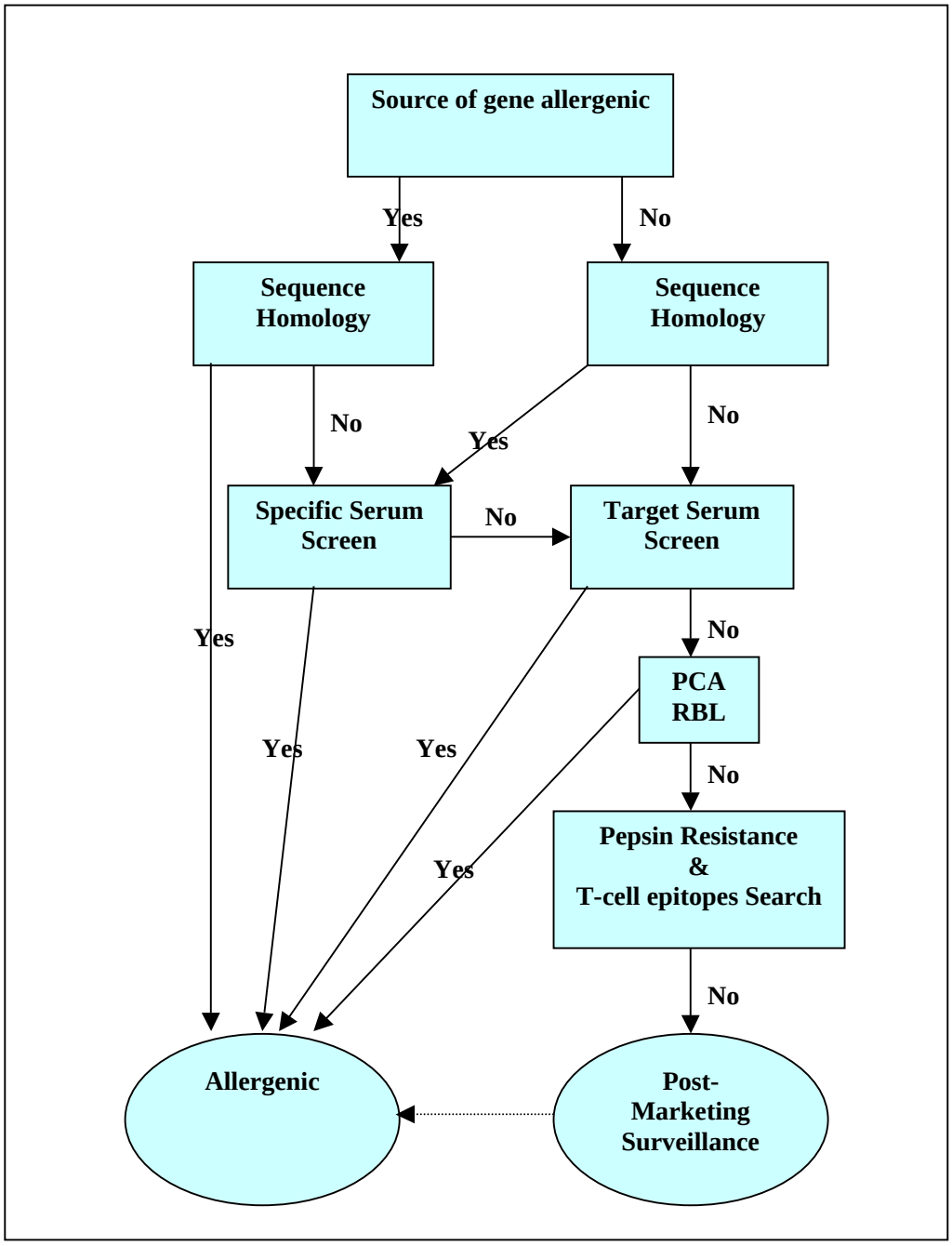
Annex 1

Decision Tree for the assessment of possible allergenicity (proteins)
(adapted FAO/WHO 2001 Decision Tree)



Feed nutrition evaluation

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1 Introduction

There has been a permanent selection for thousands of years to create new varieties of plants to supply better feeds for animal nutrition. Selection purposes may result in an increased dry matter yield per hectare, an altered dry matter composition, an improved organic matter digestibility and consequently an increased energy value, a reduction of the presence of anti-nutritional factors or a better resistance against diseases or unfavourable environmental conditions.
During the last decades, crops for livestock feeding were increasingly developed based on genetic engineering, where foreign DNA fragments with specific characteristics were inserted into the genome. The safety of these genetically modified organisms should be assessed for both livestock feeding and human nutrition. Additionally, it has to be evaluated if the DNA of inserted or modified genes (such as antibiotic resistance genes), or their products, can cause detrimental effects, if transferred into animals, or if the proteins can accumulate in the end products (milk, meat, eggs...) of animals which are fed with these novel feeds.
This chapter describes in first instance the data and information needed for the compositional analysis of the feed derived from the genetically modified crop . Next, the possible implications of genetically modified crops in the animal diet as outlined in this chapter should be considered to determine which studies are necessary for the further feed safety assessment.

2 Compositional analysis

2.1 Compositional data and methods

This section has to present the proximate analysis of the matter, to describe the sampling procedure, to refer to the analysis methods and to precise the statistical distribution of the results.

2.1.1 Major and minor constituents

A non-exhaustive checklist as presented hereafter provides information on critical parameters of feed safety and nutrition. Depending of the crop and/or derived feed product to be considered, several components may be not relevant.

Checklist for proximate composition analysis

- Moisture % of wet weight
- Protein % of dry matter (DM)
- Total fat % of DM
- Crude fibre % of DM
- Total ash % of DM
  - soluble ash
  - insoluble ash
- Other carbohydrates (nitrogen-free extractives) % of DM

In recent years the proximate analysis procedure has been replaced by other analytical procedures. Alternative procedures for fibre have been developed (Van Soest):

- Neutral Detergent Fibre (NDF), eNDF, peNDF
- Hemicellulose
- Acid Detergent Fibre (ADF)
- Lignin (ADL)
- Cellulose

Also the carbohydrate methodology has been revised:

- Non-structural carbohydrates (NSC): sugars, starches, fructans, galactans, pectins, β-glucans, etc.
- Non-starch polysaccharides (NSP): NSC minus starch and sugars

Protein can also be specified:

- NPN (non-protein nitrogen) % of DM
- Amino acids % of DM and % of total amino acids
  - Essential and semi-essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, and others according to the species of monogastric animals
  - Non-essential amino acids: alanine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, proline, serine, tyrosine

Triacylglycerols (triglycerides), which make up the major fraction of usual dietary fats, are characterised by the identity, the position and the combination of the fatty acids they contain.

Conjugated linoleic acids are synthesized from linoleic and linolenic acid in the fore-stomachs of ruminants**,** and from vaccenic acid in adipose tissue in growing ruminants and mainly in the mammary gland in lactating ruminants, or in the liver of other species; they are secreted in the milk and deposited in the meat. Anti-carcinogenic and other properties of these compounds have been reported.

