Safety and Nutritional Assessment of GM Plant derived Foods/Feed

The role of animal feeding trials

Draft report for public consultation

Deadline for comments: 31 January 2007

FOREWORD

The present report results from the self tasking activity of the Panel on Genetically Modified Organisms (GMO Panel) of the European Food Safety Authority (EFSA). This self-tasking activity on the use of animal feeding trials for the safety evaluation of *whole* GM foods/feed started its activities in January 2005 and a working group was set up consisting of the following members:

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The working group had several meetings between January 2005 and October 2006 to come to a finalized version of the document in October 2006. This document was presented to the GMO Panel at its Plenary meeting of 5-6 December 2006, prior to publication on the EFSA website for a 6-week period of public consultation. The working group and the GMO Panel are seeking views of scientific nature from interested parties, Member States and stakeholders. Comments should be submitted at the latest by 31 January 2007 by using the EFSA consultation form that can be found at:
http://www.efsa.europa.eu/en/science/gmo/gmo_consultations/gmo_AnimalFeedingTrials.html.

OVERVIEW

In this report the various elements of the safety and nutritional assessment procedure for genetically modified (GM) plant derived foods/feed are discussed, in particular the use of animal feeding trials for safety and nutritional testing.

The report is based on the general principles as described in the EFSA guidance document of the EFSA Scientific Panel on Genetically Modified Organisms for the risk assessment of GM plants and derived food and feed (EFSA, 2006a), and offers further detailed guidance for safety and nutritional testing of foods/feed derived from GM plants.

The risk assessment approach for GM plant derived foods/feed, is a *stepwise* procedure and considers two main categories of hazards, *i.e.* those related to the intended intrinsic properties and function of the introduced trait(s), and those resulting from insertion of the introduced gene(s) into the plant genome that might cause unintended effects. Key elements of the assessment procedure are the molecular, compositional, phenotypic and agronomic analysis in order to identify similarities and differences between the GM plant and its near isogenic counterpart which need further evaluation.

In ***Chapter 1*** the mandate, scope and general principles for risk assessment of GM plant derived foods and feed are discussed. Products under consideration are foods/feed derived from GM plants, such as maize, soybeans, oilseed rape and cotton, modified through the introduction of one or a few genes coding for agronomic input traits like herbicide tolerance or insect resistance, or a combination of these traits. Furthermore GM plant derived foods and feed are considered which have been obtained through extensive genetic modifications targeted at specific alterations of metabolic pathways leading to improved nutritional and/or health characteristics, such as rice containing β-carotene, soybeans with enhanced oleic acid content, or tomato with increased levels of flavonoids.

Chapter 2 provides an overview of studies performed for the safety and nutritional assessment of whole foods and feed. Relevant experience has been built up with the safety and nutritional testing of irradiated foods, as well as with long term testing of fruits and vegetables. Furthermore a review is presented of feeding trials in which foods/feed derived from GM plants were fed to laboratory or livestock animal species during prolonged periods of administration in order to assess their safety and/or nutritional characteristics. Moreover safety studies of GM foods in humans are discussed as well as the experience with post market monitoring of GM and/or novel foods.

In ***Chapter 3*** various *in silico, in vitro* and *in vivo* methods are discussed which may be applied for the safety and nutritional assessment of specific compounds present in food/feed or of whole foods/feed derived from GM plants. In particular the potential and limitations of the 90-day rodent feeding trial for the safety and nutritional testing of foods/feed are examined with respect to (i) the capacity to detect unintended effects which might occur in GM plants and derived foods/feed as a result of the genetic modification, (ii) predictivity of the test model regarding the detection of possible chronic effects of foods/feed tested, and (iii) the establishment of margins of safety between food/feed intake by test animals and humans. Furthermore target animal models for the safety and nutritional assessment of feeds derived from GM plants are discussed with respect to animal species to be used, number of animals, duration of experiments, composition of diets and biological parameters to be measured. Furthermore the feasibility and limitations of human studies with foods derived from GM plants are discussed, as well as the potential and limitations of post market monitoring to detect unintended effects of these foods.

In ***Chapter 4*** standards for test sample preparation, test materials, diet formulation and analysis are evaluated. Specific attention is paid to the choice of control diets and comparators, dietary stability, nutritional balancing of diets, restricted feeding versus *ad libitum* feeding, tolerances studies, purpose and practice of spiking diets with test compounds, and analyses and quality assurance of diets. Moreover specific recommendations are made regarding the preparation of animal diets containing GM foods/feed with enhanced nutritional properties.

Chapter 5 provides information on the collection, analysis and interpretation of data and findings obtained from animal feeding studies. Attention is paid to data generation and quality assurance, data collection, presentation and interpretation, and statistical evaluation of study results. The aim of this chapter is how the data from animal studies are evaluated in order to draw conclusions on any potential impacts that might be predicted for human and animal health, safety and nutrition.

In ***Chapter 6*** strategies are outlined for the safety and nutritional assessment of GM plant derived foods and feed, and under which conditions safety and nutritional testing of the *whole* GM plant or derived foods/feed may be considered.

Testing of the safety and nutritional value of the *whole* GM plant or derived foods/feed should be considered where the composition of the GM plant is modified substantially, or if there are any indications for the potential occurrence of unintended effects as a result of the genetic modification based on the preceding molecular, compositional, phenotypic or agronomic analysis. In such a case, the testing program should include at least a 90-day toxicity study in rodents.

Animal feeding trials in which whole foods/feed are fed to rodents, are generally sensitive and specific to detect toxicologically relevant effects of newly expressed compounds in whole foods/feed, and also to detect relevant unintentional events which may have taken place as result of the genetic modification.

The need for conducting animal feeding studies using target animals in order to evaluate the nutritional characteristics of GM plants should be carefully evaluated. Numerous feeding studies with feed derived from GM plants with improved agronomic properties, carried out in a wide range of livestock species, did not show any biologically relevant differences in the parameters tested between control and test animals. Once compositional, phenotypic and agronomic equivalence has been established, nutritional equivalence may also be assumed, and feeding trials with target animals add little to the nutritional assessment of the feed. Livestock feeding studies with target animal species should be conducted on a case-by-case basis to establish the nutritional benefits that might be expected from GM plant derived feed with claimed nutritional/health benefits.

A number of *in vitro* and *in silico* tests can be applied on a routine basis during the initial phase of the safety and nutritional assessment of GM plant derived foods/feed or ingredients. Among others, structure-activity relationship studies, structural homology searches for known toxins and allergens, biodegradation studies under simulated gastro-intestinal conditions, and application of the new genomic technologies can yield important information that will further guide the risk assessment and may possibly reduce the requirement for animal studies.