Table 1: Classification of fatty acids (% of total fat)

Saturated fatty acids	Unsaturated fatty acids		
Butyric acid	**n-9 family**	**n-6 family**	**n-3 family**
Caproic acid	Oleic acid	Linoleic acid	α-linolenic acid
Caprylic acid	Erucic acid	γ-linolenic acid	Eicosapentaenoic acid (EPA)
Capric acid		Arachidonic acid	Docosapentaenoic acid
Lauric acid			Docosahexaenoic acid (DHA)
Myristic acid			
Palmitic acid			
Stearic acid			
Aracidic acid			

- Mineral composition and trace-elements
  - Minerals (g/kg): Ca, Cl, K, Mg, Na, P, S
  - Trace-elements (mg/kg): Co, Cu, Fe, I, Mn, Mo, Se, Zn

- Vitamins
  - Fat soluble vitamins: Vitamin A (retinol) (µg/100g), Vitamin D_3 (cholecalciferol) (µg/100g), Vitamin D2 (ergocalciferol) (µg/100g), Vitamin E (α-tocopherol) (mg/100g), Vitamin K (phylloquinone) (mg/kg), β-carotene (mg/kg)
  - Water soluble vitamins: Vitamin B_1 (thiamine) (mg/kg), Vitamin B_2 (riboflavin) (mg/kg), Vitamin B_6 (pyridoxine) (mg/kg), Niacin (mg/kg), Pantothenic acid (mg/kg), Folic acid (mg/kg), Biotin (mg/kg), Vitamin B_{12} (cobalamin) (mg/kg), Vitamin C (ascorbic acid) (mg/kg)

2.1.2 Analytical methods

Reference methods must be used and mentioned. European standardized validated methods will be preferred but other official methods will be considered. Depending on the feed involved, appropriate and currently available methods are used. See Chapter VI for an extended description of analytical methods.

2.1.3 Statistical and sampling aspects

The sampling method must be explained and must take into account the requirements linked to the statistical analysis as well as the distribution of the components in the raw material.
A very important point to consider is the variability of the raw material for example by taking into account the impact of the geographical origin, the climate, the agronomic practices, the annual variations… Enough samples are to be analysed with the help of a sampling plan and the results are to be evaluated on a statistical basis.
Plants used to obtain samples for compositional analysis should be grown under conditions that represent normal practice for the crop plant. For example, studies on herbicide tolerant crops should be done on herbicide treated crops (with a waiting period afterwards). If the transgenic plant inactivates the herbicide, (metabolised) degradation products might be present in the plant.

2.2 Nutritional aspects

Whenever changes are made to the way in which a feed is produced or processed, the implications on the nutritional value require consideration. Information will be needed on any issue relating to this aspect. Feeds are usually complex mixtures of macro- and micronutrients, which provide energy and nutrients and contribute to animal welfare.

2.2.1 Identification of key nutrients

If a genetically modified crop is expected to have an important role in the diet, then appropriate information on nutritional composition is needed. Both macro- and micronutrients of nutritional value are already given. It is clear that not all these nutrients are relevant for every specific genetically modified crop. For every such crop, the place (value) within the animal diet should be determined. It is well known that different feed groups contribute in different ways to animal feeding. Depending on the composition and the (estimated) consumption of the genetically modified crop, it appears justified to limit the testing to the most relevant nutrients, which are specified in Table 2. This table should be considered as an example and not as an exhaustive list.

Table 2: Identification of relevant nutrients for different feed groups

Feed group	Key nutrients
Grass and forage crops	Energy, protein, fibre, vitamins, minerals and trace elements
Dried forages and straw	Fibre, minerals and trace
Silages	Energy, protein, fibre, minerals and trace elements
Roots tubers and related by-products	Energy, minerals and trace elements
Cereals and related by-products	Energy, minerals and trace elements
Protein concentrates	Protein, energy, minerals and trace elements
Vitamin and trace mineral premixes	Vitamins, minerals and trace elements

2.2.2 Intake

High-performing animals require a high feed intake. Feed intake capacity may not be deteriorated by the use of genetically modified feeds in the diet. As it may not be possible to predict such events, a surveillance programme should accompany the marketing of a genetically modified feed. Such a programme should encompass information on changes in the conditions for processing and preparation as well as effects of possible replacement of other feeds or feed component of dietary importance. If surveillance reveals changes in those factors, which raise concerns regarding wholesomeness, a reappraisal of the acceptability of the genetically modified feed would be required.

2.2.3 Digestion

Genetically modified feeds in animal diets come into close contact with the host within the digestive tract. Different digestive processes occur between the categories of livestock husbandry, due to different anatomical and enzymatic aspects of the digestive tract.

Mechanical, chemical and microbial activities are involved in the digestion process. Large differences exist between monogastric and ruminant animals, mainly with regard to the microbial digestion. Extensive microbial digestion occurs in the rumen, while this is restricted to the large intestine in monogastric animals, but in the latter this process is of less nutritional importance.

In monogastric animals carbohydrates are broken down by enzymes to monosaccharides and actively transported to the liver. Protein digestion results in the formation of free amino acids and peptides. Amino acids pass into the portal blood and then to the liver. Ingested DNA and RNA are rapidly cleaved into small fragments by pancreatic and gastro-intestinal enzymatic digestion and acid hydrolysis in ruminants (McAllan, 1980; Flint and Thomson, 1990). D'Mello (1982) concluded that there is an extensive catabolism of exogenous bases from nucleic acids, but the salvage of preformed purines and pyrimidines occurs widely in non-ruminant animals. The enzymes involved in DNA hydrolysis include high concentrations of DNase I. This endonuclease, with an optimal activity at neutral pH, disrupts the double stranded DNA and is produced and secreted by salivary glands, as well as the pancreas, the liver and the Paneth cells of the small intestine. DNase II, with a pH optimum of between 5.2 to 6.4, is also secreted but its primary function is in lysosomes within phagocytes, involved in the catabolism of DNA as well as the fragmentation of genomic DNA during apoptosis (Yamanaka et al., 1974). However, with more accurate analytical techniques, the degradation of DNA may be re-evaluated. Tests made with simulated gastric and intestinal conditions have confirmed that protein products of the genes introduced into current commercial crops are as rapidly degraded as other dietary proteins (Harrison et al., 1996; Wehrman et al., 1997). Nevertheless, protein fragments of Cry9C, a bacterial lectin, which, in common with a sub-group of other plant and microbial lectins and protease inhibitors, are highly resistant to proteolysis (Peferoen, 1998). With regard to transformation of DNA from genetically modified feeds, it seems likely that the sites preceding the acidic stomach, i.e. the mouth, the oesophagus, the rumen and the avian crop, might see the highest concentration of intact DNA entering with the feed.
McAllan (1982) estimated that more than 85% of the plant DNA consumed by ruminants is reduced to nucleotides or smaller constituents before entering the duodenum, with most of the larger nucleic acid fragments in the small intestine arising from rumen microbes. In addition to enzymatic digestion, low pH conditions in the stomach or the abomasum should remove most adenosine and guanine from naked DNA.
The digestion of fibre takes place in the large intestine, due to microbial activity. However, this digestion is small compared with the microbial activity taking place in the rumen. Triacylglycerols are broken down into partial glycerides and free fatty acids. These are incorporated into micelles and absorbed from the jejunum, involving bile salts. Following absorption, there is a resynthesis of triacylglycerols. They are formed into chylomicrons, which then enter the thoracic duct. It is possible to vary fatty acid composition of body tissues by altering the composition of dietary fat.

In ruminants, carbohydrates are broken down to simple sugars by the rumen flora. These simple sugars are immediately fermented to carbon dioxide, hydrogen, methane and various volatile fatty acids (VFA), depending on the composition of the diet. In the case of starch, part of it may be stable and by-pass rumen fermentation. This undegraded starch is then digested in a similar way as in non-ruminant animals. Proteins are also degraded in the rumen with peptides and ammonia as end products. When fermentable organic matter (FOS) is sufficiently available in the

rumen, ammonia is used for microbial protein synthesis. Otherwise, part of the ammonia is lost for the animal through excretion via the urine. Part of the feed protein may escape microbial degradation, depending on feed origin, processing and feeding level. This protein is then similarly digested as in non-ruminant animals. The rumen flora also enables protein synthesis from NPN.
Lipids are to a large extent hydrolysed in the rumen by bacterial lipases and unsaturated fatty acids are hydrogenated afterwards. Short-chain fatty acids are absorbed directly from the rumen, while long-chain fatty acids reach the small intestine. The formation of micelles and the absorption of long-chain fatty acids are dependent on the conjugated bile salts and the phospholipids present in bile. In contradiction with monogastrics, the predominant fatty acid of adipose tissue is stearic acid, resulting from rumen hydrogenation. However, fat can be protected, so that it bypasses the rumen and modifies body and milk fat.

In horses most microbial digestion takes place in an enlarged colon. Some microbial activity occurs in the avian crop. Rabbits take advantage of the hind-gut microbial fermentation by practising coprophagy.

2.2.4 Nutritive value

The nutritive value is obtained as a result of chemical composition and digestibility. It is mainly determined by the energy and protein value.

- Energy value
  - Net energy lactation (VEM/kg DM for dairy cattle)
  - Net energy fattening (VEVI/kg DM for beef cattle)
  - Net energy (kcal/kg DM for pigs)
  - Metabolizable energy (kcal/kg DM for poultry)

- Protein value
  - Protein digestible in the small intestine (DVE; g/kg DM for ruminants)
  - Rumen degradable protein balance (OEB; g/kg for ruminants)
  - Digestible crude protein (g/kg DM for pigs and poultry)

- Structural value (only for ruminants)

2.3 Anti-nutrients and toxicants

Information is requested with respect to the presence of anti-nutrients. This applies particularly to the key anti-nutrients for the product. The examples given below are to be considered as examples and not as an exhaustive list.

Naturally occurring toxins that are inherently present in the plant should be determined. Data on the sensitivity of the crop towards the formation of mycotoxins, pathogenic microorganisms, biogenic amines and other toxic substances or organisms formed in the product have to be given, if relevant.

2.3.1 Examples

- **Protease inhibitors** inhibit the activity of trypsin, chymotrypsin and other proteases. The are found in legumes such as beans and peas, but also in

cereals, potatoes and other products. Their presence results in impaired growth and poor feed utilisation.

- **Amylase inhibitors** have a similar activity against amylases.
- **Lectins or hemagglutinins** are glycoproteins mainly found in legumes: beans, peas, and lentils. They bind to intestinal epithelial cells. They cause agglutination of erythrocytes in vitro. Their presence results in poor food utilization and impaired growth.
- **Cyanogens** are cyanogenic glucosides found in cassava, linseed, peas, beans and other products. They may cause HCN poisoning.
- **Glucosinolates** are thioglycosides found in rapeseed meal and related species. Effects upon the thyroid function have been demonstrated.
- **Saponins** are also glycosides found in soybeans, lucerne, sugar beet and others. Haemolytic effects in vitro have been shown. Saponins may result in bloat in ruminants due to the formation of stable foam in the rumen.
- **Alkaloids** occur in certain plants. They are of particular interest since many of them have poisonous properties.
- **Gossypol** is particularly important in cottonseed. Several toxic effects have been demonstrated.
- **Phytic acid** occurs in several vegetable products. This compound has a strong chelating activity. Its presence may affect bioavailability of minerals.
- **Mycotoxins** are very diverse in chemical structure and in the characteristics of the mycotoxicoses they produce. These toxins include the aflatoxins, the tricothecenes, the fumonisins, zearalenone, moniliformin and fusaric acid.
- **Phytotoxins** (solanine, etc.).

2.3.2 Potential effects of cultivation conditions and processes

If relevant for the particular feed, information is requested about the presence of the anti-nutrient and particularly about the quantity. Moreover this information should include data about the potential effect of different cultivation conditions. In addition it is very well established that processing may have profound effects upon the level of anti-nutrients present. Two approaches at least have to be followed:

- the effect of inactivation processes
- the effect of separation processes.

In order to have a real picture of these effects, the commonly applied processing steps have to be followed. The use of flow sheets is highly recommended.
Inactivation studies under conditions equivalent to normal processing may give information about the stability of the anti-nutrient. Inactivation may be due to heat treatments, enzymatic activity, leaching or others.
Separation processes like dry milling, wet milling, extraction, centrifugation or equivalent may affect the level of anti-nutrients. In this case it is easily understood that information on the localisation of the anti-nutrient is of great help, such as the distribution in endosperm, aleurone layer, bran or germ for cereals. Data about the presence of the particular anti-nutrient in fractions for feed use are necessary.

2.4 Secondary plant and bacterial metabolites

Secondary plant metabolites are neither nutrients nor anti-nutrients. They are part of the characteristic composition of a plant. They are important for the compositional

analysis and for the comparative approach. Even more than in the previous paragraphs, an exhaustive list cannot be given.
Some of these substances may have undesirable effects, others may have beneficial effects.

2.4.1 Examples

- **Phenolic compounds** are considered to be of great importance. Detailed information about the qualitative and quantitative composition of the phenolic fractions is necessary.
- **Phyto-estrogens** naturally occur in soybeans and clover species.
- **Key enzymes** may affect the utilisation of the plant material. Information about the relevant enzymes is necessary.
- **Organic acids** are another group. This includes:
  - aliphatic plant acids like citric and malic acid and others,
  - aromatic acids like benzoic acid and analogues,
  - phenolic acids like caffeic, coumaric, ferulic acids and others.
- Additional information on **N substances**, if not covered in a previous section, is requested. This includes low molecular N substances, unusual amino acids and others.
- **Biogenic amines** are produced as a result of proteolysis during silage preservation and may induce physiological effects when toxic amounts are consumed.
- **Carbohydrates** are mainly covered in section 2.1.1 (major and minor constituents). In addition to simple sugars and polysaccharides, complex sugars such as raffinose, stachyose and verbascose have to be covered in this section.
- With respect to **lipids**, information not covered in Table 1 is requested. This includes complex lipids and others.

2.5 Derived products

Processing may have a pronounced effect upon the content and distribution of nutrients and anti-nutrients.
This aspect is, by preference, covered by means of flow sheets indicating the major steps in the processing scheme. As the global and detailed composition is already dealt with in the previous sections, further information on particular nutrients and anti-nutrients is necessary if this information is essential for the assessment of the product.
As an example it is felt that when discussing soybeans some aspects related to inactivation of anti-trypsin factors during toasting have to be included.