No progress has so far been made in reducing or replacing the use of animals in repeated dose studies, such as 28-day or 90-day studies, with the important exception for GM plant derived foods/feed stated above, that repeated dose studies in the tiered approach should normally only be undertaken when triggered by likelihood of unintended effects.

Regarding the analytical detection of unintended effects, profiling technologies such as transcriptomics, proteomics and metabolomics are promising tools, which will broaden the spectrum of detectable

compounds and supplement current targeted analytical approaches. These technologies are still under development, and need validation before they can be used for routine safety assessment purposes.

In ***Chapter 7*** conclusions and recommendations are reported regarding:

- the comparative approach to safety and nutritional testing of foods/feed derived from GM plants;
- the experience from testing of non-GM and GM whole foods;
- the *in silico* and *in vitro* tools available for safety and nutritional testing of GM plant derived foods/feed;
- the testing of defined single substances from GM plant derived foods/feed in *in vivo* studies;
- the testing of whole GM plant derived foods/feed in animal feeding studies;
- the importance of a structured approach for development of data for the pre-market safety and nutritional testing of GM plant derived foods/feed;
- the role of post market monitoring.

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1. INTRODUCTION

1.1 Terms of reference and mandate

In order to arrive at a high level science-based risk assessment of chemicals in food and diet, and of *whole* foods and feed where appropriate, animal studies may still be needed, although extensive efforts are ongoing to develop and validate alternative testing systems (FOSIE, 2002). To this end EFSA is taking a pro-active approach in animal welfare issues by stimulating and participating in the development of new food and feed assessment approaches that would refine, reduce or replace the use of experimental animals (EFSA, 2004a).

The EFSA GMO Panel has issued a Guidance Document (EFSA, 2006a) which provides guidance for the risk assessment of genetically modified (GM) plants and derived food and feed submitted within the framework of the Regulation (EC) No. 1829/2003 on GM food and feed (EC, 2003a), or Directive 2001/18/EC on the deliberate release into the environment of GMOs (EC, 2001). Depending upon the available information on the donor and recipient organisms, specific studies, including animal studies, may be needed to assess the toxicity and allergenicity of newly expressed proteins, metabolites and of the whole GM plant. Such studies should be conducted according to internationally accepted test methods and protocols as developed by OECD and adopted by the European Union.

With respect to the safety testing of the GM foods/feed, the Guidance Document states (Chapter III 7.8.4, EFSA, 2006a): "If the composition of the GM plant is modified substantially, or if there are any indications for the potential occurrence of unintended effects, based on the preceding molecular, compositional, phenotypic or agronomic analysis, not only new constituents, but also the whole GM foods/feed should be tested". In these cases, the testing programme should include at least a 90-day toxicity study in rodents, while further comparative growth studies may be conducted with target species or categories of adequate food producing animals.

In contrast to the testing of single food chemicals, e.g. additives, no detailed test protocols for diet preparation and animal testing of foods/feed are available. Performance of animal studies with foods/feed faces many difficulties regarding diet preparation, dose levels to be administered, nutritional imbalances in the diet, food matrix effects etc.

Evaluation of foods/feed animal safety studies, submitted by applicants in the framework of Directive 2001/18/EC and Regulation (EC) 1829/2003, shows that the above mentioned challenges may lead to differences in experimental performance, data analysis and processing and interpretation. This has sometimes resulted in different scientific opinions of expert committees in the EU Member States, and as a consequence occasionally in different views of national authorities involved in the EU regulatory framework. The EFSA GMO Panel has been and will probably in the future be confronted with differences in scientific assessments of GM foods/feed.

Furthermore from the comments of stakeholders on the EFSA Guidance Document (EFSA, 2006a), which was prepared by the EFSA GMO Panel, it became clear that different views exist regarding the necessary duration of animal feeding trials, *i.e.* varying from 28 days to 6 months. A clear need exists to examine this issue and if possible to indicate criteria for optimal duration of this type of experiment.

From a scientific risk assessment (and risk communication) point of view it is of utmost importance to arrive at a more standardised and harmonised approach in risk assessment strategies aimed at the safety assessment of GM foods/feed.

Mandate

The GMO Panel agreed to:

- examine the potential and limitations of animal feeding studies for the safety and nutritional assessment of (GM) foods/feed;
- describe principles and provide guidance for the preparation of animal diets and for the performance of the animal tests;
- provide guidance for data collection and data analysis;
- provide guidance for data interpretation and risk characterisation (*i.e.* biological significance of results, margins of exposure and safety, extrapolation of data, confounding factors, remaining uncertainties etc.);
- indicate how existing animal models can be improved, supplemented and/or replaced by specific cell based *in vitro* and *ex vivo* models, and/or modern gene expression, gene translation and metabolomics technologies;
- develop criteria and provide guidance to applicants and risk assessors on conditions for carrying out animal feeding trials in combination with alternative complementary methods for safety and nutritional testing of (GM) foods/feed;
- consult stakeholders (national experts, biotech companies, non-governmental organisations) in order to try to establish a consensus.

1.2 Background

GM plants and derived foods/feed that are currently on the market, have been modified through insertion of single or a few genes which express traits, such as providing herbicide tolerance and/or insect resistance. Apart from the intended alterations in their composition, these plants show no evidence for alterations in phenotype and basal composition.

GM plants are now under development, in which significant *intended* alterations in composition have been achieved in order to improve the agronomic properties (e.g. drought resistance, salt tolerance etc.), or to enhance the nutritional or health beneficial properties. Examples of GM plants with nutritionally improved traits intended to provide health benefits to consumers and domestic animals are given in Table 1.

GM plant safety assessment is particularly stringent as it focuses on two elements, the safety of the intended effects of the genetic modification as well as on the sum of all the possible modifications by carefully analysing the whole plant and its performance in a range of studies.

Safety and nutritional assessment of GM plant derived foods/feed

Risk assessment is defined as the evaluation of the probability of known or potential adverse health effects arising from human or animal exposure to the identified hazards (FAO/WHO, 1996, 2000; Chapter IV of EFSA, 2006a).

The safety assessment of GM plants and derived foods/feed follows a *comparative* approach, *i.e.* the foods/feed are compared with their non-GM counterparts in order to identify intended and unintended

differences which subsequently are assessed with respect to their potential impact on the environment, safety for humans and animals, and nutritional quality (Concept of Substantial Equivalence or Comparative Safety Assessment, Concept of Familiarity; OECD, 1993; EC, 1997a; WHO, 1995; FAO/WHO, 2000; Codex Alimentarius Commission, 2003; ENTRANSFOOD, 2004; EFSA, 2006a).