3 Implications of genetically modified crops in animal diets

3.1 Effects on animal performance and animal health

As regards genetically modified crops subdivision into two classes can be made between first and next-generation plants (see Introduction, Chapter I). Agronomic characteristics are mainly improved in first-generation plants, while changes in the content of major or minor constituents are intended by next-generation plants. Results from several experiments indicate that the current genetically modified feeds are substantially equivalent to their conventionally counterpart in composition, are

similar in digestibility, and have a similar feeding value for livestock (Clark and Ipharraguerre, 2001). Genetic engineering may however lead to enhanced levels of some essential amino acids and increased nutritive values (Molvig et al., 1997). Other studies showed that the nutritional value can be improved through recombinant techniques, resulting in improved daily gain and feed efficiency as reported by von Wettstein et al. (2000) for broiler feed. Furthermore, the number of chickens with adhering sticky droppings was drastically reduced. Panaccione et al. (2001) reported that alkaloid toxicoses in livestock could be reduced by genetic modification of the endophyte responsible for ergovaline production in perennial ryegrass. Piva et al. (2001) reported that pigs fed Bt maize had 2.8% higher final weights compared to isogenic maize, which may be attributed to lower levels of fumosin (69%) and deoxynivalenol (14.4%). On the other hand Leeson (1998) reported an increased mortality of male broilers fed with genetically modified maize. Ewen and Pusztai (1999) reported that snowdrop lectins in genetically modified potatoes altered the mucosa of the gastrointestinal tract of rats.

However, if a genetically modified feed is expected to have an important role in the animal diet, then appropriate assessment data are needed. Attention should be paid to the particular physiologic characteristics and metabolic requirements. Information will be needed on long term as well as on short-term effects of the consumption of the genetically modified feed.

Nucleotides are generally abundant in feed. Assuming that 85% of plant DNA is degraded before entering the small intestine (McAllan, 1982), a small proportion of the plant or microbial DNA fragments could potentially be absorbed from the digesta through the intestinal mucosa, either directly by epithelial cells or by antigen presenting cells of the immune system. Beever and Kemp (2000) suggested that most of this DNA is phagocytised by tissue macrophages. Nevertheless, it is conceivable that micro–environments exist where DNA is not degraded. Klotz and Einspanier (1998) reported the detection of a plant DNA fragment in white blood cells of a cow fed a diet containing genetically modified soybean meal. In other studies (Doerfler et al., 1997; Schubbert et al., 1994; 1997; 1998) viral DNA not only survived the passage through the gastrointestinal tract of mice, but was detected in host tissues (see also annex 1 to this chapter).
Gene transfer between bacteria is very extensive in natural ecosystems, so that it is thought that any transfer of transgenes may be negligible. Rare transfer events can have an enormous significance and can be amplified very rapidly under favourable circumstances. With regard to transgenes, pertinent questions are whether the release of a modified organism is likely to create a new route of acquisition of novel genes by organisms that are unlikely to have been able to acquire them naturally, and whether such acquisition could have detrimental consequences.
There are several reasons to assume that the rumen environment is more favourable for inter and intraspecies gene transfer (Forano and Flint, 2000). The microbial population in the rumen is very dense. The bacteria live in adhesion with feed particles, so that it is likely to have a permanent contact with exogenous free DNA. Furthermore, plasmids and bacteriophages have been found in rumen bacterial species.

Fragmentation of DNA is affected by feed processing. Chiter et al. (2000) reported that temperatures of 95°C or above for more than a few minutes are sufficient for degradation of DNA to take place to the extent that it should be incapable of transmitting genetic information. Furthermore, they mentioned that chemical

expulsion and extrusion of oilseeds resulted in residues with completely degraded genomic DNA. For feeds that are not subjected to high temperatures, such as wet sugarbeet pulp silage and cereal grain, is intact DNA still present and potentially taken up by microbes in the digestive tract (Chiter et al., 2000). This is in agreement with earlier findings of Hupfer et al. (1999), where amplification of a 211 bp sequence of the cry1A(b) gene was detected in maize seven months after ensiling. Duggan et al. (2001) concluded that free DNA from transgenic maize could survive in rumen fluid and saliva under in vitro condition. Extended exposure of maize grain to steepwater could result in some DNA degradation (Gawienowski et al., 1999).

There is no evidence to date that feeding genetically modified crops has detrimental effects on animal welfare. However, it can not be completely excluded, if foreign DNA is partly absorbed.
On the other hand, genetically modified plants may exert a positive effect on health: reduced fumosin concentrations may be expected in transgenic Bt-maize due to a decreased incidence of Fusarium ear rot (Munkvold et al., 1997 and 1999). Gregg et al. (1998) reported markedly reduced toxicological symptoms after fluoroacetate poisoning in sheep inoculated with ruminal bacteria, transformed with a gene encoding fluoroacetate dehalogenase.

The agro-industrial processing of genetically modified plants may increase transgenic protein in the involved end-products. For instance, the protein content in full fat soybeans used in ruminant diets may increase from ±400 g crude protein per kg DM to about 900 g in soy isolates used in milk replacers.
Many proteins are degraded by heat. However, Peferoen (1998) found no effect of a heat treatment at 90°C for 10 minutes on Cry9C activity. Van Wert and Noteborn (1999b) have shown that the Cry9C protein is resistant to the digestive enzymes from the stomach (pepsin) and the pancreas (trypsin), besides its heat resistance. Faust (2000) reported the detection of transgenic protein in soybean meal and maize silage.

3.2 Effects on the quality of end products

Data dealing with the effect of genetically modified crops in animal nutrition on the quality of the end product are rather scarce. Effects on milk composition are reported in the literature (Vilotte et al., 1997), but this is a consequence of the use of transgenic animals rather than feeding of genetically modified crops. Faust (2000) reviewed the literature on the composition and detection of transgenic protein and DNA in a range of livestock products. It was concluded that transgenic protein and transgenic DNA had not been found in milk, meat and eggs. Ash et al. (2000) neither found genetically modified protein in whole egg, egg white, liver and faeces of laying hens. Einspanier et al. (2001) reported that only short DNA fragments (<200 base pairs) derived from plant chloroplasts could be detected in the blood lymphocytes of cows. In all other cattle organs investigated (muscle, liver, spleen, kidney) plant DNAs were not found, except for faint signals in milk. However, in all chicken tissues (muscle, liver, spleen, and kidney) the short maize chloroplast gene fragment was amplified. In contrast to this, no foreign plant DNA fragments were found in eggs. Bt-gene specific constructs originating from recombinant Bt-maize were not detectable in any of these poultry samples either (see also annex 1 to this chapter).

4 Feed safety assessment

As described in Chapter I, three outcomes of the comparative approach can be considered (FAO,1996). The first category is this, which have the same composition as the parent crop (substantially equivalent). The second class has the same composition as the parent crop with the exception of a well-defined trait (substantially equivalent apart from defined differences). Finally, there are genetically modified crops, which are different from the parent crop (not substantially equivalent).
The concept of substantial equivalence has been accepted as a useful framework for the hazard assessment of genetically modified feed. Data for comparison should take into account agronomic, molecular and compositional aspects and the need for further studies depend on the nature of the differences and whether or not they are well characterised. Anti-nutritive factors should be determined when an altered composition is envisaged by the genetic modification. Nutritive value can be screened with in vitro tests. However, in vivo digestibility trials and balance studies in target animal should confirm results of in vitro studies. Long term feeding experiments, with ad lib. feeding of diets containing a high content of the genetically modified feed, are necessary to verify if there is no harmful effect on feed intake capacity, growth rate, feed efficiency, or yield of milk or eggs, animal health, reproduction, quality of end products and fate of modified protein and /or DNA. Special attention is necessary in case of young animals, which are more susceptible to deleterious effects because of an immature immune system.
Unintended effects are considered to be consistent differences between the genetically modified crop and its conventional counterpart, which go beyond the primary expected effect(s) of the introduced target gene(s). There remains a remote possibility that unintended effects in the plant are not detected by the approach of substantial equivalence. Novel methods and concepts are needed for the safety evaluation of next generation genetically modified crops to probe into compositional, nutritional, toxicological and metabolic differences between genetically modified and conventional crops.
Integrity of recombinant DNA should be studied with regard to processing (heating, flaking, crushing, ensiling,…). Industrial by-products of genetically modified crops involved in animal feeding should be subjected to a similar investigation as genetically modified crops.
A decision tree for the nutritional assessment can be recommended (see Annex 2 to this chapter), as proposed by Flachowsky et al. (2002).