The rationale for the comparison of the GM plant derived foods/feed with non-GM plant derived foods/feed is based on the assumption that traditional varieties from which GM plants have been derived, are generally regarded as safe to eat, because of their history of use. The appropriate comparators have all traits in common except for the newly introduced ones. The OECD concluded that a food is safe if "there is reasonable certainty that no harm will result from its consumption under anticipated conditions of use" (OECD, 1993).

Due to the complexity of whole foods, the goal of the assessment is to provide the same level of safety as accepted for traditional foods. The criterion is therefore not *absolute* safety, but *relative* safety based on comparison with traditional foods, in other words, to establish whether the food from the GM plant is as safe as its traditional counterpart.

The comparative approach to assess whether the GM plant is "as safe and nutritious as" its comparator plant, encompasses several aspects of evaluation, including both toxicological, nutritional, microbiological and environmental effects, and is often called an assessment of "wholesomeness" (Dybing, 2002).

A detailed and *stepwise* procedure for the safety assessment has been developed within the EU project ENTRANSFOOD (König *et al.*, 2004, see Figure 1), and is described in EFSA (2006a).

Background knowledge is required on:

- parent plant (history of safe use, phenotype, chemical composition);
- transformation process (source of transferred gene(s), DNA construct, consequences of DNA insertion);
- newly expressed proteins and other constituents (potential toxic or allergenic effects);
- GM plant (agronomic performance, phenotypic appearance, composition, safety and nutritional characteristics, ability to transfer genetic material to other organisms);
- anticipated intake/extent of use;
- nutritional properties;
- food processing characteristics.

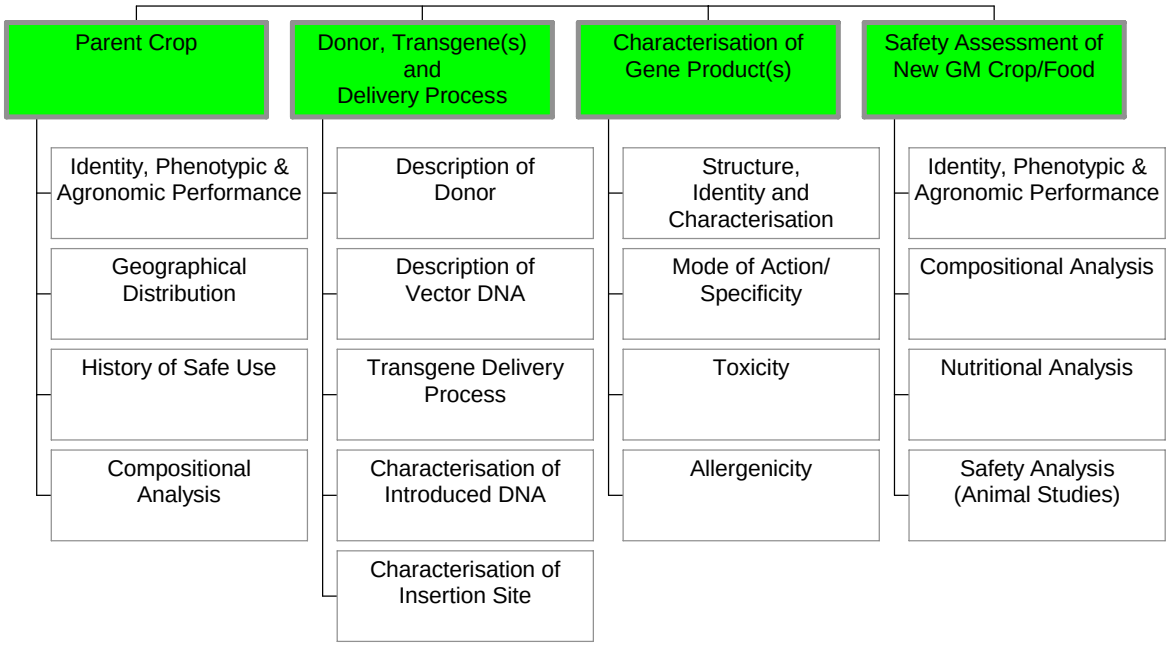


Figure 1: A fully integrated and iterative approach to the hazard assessment and characterisation of all elements involved in producing a new GM variety (from König *et al.*, 2004).

The safety assessment is focussed on the presence and characteristics of newly expressed proteins and other new constituents and possible changes in the level of natural constituents beyond normal variation, and on the characteristics of the GM foods/feed.

The toxicological assessment of individual gene products is done using standardized toxicological methodology designed for the assessment of defined chemical substances, and standard guidelines for the conduct of toxicity tests are clearly described in the OECD Guidelines for Testing of Chemicals or in the most up-to-date European Commission Directive on dangerous substances (OECD, 1995; EC, 2002). Furthermore these tests should be carried out following Good Laboratory Practice (GLP) principles (EC, 2004).

Compositional analysis of GM plants and derived foods/feed is a key element of the comparative safety assessment approach in order to identify similarities and potential differences between the GM product and its conventional counterpart. The analysis encompasses: proximate analysis of macro-nutrients, analysis of micro-nutrients, and analysis of inherent toxins, allergens, and anti-nutrients. For specific crop plants Consensus Documents with lists of parameters to be measured have been developed by the OECD (OECD, 2001a,b; 2002, a,b,c). Validated analytical methods for single compounds (targeted analysis) should be used.

Most if not all proteins are immunogenic and, while tolerance is usually evident after ingestion of these proteins, sometimes immunogenicity leads to hypersensitivity reactions, *i.e.* allergy. Sometimes such reactions are of a cellular nature, as is the case with gluten; more often such hypersensitivity is of an immediate type. Assessment of the potential allergenicity of newly expressed proteins and of a possible alteration of the allergenicity of the whole GM plant and derived foods is carried out taking an integrated, stepwise, case-by-case approach as described in the EFSA Guidance Document (EFSA, 2006a). This approach is in line with the Guidelines developed by Codex (Codex Alimentarius, 2003). At present a Working Group of the EFSA GMO Panel is further addressing the assessment of potential allergenicity of GM plant derived foods/feed, taking into account the latest developments in this area.

Occurrence and identification of unintended effects

As in the case of traditionally bred crops, the potential occurrence of "unintended effects" in GM plants as a direct or indirect result of the genetic modification is one of the issues to be considered during the safety assessment of GM plants and derived foods/feed. Unintended effects can be defined as "consistent differences between the GM plant and its appropriate control lines, which go beyond the primary expected effect(s) of introducing the target gene(s)" (EFSA, 2006a). Such effects may occur due to genetic re-arrangements or disruptions of metabolic pathways in the recipient plant through gene insertion. Changes may include alterations in metabolic pathways resulting in increased levels of endogenous toxins or allergens, or lower levels of essential nutrients, or expression of previously silent genes encoding toxins or allergens.

The occurrence of unintended effects is not a phenomenon specific to genetic modification. In classical breeding extensive backcrossing, selection of favourable lines and discarding lines with unwanted properties is common practice in order to remove unintended effects.

Strategies for the detection of unintended (unexpected) effects in GM plants and derived foods/feed have been described by ENTRANSFOOD (Cellini *et al.*, 2004). In order to identify possible unintended effects in GM plants due to the genetic modification, a comparative phenotypic and molecular analysis of the GM plant and its near isogenic counterpart is carried out, in parallel with a *targeted* analysis of single specific compounds, which represent important metabolic pathways in the plant like macro and micro nutrients, known anti-nutrients and toxins. Significant differences between the GM plant and its appropriate control line identified on the basis of phenotypic, molecular or compositional analysis, may be indicative of the occurrence of unintended effects, and require further investigation.

In order to assess the biological relevance of possibly identified differences in composition between the GM plant and its non-GM near isogenic counterpart, information on natural ranges of variation of specific compounds is essential. To this end an OECD Task Force is compiling information on the composition of major foods/feed crops in Consensus Documents (OECD, 2001a,b; 2002, a,b,c), as well as an ILSI Task Force which is setting up a database with compositional data of crops (ILSI, 2003a).

Safety and nutritional testing of GM foods/feed

Testing the safety and nutritional value of *whole* GM foods/feed should be considered in cases where the composition of the GM plant is modified substantially, or if there are any indications for the potential occurrence of *unintended* effects as result of the genetic modification (EFSA, 2006a). It is realised that laboratory animal studies of whole foods are not easily performed, since whole foods are complex mixtures of compounds with very different biological characteristics. Moreover foods are bulky and have an effect on the satiety of animals and can therefore only be fed at relatively low multiples compared to their typical presence in the human diet. Moreover there is a possibility that in attempting to maximise the dietary content of the foods/feed under investigation, nutritional imbalances may occur. These could lead to the appearance of effects which may not be related to the properties of the whole food being tested. Strengths and weaknesses of this type of testing are discussed in details in the following chapters.

Once compositional equivalence of the GM plant has been demonstrated, work may then be focused, where necessary, on livestock feeding studies to confirm nutritional equivalence, and to obtain further information on the safety. Livestock feeding studies with target species are sometimes conducted to establish the effect of a new feed material on animal performance with endpoint measurements such as feed intake, animal performance, feed conversion efficiency, animal health and welfare, efficacy, and acceptability of the new feed material. The extent and type of livestock feeding studies conducted will

depend on the type of feed material developed, and their need should be determined on a case-by-case basis.

This report addresses the issues regarding the potential and limitations of performing animal tests in order to characterise the safety and nutritional properties of GM foods/feed, and examines whether current strategies for the safety/nutritional testing can be improved by the additional or alternative use of modern *in vitro*, *ex vivo* methods.

1.3 Scope of the report

The scope of this report is:

1. to review the experience gained with testing (GM) foods/feed regarding human and animal safety;
2. to examine models for safety and nutritional testing of GM foods and feed, including laboratory and target animal models, *in silico* and *in vitro* models and human studies;
3. to further develop an integrated risk assessment paradigm for testing of the safety and nutritional properties of GM foods/feed.

The emphasis in this document is on the safety and nutritional assessment of plant derived GM foods/feed and of derived complex mixtures of nutrients and non-nutrients, because of the relatively urgent need for guidance in the area of safety assessment of whole GM plant derived foods/feed within the regulatory framework. It does not cover the safety and nutritional assessment of foods/feed derived from GM animals or from GM microorganisms (GMM). Nevertheless many of the principles described in this document may equally apply to any foods/feed of complex nature. For the assessment of foods/feed derived from GMMs, the reader is referred to the EFSA Guidance Document for the safety assessment of foods/feed derived from GM microorganisms (EFSA, 2006b).

1.4 GM plant derived foods/feed to be considered

Products under consideration are for example whole foods/feed derived from GM plants, such as maize, soybeans, oilseed rape and cotton, modified through the introduction of one or a few genes coding for herbicide tolerance, insect resistance or a combination of these traits. In these plants the DNA insert leads to the synthesis of a gene product, which does not interfere with the overall metabolism of the plant cell. Examples are proteins such as phosphinothricin acetyltransferase (PAT) conferring glufosinate-ammonium tolerance to the plant, 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) making the GM plant glyphosate-tolerant, and CRY proteins making plants insect-resistant.

More extensive genetic modifications of plants are targeted at specific alterations of the plant's metabolism leading to improved responses to environmental stress conditions, like salt or metal tolerance, or drought resistance.

Currently GM plants with "quality" or "output" traits with the purpose to improve human or animal nutrition and/or health are under development. In these cases relatively complex genetic modifications are applied through, for instance, the insertion of multiple gene cassettes, leading to substantial changes in the metabolism and composition of the GM plants and derived foods/feed. Examples are rice with β-carotene and maize and soybean with altered amino acid or fatty acid composition (see further Table 1).

Table 1 Examples of GM plants with improved characteristics intended to provide nutritional or other health benefits to consumers and/or domestic animals[1]