4.1 Nutritional assessment of genetically modified feeds from a physiological point of view

The many manufactured feeds for animals make use of the same crops (or by-products of the same crops) used for human food. The safety assessment of animal feeds must take into account any risk to the animals consuming the feed and any indirect risk to the consumer of animal products (meat, milk and eggs).
An important question in this discussion concerns the definition of “safety of genetically modified feeds for animals”. A much wider range of factors than merely the aspect of “safety for animals” has to be taken into consideration but environmental aspects (biodiversity, soil, water), antibiotic resistance and human beings as consumers of these foodstuffs of animal origin should be included. As it is unlikely that any introduced protein will become directly incorporated into animal products, it is not considered necessary to test routinely for the presence of introduced genes or their products unless their characteristics suggest cause of

concern. The evaluation of the environmental aspects is beyond the scope of this report.

Studies on the composition of feeds, digestibility, feeding experiments, animal health and performance, quality of livestock products and fate of DNA demonstrate no significant differences between feeds from isogenic and transgenic plants where the genetic modification has introduced an agronomic characteristic (so called “first-generation plants”).

Although there is a growing body of scientifically valid information available that indicates no significant risk associated with the consumption of DNA or the resulting proteins from any genetically modified crops that are registered yet, a more complex nutritional assessment will remain necessary for genetically modified plants where the genetic modification has changed the feed composition (so called “next-generation plants”). They cannot be considered substantially equivalent to isogenic plants, as far as substantial changes in composition and nutritive value of those products are incorporated. A combination of nutritional and safety assessment in animal experiments is necessary and next-generation of genetically modified plants should be subject to the full range of physiological-nutritional studies with representative groups of animals. This requirement should apply for any new genetically modified plant when first introduced and it should include besides choosen animal test species and/or age categories, also feeding trials with the species concerned or for those categories of animals for which the modification is significant.
Indeed, farm animal species are not necessarily equally sensitive to possible toxic effects, their lifespan may be very different from one species to another or according to their production aim and moreover the quantity of a certain product consumed on daily basis and per kg BW may also be very different.

Nutritional efficacy is a legitimate indicator of product (genetically modified plant) quality, and therefore, wholesomeness. Animal performance studies are sensitive methods to measure feed quality and safety since one of the first, if not the first, indicator of a health problem of the animal is reduced performance.
However in the context of far reaching selection for performance in farm animals, subacute toxicity may be masked or overruled by their genetic predisposition for specific production goals (growth, milk, egg production) and additional safety studies have to be performed in addition to performance or wholesomeness studies.

Besides the determination of important ingredients in genetically modified feeds (see section 2, Compositional Analysis), the following parameters should be studied:

- digestibility of total novel feed
- balance studies
- availability of modified nutrients in target animals
- animal performance, health, welfare
- quality of foods of animal origin

Further in vitro studies to assess nutritional value or to study the fate of modified protein and/or DNA could be useful and of scientific value but is not necessary for risk assessment in farm animals.
As for the further physiological studies in case of new feeds or feed products, where there is expression of new proteins or other substances this can be regarded as foreign substances, hence possible toxic products.

Therefore the physiological tests should be equivalent as for chemicals or products with sub-acute oral toxicity in the worst case. The following test proposal is in first instance designed for the next-generation of genetically modified crops, taking the limitations of extrapolating results to other animal species into account. Special attention must be paid to the avoidance of problems of nutritional imbalance.

4.2 Proposal of test method

The genetically modified feed is administered orally in daily graduated doses to several groups of experimental animals for an appropriate period (28 or 90 days); the genetically modified feed should be performance tested in target species that would normally consume the plant products, besides rats as the preferred species as an experimental animal.
As a general rule, younger animals are more sensitive to nutritional imbalances or performance enhancement compared with older animals. On this basis it can be argued from a scientific point of view that young animals could serve as a bioassay model for their species.
However, from an industry acceptance point of view, producers are most interested in phases of performance in which the greatest quantities of feed are utilized. These are typically grower/finisher types of studies. As more data become available which support that such residues are not in marketed products, use of younger animal models may become more appropriate. Therefore, commonly used laboratory strains of young healthy rats, less than 6 weeks old and certainly not older than 8 weeks should be used. Perhaps the ideal animal model, when appropriate, is the broiler chicken, which allows testing over the full life of an animal in a rapidly growing and sensitive species. At the commencement of the study, weight variation in the animals used, whatever the species, should not exceed ± 20% of the mean value and preferably be less. The number of animals to be tested should be depended on the variation in the animals at the start of the trial for the variable to be tested in order to be able to detect significant effects.

The subacute oral toxicity test should be carried out in the target species according to the appropriate OECD protocol (see also section 3.4.7 of Chapter III).

5 Conclusion

In order to subject genetically modified feeds to a thorough safety assessment, notifiers should submit data as presented in this chapter. This includes investigation of the nutritional and physiological aspects of the genetically modified feed on a case-by-case basis, taking into account following considerations:

- the category of the genetically modified crop (first or next-generation) and a thorough description of the genetic modification; the potential effects of cultivation conditions and processes on the quality of the end product and the influence of the fate of modified DNA/protein on the composition;
- the concept of substantial equivalence as a useful framework for the safety assessment of first-generation products; next-generation of genetically modified plants should be subjected to physiological-nutritional studies;
- the nutritional and the food safety aspects of either feed or food derived from genetically modified crops, as well as health and welfare aspects of the animal. Investigation of the physiological aspects is important for the detection of possible unintended effects. The integrity of the animal should be

unaffected and the animal should function in the same manner as the animal that has been fed with the same non-genetically modified counterpart. Additionally the ecological safety of the genetically modified crop should be considered, which implies the evaluation of the effects of the genetically modified feed on the gut flora;

- the full range of physiological-nutritional studies should be carried out with representative groups of animals and special attention should be given to the target species itself.