Plant/Species	Altered characteristic	Transgene	Reference
Alfalfa	Sulphur amino acids (cysteine, methionine) ↑	Cystathionine γ-synthase	Avraham et al., 2005
Canola	Vitamin E ↑	γ-Tocopherol methyl transferase	ILSI, 2004
Canola	Lauric acid ↑	Lauroyl ACP thioesterase	ILSI, 2004
Canola	γ-Linolenic acid ↑	Δ^6- and Δ^{12} Desaturases	ILSI, 2004
Canola	+ ώ-3 Fatty acid	Δ^6 Desaturase	ILSI, 2004
Canola	+ β-Carotene	Phytoene synthase, phytoene desaturase, lycopene cyclase	ILSI, 2004
Canola	+ Resveratrol glucoside	Stilbene synthase and silencing of alternative pathway involving sinapate glucosyltransferase	Hüsken et al., 2005
Cassava	Cyanogenic glycosides ↓	Hydroxynitril lyase	ILSI, 2004
Cassava	Cyanogenic glycoside ↓ (linamarin)	Silencing of P450 enzymes CYP79D1 and CYP79D2	Siritunga and Sayre, 2004
Coffee	Caffeine ↓	Antisense xanthosine-N-7-methyl transferase	ILSI, 2004
Fescue grass	Lignin ↓; lignin digestibility ↑	Sense downregulated caffeic acid O-methyltransferase	Chen et al., 2004
Indian mustard	Very long chain polyunsaturated fatty acids ↑ (including arachidonic, eicosapentaenoic, and docosahexaenoic acids)	Fatty acid desaturases (Δ^4, Δ^5, Δ^6, Δ^{12}, ω-3), fatty acid elongases (Δ^6, C_{20}), and lysophosphatidic acid acyltransferase	Wu et al., 2005
Maize	Fumonisin ↓	De-esterase and de-aminase	ILSI, 2004
Maize	Protein with favorable amino acid profile ↑	α-Lactalbumin	ILSI, 2004
Maize	Vitamin C ↑	Dehydroascorbate reductase	ILSI, 2004
Maize	Bioavailable iron ↑	Ferritin and phytase	Drakakaki et al., 2005
Potato	Starch ↑	ADP glucose pyrophosphorylase	ILSI, 2004
Potato	Inulin ↑	Sucrose: sucrose 1-fructosyltransferase and fructan : fructan 1-fructosyltransferase	ILSI, 2004

Plant/Species	Altered characteristic	Transgene	Reference
Potato	+ Sulphur-rich protein	Non-allergenic seed albumin	ILSI, 2004
Potato	Solanine ↓	Antisense sterol glycotransferase	ILSI, 2004
Rice	+ β-Carotene	Phytoene synthase, phytoene desaturase, and lycopene cyclase	ILSI, 2004
Rice	Iron ↑	Ferritin, metallothionein, and phytase	ILSI, 2004
Rice	Allergenic protein ↓	Antisense 16kDa allergen	ILSI, 2004
Soybean	Sulphur amino acids ↑	Overexpression of maize 15 kDa zein	ILSI, 2004
Soybean	Oleic acid ↑	Sense suppression of Δ^{12} desaturase	ILSI, 2004
Soybean	Oleic acid ↑	Ribozyme termination of RNA transcripts down-regulate seed fatty acid	ILSI, 2004
Soybean	Immunodominant allergen ↓	Gene silencing of cysteine protease P34 (34kDa)	ILSI, 2004
Soybean	Isoflavones ↑	Isoflavone synthase	ILSI, 2004
Soybean	Tocochromanols ↑ (including tocotrienol)	Chorismate mutase-prephenate dehydrogenase, homogentisate phytyltransferase, and *p*-hydroxyphenylpyruvate dioxygenase	Karunanandaa et al., 2005
Sweet Potato	Protein content ↑	Artificial storage protein (ASP-1)	ILSI, 2004
Tomato	Provitamin A ↑ and lycopene ↑	Lycopene cyclase and phytoene desaturase	ILSI, 2004
Tomato	Flavonoids ↑	Chalcone isomerase	ILSI, 2004
Tomato	Provitamin A ↑, lycopene ↑, and flavonoids ↑	RNAi-mediated silencing of photomorphogenesis-related transcription factor TDET1	Davuluri et al., 2005

1 [1] Data collected by Dr. G.A. Kleter, RIKILT.
2 ↑/↓ Arrows reflect an increased or decreased characteristic; + reflects an added characteristic.

3

2. PRESENT EXPERIENCE WITH THE SAFETY AND NUTRITIONAL ASSESSMENT OF FOODS AND FEED INCLUDING GM FOODS AND FEED

2.1 Safety assessment of foods and feed

Traditional foods are seldom evaluated for safety as they have an established history of use. On the other hand, irradiated foods were subjected to a comprehensive safety assessment. In addition, according to Regulation (EC) No 258/97, novel foods and novel food ingredients which do not have a history of safe use are required to be evaluated for safety (EC, 1997b). Much can be learned from the experience gained in the safety assessment of these foods over the last half century. The basic concepts are built on the science of toxicology and the protocols used for testing single defined chemical substances. Clearly the evaluation of bulky whole foods containing tens of thousands of single substances requires a modified approach and the following section sets out to explain the history and evolution of whole food testing, exemplifying different products and the associated lessons learned. Safety assessment of GM plants and the derived food and feed is in turn developed further based on this experience. The rapid pace of toxicological development will ensure that current studies are continually evaluated in order for any refinements that are found necessary can be introduced in an agreed and harmonised manner.

2.1.1 Wholesomeness testing of irradiated foods

Extensive experience has been built up with the safety and nutritional testing of irradiated foods. The safety of high-dose irradiated foods has been evaluated in many feeding studies conducted over the past four decades involving a variety of laboratory diets and food components given to a broad selection of animal species, including rats, mice, dogs, quails, hamsters, chickens, pigs and monkeys. These investigations, which have included subacute, chronic, reproductive, multigeneration and carcinogenicity studies, have been conducted under a variety of experimental protocols and have covered a range of doses. In addition, a large number of evaluations for mutagenicity have been conducted in *in vitro* and *in vivo* systems.

In the early 1980s, the US Food and Drug Administration (FDA) reviewed all available animal studies to determine their adequacy and to evaluate the toxicological evidence (FDA, 1986). This review of over 400 studies resulted in over 250 being “accepted” or “accepted with reservation”, and about 150 being “rejected”. Studies were rejected for one or more reasons: the radiation dose was not reported; the number of animals per group was not reported; the number of animals per group was small (less than five); the study was conducted without controls fed a non-irradiated diet; the diet fed was determined to be nutritionally inadequate; and the studies were conducted at a laboratory that was considered by the FDA to be in violation of good laboratory practice.

The Joint FAO/IAEA/WHO Study Group convened in 1997 considered that in terms of extrapolation to humans, the data derived from the animal studies are especially relevant because of the composite nature of the food materials used and the manner in which the diets were administered (Joint FAO/IAEA/WHO, 1999). In the opinion of the FAO/IAEA/WHO Study Group the extensive collection of animal data demonstrates that irradiated foods using a variety of radiation sources under a variety of radiation conditions are toxicologically safe. Neither the carcinogenicity nor the mutagenicity studies with irradiated foods and feed have demonstrated treatment-related effects.

The results from the animal studies on irradiated foods have always been used in concert with the chemical studies on the same types of irradiated foods and the data from *in vitro* studies on similar foods

in such a way that the animal data have covered the issue of wholesomeness of irradiated foods for human consumption in the overall *weight-of-evidence* equation.