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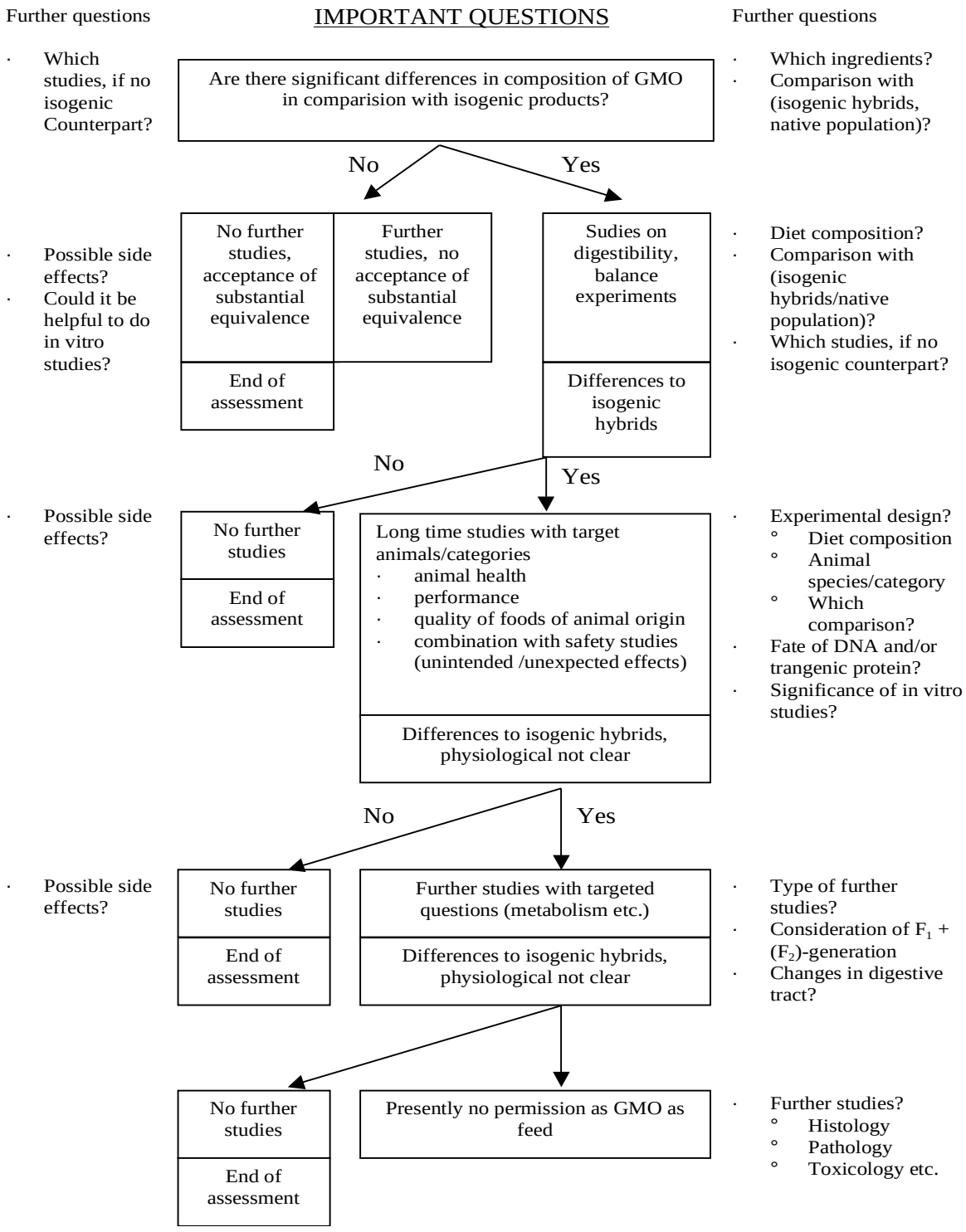
Annexes

Annex 1: Studies on the transfer of foreign DNA fragments from feed into the animal (translated from Flachowsky and Aulrich, 2001b)

Authors	DNA-source	Animal species	Results
Schubbert et al. (1994)	Phage-DNA	Mice	DNA-fragments in blood
Schubbert et al. (1997)	Phage-DNA	Mice, pregnant	DNA-fragments till 8 h in leukocytes, till 24 h in kidney and liver
Schubbert et al. (1998)	Phage-DNA	Mice, pregnant	DNA-transfer via placenta into foetus
Klotz and Einspanier (1998)	Soy beans	Dairy cows	Plant-DNA fragments in leukocytes, nothing in milk
Aeschbacher et al. (2001)	Bt-maize	Broilers	No plant-DNA fragments in the animal body
Einspanier et al. (2001)	Bt-maize (seeds and silage)	Broilers Laying hens Beef cattle Dairy cows	Plant-DNA fragments in muscle, liver, spleen, kidney of broilers and laying hens; no identification in blood, muscle, liver, spleen, kidney of bulls, eggs and excrements of broilers and layers; no identification of transgenic DNA-fragments in excrements of dairy cows
Hohlweg and Doerfler (2001)	Soy bean leaves	Mice	Plant-DNA fragments till 121 h in faeces, till 330 bp in liver and spleen samples
Khumnirdpetch et al. (2001)	Gt- soy beans	Broilers	No identification of DNA-fragments in tissue of muscles
Phipps et al. (2001)	Bt-maize	Dairy cows	No identification of transgenic DNA-fragments in the milk
Reuter et al. (2001)	Bt-maize	Pigs	Plant-DNA fragments in organs and tissues, no identification of transgenic DNA

Annex 2: Proposal for a decision tree for the nutritional assessment in combination with the safety assessment of genetically modified feeds (Flachowsky et. al., 2002)



Food nutrition evaluation

Working Group "Nutritional aspects of genetically modified foods": André Huyghebaert, Michel Paquot, Greet Vansant; Secretariat: Ellen Van Haver

1 Introduction

The variation in nutrient content is important because of the effects it can have in meeting nutritional requirements. It is therefore important to analyse the composition of the genetically modified food and to establish the extent to which the product is equivalent to its non-genetically modified counterpart. Statistically significant differences in composition may warrant closer examination during the safety assessment process of the food product with respect to the genetic modification. Furthermore, an estimate of the expected intake is necessary for the safety evaluation of the genetically modified food and for the evaluation of its nutritional significance.

2 Compositional analysis

2.1 Compositional data and methods

This section has to present the proximate analysis of the matter, to describe the sampling procedure, to refer to the analysis methods and to precise the statistical distribution of the results.

2.1.1 Major and minor constituents

A non-exhaustive checklist as presented hereafter provides information on critical parameters of food safety and nutrition. Depending of the crop and/or derived food product to be considered, several components may be not relevant.

Checklist for proximate composition analysis

- o Moisture % of wet weight
- o Protein % of dry weight
- o Total fat % of dry weight
- o Ash % of dry weight
- o Total carbohydrates % of dry weight

- o Amino acids % of dry weight and % of total amino acids
  - Essential and semi-essential amino acids: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine
  - Non-essential amino acids: alanine, asparagine, aspartic acid, cysteine, cystine, glutamic acid, glutamine, glycine, proline, serine, tyrosine

- o Fatty acids % of dry weight and % of total fat

  - See Table 1 of Chapter V for the classification of fatty acids.

- o Carbohydrates % of dry weight
  - Reducing sugars
  - Mono and disaccharides
  - Starch
  - Other polysaccharides

- o Dietary fibre % of dry weight
  - soluble fibre, with further specification if relevant
  - insoluble fibre, with further specification if relevant

- o Mineral composition and trace-elements
  - Na, K, Ca, P, Mg, Fe, Mn, Cu, Zn, S, I (mg / 100g)
  - Se, Cl, Pb, Co, Cr, Cd, Hg (mg / kg)

- o Vitamins
  - Fat soluble vitamins: Vitamin A (retinol) (µg/100g), Vitamin D_3 (cholecalciferol) (µg/100g), Vitamin D2 (ergocalciferol) (µg/100g), Vitamin E (α-tocopherol) (mg/100g), Vitamin K (phylloquinone) (mg/kg), β-carotene (mg/kg)
  - Water soluble vitamins: Vitamin B_1 (thiamine) (mg/kg), Vitamin B_2 (riboflavin) (mg/kg), Vitamin B_6 (pyridoxine) (mg/kg), Niacin (mg/kg), Pantothenic acid (mg/kg), Folic acid (mg/kg), Biotin (mg/kg), Vitamin B_{12} (cobalamin) (mg/kg), Vitamin C (ascorbic acid) (mg/kg)

2.1.2 Analytical methods

Reference methods must be used and mentioned. European standardized validated methods will be preferred but other official methods will be considered.