The role of the animal studies is the comparative testing of irradiated and non-irradiated foods in order to establish or to confirm that the *Margins-of-Safety (MOS)* both for toxicity and for nutritional adequacy are similar for the irradiated foods and its non-irradiated counterpart. The *sensitivity* and the *specificity* of animal feeding studies for the detection of potential unintended effects of the whole irradiated foods have been deemed second-to-none by most safety experts in the field of food irradiation. So far no alternatives to replace the animal repeat dose dietary testing in the overall safety assessment of food irradiation have been identified.

The testing of irradiated foods in repeat dose feeding studies never had the purpose to test the toxicity of a known chemical entity in the irradiated foods, but rather to assess the overall safety, when any specific safety issues have been settled in separate studies. Therefore this review of the experiences from safety assessment of irradiated foods is relevant for the discussion of the safety testing of foods.

2.1.2 Long term safety testing of food components (fruits and vegetables)

An overview of laboratory animal studies investigating the effects of whole foods or major food constituents in vegetables and fruits on carcinogenesis appeared in the IARC Handbooks of Cancer Prevention (2003). In thirty animal experiments the effects of high quantities of 13 different fruits or vegetables on chemically-induced carcinogenesis were examined. In six experiments effects of low amounts of a mixture of fruits and vegetables on colon cancer were evaluated. Four studies indicated preventive effects of the fruit/vegetables mixtures, and two studies showed adverse effects.

Biomarkers used related to carcinogenic risk were the uptake, chemical activation/de-activation, DNA-binding, DNA-repair, cytogenetic markers, oxidative damage, cell turnover, apoptosis, intercellular communication or gene expression.

No conclusion was drawn concerning the *design* of animal experiments testing whole foods. However, studies with no compensation for vegetables/fruit in the control diets were flagged or excluded; furthermore, lack of energy balance throughout the test diets was mentioned as a serious flaw.

The following conclusions can be drawn from these studies:

- only a low incidence of adverse effects were found;
- most studies indicated a preventive effect of vegetables or fruit items on the appearance of cancer in test animals.

It should be noted that conclusions drawn regarding the effects of fruits and vegetables on human carcinogenesis are mainly (90% of the studies) based on epidemiological and intervention studies on humans.

2.1.3 Safety assessment of novel foods

Foods and food ingredients which have not been used for human consumption to a significant degree within the EU before 15 May 1997 fall within the scope of Regulation (EC) No 258/97 (EC, 1997b). These novel foods comprise a broad spectrum of various products, including single substances and simple

or complex mixtures obtained from plant or microbial sources (e.g. phytosterols or oils), as well as complex foods traditionally consumed outside the EU (e.g. fruits and cereals) and foods produced using novel processes. Originally, the Regulation also covered foods and food ingredients containing, consisting or produced from GMOs. However, when the specific legislation on GM foods and feed came into force (EC, 2003a, 2003b), these products were excluded from the novel foods Regulation.

In the safety assessment of novel foods, a case-by-case approach is applied (EC, 1997a). Information is normally required on the source, composition and other nutritional characteristics, any previous human exposure, expected use and exposure of the novel food, as well as potential toxicity and allergenicity.

Single substances and simple or complex mixtures like phytosterols, for which no traditional counterpart is available, are tested and evaluated like food additives according to the respective guidelines (SCF, 2001). In these cases, the toxicological testing normally includes studies on metabolism and toxicokinetics, subchronic toxicity (90-day feeding study in rodents), genotoxicity, chronic toxicity and carcinogenicity, reproduction and developmental toxicity. Depending on the outcome of these investigations, additional studies may be required.

If a traditional counterpart is available, e.g. in the case of oils obtained from novel sources, a comparative approach may be applied. If it can be shown by adequate analytical studies that the composition of the novel food or food ingredient does not differ significantly from this counterpart, and the available information on the source does not raise concerns, further toxicological and nutritional testing is not required and the product is regarded as safe as the traditional counterpart.

For complex novel foods like fruits or cereals, however, a traditional counterpart normally does not exist. The approach applied in the evaluation of food additives, aimed at deriving an acceptable daily intake (ADI), which usually offers a large safety margin relative to the expected exposure, cannot be applied (EC, 1997a; Edwards, 2005). In these cases, comprehensive information is required on the source of the novel food, the composition (macro- and micronutrients, secondary plant metabolites, in particular anti-nutritional factors and toxicants, as well as potential allergens). Data on the experience gained with the food product in countries outside the EU, including traditional procedures of food preparation, as well as any information on other uses, e.g. in traditional medicines, are also required. In some cases, a conclusion about the safety of the food may be reached on the basis of this information alone, whereas in other cases, it will serve to determine whether any further nutritional or toxicological testing will be required. If there is any doubt regarding the safety, a minimum 90-day feeding study in a rodent species with the whole food should be conducted, whereby special attention should be paid to the determining of the doses and the avoidance of problems of nutritional imbalance. The outcome of this study will determine whether there is a need for further investigations (EC, 1997b; Howlett *et al.*, 2003).

2.1.4 Safety testing of GM foods in laboratory animal species

Examples of safety studies with GM foods/feed are given in Table 2. In different experiments foods/feed derived from GM plants, mixed in animal diets have been fed to rats or mice during different periods of administration, and parameters such as body weight, feed consumption, blood chemistry, organ weights, histopathology, etc., have been measured.

Table 2. Safety studies performed on laboratory animals with GM plant derived foods [1]

Plant	*Trait*	*Species*	*Duration*	*Parameters*	*Reference*
Oilseed rape	High γ-linolenic acid (Δ^6- and Δ^{12}-desaturases from *Mortierella alpina*)	Mouse	2 generations, 28 days after birth	Maternal characteristics, litter size, pup weight Brain weight and lipid chemistry, pup behaviour Pup maze test	Wainwright et al., 2003
Maize	Cry3Bb1 endotoxin (*Bacillus thuringiensis* var kumamotoensis)	Rat	90 days	Feed consumption, body weight gain, organ weights Blood cell count, blood chemistry, urine chemistry Histopathology	Hammond et al., 2006a
Maize	Cry1Ab endotoxin (*Bacillus thuringiensis* var kurstaki)	Rat	90 days	Feed consumption, body weight, organ weights Blood cell count, blood chemistry, urine chemistry Histopathology	Hammond et al., 2006b
Maize	CP4 EPSPS (*Agrobacterium*)	Rat	90 days	Feed consumption, body weight, organ weights Blood cell count, blood chemistry, urine chemistry Histopathology	Hammond et al., 2004
Maize	Cry1Ab endotoxin (*Bacillus thuringiensis* var kurstaki)	Mouse	2-4 generations; 87 days after birth (2nd generation), and 63 days after birth (4th generation)	Litter size, body weight Testicular cell populations	Brake et al., 2004
Potato	Lectin (*Galanthus nivalis*)	Rat	10 days	Histopathology of intestines	Ewen and Pusztai, 1999