Following considerations may be useful for the choice of these methods. There is not a unique model for chemical and nutritional analysis of a food product. The nature

and finality of the product are to be taken into account. Nevertheless the choice of the analytical method is crucial for the validation and the signification of the result.

Proteins
The "conventional" test for protein measurement is based on the N content (Kjeldhall method). AOAC has proposed the modalities for nitrogen analysis in grains, animal products and milk products. In some cases (e.g. milk products) it is recommended to precipitate proteins (with trichloroacetic acid) in order to estimate the real protein nitrogen quantity and not the total nitrogen quantity. Nutritional values are not the same for proteins and non proteic nitrogen compounds. In the USA the Dumas method is an official method for protein measurement in cereals. A strong correlation exists between the two methods (Kjeldhall and Dumas).

Amino acids
Total amino acids composition is obtained after acid hydrolysis of peptidic links and separation by ion exchange chromatography. Conventional methods can be applied to all amino acids with the exception of tryptophan (totally destroyed) and sulfur amino acid that are oxidized. Asparagine and glutamine are transformed in aspartic acid and glutamic acid. Alkaline hydrolysis is the obligatory pathway for tryptophan analysis. Sulfur amino acids can be estimated after oxidation.

Fatty acids
Lipids are mainly composed of hydrophobic units. Their solubility characteristics, rather than a common structural feature, are unique for this class of compounds. The majority of lipids are derivatives of fatty acids.
Some lipids act as building blocks in the formation of biological membranes. They occur in food but usually at less than 2%. Nevertheless, even as minor food constituents they must receive particular attention, since their reactivity may strongly influence the organoleptic quality.
Triacylglycerols (also called triglycerides) are deposited in several animal tissues and organs of some plants. Lipid content in such storage tissues are a commercial source of lipids that can rise up to 20%.
The class of lipids (see Table 1: classification of lipids according to “acyl residue” characteristics) also includes some important food aroma substances or precursors, as well as amphiphilic substances, pigments, vitamins, colorants….

Table 1 : Classification of lipids according to “acyl residue” characteristics

Class of lipids	**Constituents**
Simple lipids (not saponifiable)	Free fatty acids, isoprenoid lipids (steroids, carotenoids, monoterpenes), tocopherols
Acyl lipids (saponifiable)	
Mono-, di-, triacyl-glycerols	Fatty acids, glycerol
Phospholipids (phosphatides)	Fatty acids, glycerol or sphingosine, phosphoric acid, organic base
Glycolipids	Fatty acids, glycerol or sphingosine, mono-, di- or oligosaccharide
Diols lipids	Fatty acids, ethane, propane, or butane diol
Waxes	Fatty acids, fatty alcohols
Sterol and stanol esters	Fatty acids, sterols, stanols

Total fat can be determined after extraction and gravimetric estimation. Some methods (e.g. Folch) allow to identify the different categories of extracted lipids afterwards. Hydrolysis methods (e.g. acid hydrolysis) have been standardised but these methods do not allow to characterise the individual lipid classes, only the total fatty acids.. A lot of specific methods have been standardized with regard to the kind of raw materials (grains, oleaginous crops…).
Lipids in fats are a very heterogeneous category of components. The extraction procedure is very important in order to obtain all the fractions and to prevent the extraction of other materials (hydrophobic proteins…). Fatty acids can be determined by gas chromatography after saponification and esterification.
It will be very useful to distinguish the ω6 and the ω3 families for nutritional aspects.

Carbohydrates
Carbohydrates are commonly divided into monosaccharides, oligosaccharides and polysaccharides.
Total reducing power after chemical hydrolysis gives an approximation of the digestible glucids (sugars + starch). Chromatographic methods (CPG and HPLC) allow to obtain individual sugars.
Starch can be measured after gelatinization, liquefaction and hydrolysis into glucose.

Dietary fibres
Dietary fibres may be composed of soluble and insoluble constituents. Because of the large diversity of undigestible materials, analysis is difficult. Enzymatical methods are preferable to the Van Soest method even if this last technique has been standardized in some countries, especially for cereals.
The Van Soest method gives values similar to those obtained in vivo from digestibility studies with animals. This technique allows to determine the concentration of cellulose, lignin and hemicelluloses. Nevertheless, the Van Soest method does not correspond to the actual notion of dietary fibres including a lot of other constituents as soluble and insoluble fibres are not distinguished.
With enzymatical methods the digestible constituents (1-4 β-glucans, proteins) in the defatted sample are enzymatically hydrolysed (heat stable α-amylase, gluco-amylase, protease). Water soluble fibres are isolated by precipitation with ethanol. The proteins and mineral matter still remaining with the soluble and insoluble dietary fibres are deducted.

Minerals and Trace-elements
Minerals are the constituents remaining as ash after calcination. They may be divided into two categories: main elements (Ca, P, K, Cl, Na, Mg) and trace elements (Fe, Zn, Cu, Mn, I, Mo…).
The main elements and number of trace elements are essential because they have a biological role. In the same food raw material, the content can vary greatly according to genetic and climatic factors, agricultural procedures…
A lot of food constituents (protein, organic acid, polysaccharides…) bind minerals and influence their biodisponibility.

Several trace elements may be toxic depending of the food intake.

Vitamins
Vitamins are minor but essential constituents of food. They are usually divided into fat-soluble and water soluble vitamins.

Several methods can be used to measure their content. Chromatographic methods are often possible. Attention must be paid to extraction procedures before analysis.

2.1.3 Statistical and sampling aspects

The sampling method must be explained and must take into account the requirements linked to the statistical analysis as well as the distribution of the components in the raw material.
A very important point to consider is the variability of the raw material for example by taking into account the impact of the geographical origin, the climate, the agronomical practices, the annual variations…
Enough samples are to be analysed with the help of a sampling plan and the results are to be evaluated on a statistical basis.
Plants used to obtain samples for compositional analysis should be grown under conditions that represent normal practice for the crop plant. For example, studies on herbicide tolerant crops should be done on herbicide treated crops (with a waiting period afterwards). As the transgenic plant inactivates the herbicide, (metabolised) degradation products might be present in the plant.

2.2 Nutritional aspects

Whenever changes are made to the way in which a food is produced or processed or uses non-conventional ingredients, the implications on the nutritional value require consideration. Information will be needed on any issue relating to this aspect. Foods are usually complex mixtures of macro- and micronutrients which provide energy and nutrients and contribute to human well-being.

2.2.1 Identification of key nutrients

If a genetically modified crop is expected to have an important role in the diet then appropriate information on nutritional composition is needed. Both macro- and micronutrients of nutritional value are already given in section 2.1.1. It is clear that not all these nutrients are relevant for every specific genetically modified crop. For every such crop, the place (value) within the human diet should be determined. It is well known that different food groups contribute in different ways to human nutrition. Depending on the composition and the (estimated) consumption of the genetically modified food, it appears justified to limit the testing to the most relevant nutrients, which are specified in Table 2. This table should be considered as an example and not as an exhaustive list.