Plant	*Trait*	*Species*	*Duration*	*Parameters*	*Reference*
Potato	Cry1 endotoxin (*Bacillus thuringiensis* var kurstaki HD1)	Mouse	14 days	Histopathology of intestines	Fares and El Sayed, 1998
Potato	Glycinin (Soybean [*Glycine max*])	Rat	28 days	Feed consumption, body weight, blood chemistry, blood count, organ weights, liver- & kidney histopathology	Hashimoto *et al.*, 1999a; Hashimoto *et al.*, 1999b
Potato	CryV endotoxin (*Bacillus thuringiensis*)	Rat	30 days	Feed consumption, body weight, blood chemistry Organ weights	El Sanhoty et al., 2004
Potato	Phosphinothricin acetyltransferase (*bar* gene, *Streptomyces hygroscopicus*)	Rat	5 generations; 70-day intervals before reproduction	Feed consumption, body weight Reproductive performance, development and viability of progeny Organ weights Skeletal and visceral deformations Histopathology	Rhee et al., 2005
Potato	Polymerase and non-coding DNA sequences derived from potato virus Y (PVY)	Rat	21 days	Serum chemistry, non-specific immunity, caecal wall and digesta characteristics	Zdunczyk et al., 2005
Rice	Glycinin (Soybean [*Glycine max*])	Rat	28 days	Feed consumption, body weight, blood chemistry, blood cell count, organ weights Liver- and kidney histopathology	Momma et al., 2000

Plant	*Trait*	*Species*	*Duration*	*Parameters*	*Reference*
Rice	Cry1Ab endotoxin (*Bacillus thuringiensis* var kurstaki)	Rat	98 days	Feed consumption, body weight, blood chemistry, blood cell count, organ weights, histopathology	Wang et al., 2002
Soybean	CP4 EPSPS (*Agrobacterium*)	Mouse	2-4 generations; 87 days after birth (2nd generation), and 63 days after birth (4th generation)	Litter size, body weight, testicular cell populations	Brake and Evenson, 2004
Soybean	CP4 EPSPS (*Agrobacterium*)	Rat	91 days	Feed consumption, body weight, organ weights, blood cell count, blood chemistry, urine chemistry, histopathology	Zhu et al., 2004
Soybean	CP4 EPSPS (*Agrobacterium*)	Mouse	240 days	Histocytochemistry of hepatocytes, pancreatic acinar and testicular cells Enzyme chemistry of serum, liver, and pancreas	Malatesta et al., 2002a, b; 2003 Vecchio et al., 2004
Soybean	CP4 EPSPS (*Agrobacterium*)	Mouse	30 days	Histocytochemistry of hepatocytes	Malatesta et al., 2005
Soybean	CP4 EPSPS (*Agrobacterium*)	Rabbit	40 days	Body weight, organ weights, serum and tissues enzyme chemistry	Tudisco et al., 2006
Sweet pepper, tomato	Cucumber mosaic virus coat protein (CMV-CP)	Rat	30 days	Feed consumption, body weight, organ weights, blood cell count, blood chemistry, histopathology Genotoxicity	Chen et al., 2003

Plant	*Trait*	*Species*	*Duration*	*Parameters*	*Reference*
Tomato	Cry1Ab endotoxin (*Bacillus thuringiensis* var kurstaki)	Rat	91 days	Feed consumption, body weight, organ weights, blood chemistry, histopathology	Noteborn *et al.*, 1995

[1] Data collected by Dr. G.A. Kleter, RIKILT, partly derived from Kuiper *et al.*, 2001, Table 4).

In the case of GM tomatoes containing Cry1Ab, a semi-synthetic diet was supplemented with 10% (w/w) of lyophilised GM or control tomato powder and fed for 91 days to rats. The average daily intake was approximately 200 g of tomatoes/day/rat, corresponding to a daily human consumption of 13 kg. No clinical, toxicological or histopathological abnormalities were observed. The 10% (w/w) tomato content of the diet was chosen as the highest level, because of the relatively high potassium content of tomatoes (40-60 g/kg); higher amounts of potassium could have caused renal toxicity (Noteborn and Kuiper, 1994).

GM potatoes and rice modified with native and synthetic (four additional methioninyl residues) soybean glycinin were fed to rats during 28 days (Hashimoto *et al.*, 1999a,b; Momma *et al.*, 2000). Results indicated that a daily administration of 2 g potatoes - and 10 g rice - per kg body weight to rats did not induce pathological or histopathological abnormalities in liver and kidney.

Teshima *et al.* (2000) fed either heat-treated GM soybean meal containing the *cp4-epsps* gene or control soybean meal to Brown Norway rats and B10A mice. These experimental animals were employed based on their immunosensitivity to oral challenges. The semi-synthetic animal diet was supplemented with 30% (w/w) of heat-treated soybean meal and fed during 105 days. Both treatments failed to cause an immunotoxic activity nor did they increase the IgE in serum of either rats or mice. Moreover, no significant abnormalities were observed histopathologically in the mucosa of the small intestine of animals fed either GM or non-GM soybean meal.

In some cases adverse effects have been reported. Fares and El Sayed (1998) reported that mice fed for 14 days with fresh potatoes immersed in a suspension of delta-endotoxin of *Bacillus thuringiensis* var. kurstaki strain HD1 showed an increase in hyperplastic cells in their ileum. Feeding with fresh GM potatoes expressing the *cryI* gene caused mild adverse changes of the various ileac compartments as compared to the control group fed with fresh non-GM potatoes. No details of the intake of CryI protein or of the dietary composition were given, which limits interpretation of this study.

Ewen and Pusztai (1999) reported that rats fed GM potatoes containing *Galanthus nivalis* agglutinin (GNA) lectin showed proliferative and antiproliferative effects in the gut. These effects were presumed by the authors to be due to (unknown) alterations in the composition of the GM potatoes, rather than to the newly expressed gene product. However, various shortcomings of this study were noted, such as protein deficiency of the diets and lack of control diets (Kuiper *et al.*, 1999; Royal Society, 1999).

Ninety-day rat studies with GM maize grain expressing either the insecticidal CRY3Bb1 protein from *Bacillus thuringiensis* (MON863) or CRY1Ab (MON810) have been performed (Hammond *et al.*, 2006a,b) and were evaluated by the EFSA GMO Panel (EFSA, 2005a,b). These studies were carried out in accordance with OECD protocols.