Table 2: Identification of relevant nutrients for different food groups

Food group	Key nutrients
Cereals	Carbohydrates (simple and complex), dietary fibre, B-vitamins, minerals and trace elements, proteins and amino acids (if present)
Fruits and vegetables	Water-soluble vitamins, dietary fibre, carbohydrates, minerals and trace elements
Milk and milk-products	Total protein content and specific amino acid composition, fatty acid composition, fat-soluble vitamins, calcium, other relevant minerals and trace elements
Meat, Poultry, Fish and Meat-replacers	Total fat and fatty acid composition, total protein (for meat replacers also specific amino acids), fat-soluble vitamins, vitamin B12, trans fatty acids
Fats and oils	Fatty acid composition, fat-soluble vitamins, total fat, trans fatty acids
Extras	Macro-nutrient composition

2.2.2 Intake

The consumption pattern may show a major change when a genetically modified food is included in the diet and thus affects human nutritional status. As it may not be possible to predict such events, a surveillance programme should accompany the marketing of a genetically modified food. Such a programme should encompass information on changes in the conditions for processing and preparation as well as effects of possible replacement of other foods or food component of dietary importance. If surveillance reveals changes in those factors which raise concerns regarding wholesomeness, a reappraisal of the acceptability of the genetically modified crop would be required.

2.3 Toxicants and anti-nutrients

Naturally occurring toxins that are inherently present in the plant should be determined. Data on the sensitivity of the crop towards the formation of mycotoxins, pathogenic microorganisms, biogenic amines and other toxic substances or organisms formed in the product have to be given, if relevant.

Information is requested with respect to the presence of anti-nutrients. This applies particularly to the key anti-nutrients for the product. The examples given below are to be considered as examples and not as an exhaustive list.

2.3.1 Examples

- **Protease inhibitors** inhibit the activity of trypsin, chymotrypsin and other proteases. They are found in legumes such as beans and peas, but also in cereals, potatoes and other products. Their presence results in impaired growth and poor food utilization.
- **Amylase inhibitors** have a similar activity against amylases.
- **Lectins or hemagglutinins** are glycoproteins mainly found in legumes: beans, peas, lentils. They bind to intestinal epithelial cells. They cause agglutination of

erythrocytes in vitro. Their presence results in poor food utilization and impaired growth.

- **Cyanogens** are cyanogenic glucosides found in cassava, linseed, peas, beans and other products. They may cause HCN poisoning.
- **Glucosinolates** are thioglycosides found in cabbage and related species. Effects upon the thyroid function have been demonstrated.
- **Saponins** are also glycosides found in soybeans, peanuts, sugar beets and others. Haemolytic effects in vitro have been shown.
- **Gossypol** is particularly important in cottonseed. Several toxic effects have been demonstrated.
- **Phytic acid** occurs in several vegetable products. This compound has a strong chelating activity. Its presence may affect bioavailability of minerals.
- **Mycotoxins** are very diverse in chemical structure and in the characteristics of the mycotoxicoses they produce. These toxins include the aflatoxins, the tricothecenes, the fumonisins, zearalenone, moniliformin and fusaric acid.
- **Phytotoxins** (solanine, etc.).

2.3.2 Potential effects of cultivation conditions and processes

If relevant for the particular food, information is requested about the presence of the anti-nutrient and particularly about the quantity. Moreover this information should include data about the potential effect of different cultivation conditions.
In addition it is very well established that processing may have profound effect upon the level of anti-nutrients present. Two approaches at least have to be followed:

- the effect of inactivation processes
- the effect of separation processes.

In order to have a real picture of these effects, the commonly applied processing steps have to be followed. The use of flow sheats is highly recommended.
Inactivation studies under conditions equivalent to normal processing may give information about the stability of the anti-nutrient. Inactivation may be due to heat treatments, enzymatic activity , leaching or others.
Separation processes like dry milling, wet milling, extraction, centrifugation or equivalent may affect the level of anti-nutrients. In this case it is easily understood that information on the localisation of the anti-nutrient is of great help, such as the distribution in endosperm, aleurone layer, bran or germ for cereals. Data about the presence of the particular anti-nutrient in fractions for food and for feed use are necessary.

2.4 Secondary Plant Metabolites

Secondary plant metabolites are neither nutrients nor anti-nutrients. They are part of the characteristic composition of a plant. They are important for the compositional analysis and for the comparative approach. Even more than in the previous paragraphs, an exhaustive list cannot be given.
Some of these substances may have undesirable effects. Others may have beneficial effects for human health or in terms of resistance to mould growth.

2.4.1 Examples

- **Phenolic compounds** are considered to be of great importance. Detailed information about the qualitative and quantitative composition of the phenolic fractions is necessary.
- **Key enzymes** may affect the utilization of the plant material. Information about the relevant enzymes is necessary.
- **Organic acids** are another group. This includes:
  - aliphatic plant acids like citric, malic and others,
  - aromatic acids like benzoic acid and analogues,
  - phenolic acids like caffeic, coumaric, ferulic acids and others.
- **Carbohydrates** are mainly covered in section 2.1.1 (major and minor constituents). In addition to simple sugars and polysaccharides, complex sugars such as raffinose, stachyose and verbascose have to be covered in this section.
- Additional information on **N substances**, if not covered in a previous section, is requested. This includes low molecular N substances, unusual amino acids and others.
- With respect to **lipids**, information not covered in Table 1 is requested. This includes complex lipids and others.

2.5 Derived Products

Processing may have a pronounced effect upon the content and distribution of nutrients and anti-nutrients.
This aspect is, by preference, covered by means of flow sheats indicating the major steps in the processing scheme. As the global and detailed composition is already dealt with in the previous sections, further information on particular nutrients and anti-nutrients is necessary if this information is essential for the assessment of the product.
As an example it is felt that when discussing soya-beans some aspects related to inactivation of anti-trypsin factors during toasting have to be included

3 Implications of genetically modified crops to human nutrition

If a genetically modified food is expected to have an important role in the diet then appropriate human nutritional assessment data are needed. Attention should be paid to the particular physiologic characteristics and metabolic requirements of specific groups of the population (infants, pregnant and lactating women, elderly) and to persons with chronic diseases (like diabetes). Information will be needed on long term as well as on short term effects of the consumption of the genetically modified food.

4 Conclusion

The assessment of possible compositional changes as a result of the genetic modification has to be carried out for genetically modified crops and derived products as presented in this chapter. This includes the analysis of the major and minor constituents, the anti-nutrients, the secondary plant metabolites and the possible

occurrence of toxicants. Investigation to what extent cultivation conditions and processes can lead to the concentration or to the elimination of the constituents in the final product should also be carried out. For every genetically modified crop, the place (value) within the human diet should be determined. As the genetic modification could change the overall nutrient profile of the crop and consequently affect the nutritional status of individuals consuming the food, a surveillance programme should accompany the marketing of such crops.

List of experts

Within the framework of the Biosafety Council, which is the Belgian advisory board on genetically modified organisms, different working groups have contributed to the development of this document. The secretariat and the co-ordination of these working groups was carried out by the Section of Biosafety and Biotechnology (SBB) of the Scientific Institute of Public Health.

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