Grain from MON863 and its non-GM near isogenic control were included in rat diets at levels of 11% and 33% (w/w). Additionally, six groups of rats were fed diets containing grain from different conventional (non-GM) reference varieties. Overall health, body weight gain, food consumption, clinical pathology parameters, organ weights, gross and microscopic appearance of tissues were measured. Some differences

were observed in haematological parameters in the male and female test group (at the higher MON863 maize inclusion level), but these were not considered to be biologically meaningful since they fall within the standard deviation of the reference population. Individual kidney weights of male rats fed with feed containing 33% MON863 maize were statistically significantly lower compared to those of animals on control diets, but fell within the range of values of the reference population. Analysis of microscopic pathology data of a large number of organs and tissues showed no statistically significant differences between test and control groups, except for a statistically significantly lower incidence of mineralized kidney tubules on rats fed 33% MON863 maize diet compared to those fed the control maize. These findings were observed only in females, were of minimal grade severity, and were considered as incidental and not treatment-related.

In the case of MON810 maize lines were included in the diets at the 33% level, while GM and control diets were also included at the 11% level (and supplemented with other non-GM maize to 33%). Analysis was performed on feed consumption, body weight, clinically observable adverse effects, clinical pathology during the experimental period, as well as organ weights and histopathology after study termination. For rats fed 33% MON810 maize, a statistically significantly lower albumin/globulin ratio was observed at study termination compared with control and overall reference maize lines. Rats fed one reference line showed similar values to those fed MON810. Thus, results of this rodent study do not indicate adverse effects from consumption of maize derived from MON810.

In cases in which genetic modification events are combined by the interbreeding of two or more GM parents, a risk assessment is required within the European Union if such combinations are not already covered by the consent for the single events (EC, 2001). The need for further assessment will depend, on a case-by-case basis, on the nature of the genetic modifications involved (EFSA, 2006a). Key elements of the evaluation process are (i) assessment of the intactness of the inserted loci and phenotypic stability, and (ii) assessment of potential interactions between combined events.

Recently the EFSA GMO Panel has evaluated for import and processing the hybrid maize MON863 x MON810, produced by a conventional cross between maize inbred lines containing MON863 and MON810 events (EFSA, 2005a,b). The molecular analysis of the DNA inserts present in the MON863 x MON810 hybrid confirmed that the insert structures and loci of insertion were retained. The safety of the whole product derived from kernels of maize MON863 x MON810 was tested in a 90-day toxicity study with rats. The design and execution of this study complied with OECD Guideline 408. Three groups of rats consisting of 20 rats per sex within each group received diets *ad libitum* for 90 days, containing either 33% MON863 x MON810 maize, or 11% MON863 x MON810 maize supplemented with 22% control maize, while a control group was administered a diet containing 33% control maize.

All animals were examined daily for appearance, morbidity, and mortality. Individual body weights and food consumption were also recorded weekly. At the end of the experiment, an extensive clinical pathological evaluation was performed, including haematology, serum chemistry, and urine analysis. In addition, a complete necropsy was carried out, including both macroscopic examinations and histopathology.

Small deviations in food consumption by females on test diets containing MON863 x MON810 were observed and most of the clinical chemistry data showed no differences. Nevertheless, analysis of the clinical chemistry data showed statistically significant decreases in mean corpuscular haemoglobin concentration in male animals in the 11% and 33% test diet groups, but these values were not dose-related. Some statistically significant differences were observed in organ weights, but these differences did not exhibit a dose-response relationship and microscopic observations showed no abnormalities either.

MON863, MON810, and MON863 x MON810 maize have also been studied in separate nutritional feeding studies with broilers. These animals grow rapidly within six weeks to full size and are therefore a sensitive model to detect any nutritional imbalances that might be present in the GM maize lines. Both performance (weight gain, feed consumption) and carcass parameters (weight, weight of carcass parts and compositional analysis of breast and thigh muscles) were measured. None of these studies showed adverse effects in animals fed the test diets.

Multigeneration studies

Multigeneration studies have been performed on GM maize and soybean in mice and GM potato in rats. With regard to herbicide-tolerant GM maize and soybean expressing CP4 EPSPS, testicular cell populations in progeny of mice were measured, as well as litter size and body weights, in the second and fourth generations. Flow cytometry was used to distinguish between haploid, diploid, and tetraploid cells. Some differences were noted at intermediate measuring points in the fractions of diploid and tetraploid cells in GM maize fed animals compared to those fed conventional maize. The authors related these differences to slight differences (up to 32 hours) in age of the animals (Brake *et al.*, 2004). A similar reasoning was given by the authors of the study on mice fed GM soybean for variation in haploid cells at an intermediate time point (26 days; Brake and Evenson, 2004).

The study on the reproductive toxicity of herbicide-tolerant GM potato expressing the PAT enzyme in rats focused on the reproductive performance, development and viability of pups, organ weights of weaning rats, and skeletal and visceral malformations. Reproductive performance measurements included mating, fertility, gestation, delivery, litter size, oestrous cycle, and sperm motility. Developmental studies included, among others, genital development. In addition, histopathology was carried out on reproductive tissues. No GM-potato-related effects were observed (Rhee *et al.*, 2005).

Wrainwright *et al.* (2003) performed a reproductive toxicity study on various oils fed to mice, including GM canola oil, either pure or mixed with other oils, as well as borage oil and maize oil. Maternal animals were tested for weight and characteristics related to pregnancies and litter size. Two generations of progeny were tested for body weight, behavorial development using sensorimotor and maze tests on 12-day old pups, and brain fatty acid composition of 28-day old animals. Differences that occurred between the groups fed GM canola oil and both other groups included a lower body weight for pups aged 26 days, which, according to the authors, relates to an effect of γ-linolenic acid (GLA) that probably had greater bioavailability from GM canola than from borage oil. In addition, n-3 fatty acids, including docoshexaenoic acid (DHA; 22:6n-3), were decreased in brains from animals fed GM canola oil, whereas a specific n-6 fatty acid (22:4n-6) was increased. The effects on fatty acid composition of the diet containing a mixture of GM canola oil were greater than those of borage oil, although both contained similar levels of GLA. Similarly to Liu *et al.* (2004), these authors linked the observed effects with the increased bioavailability of GLA from GM canola oil.

A series of articles have appeared summarizing the results of several studies in which histocytochemistry was performed on cells of specific organs, such as liver, pancreas, and testis, of mice fed diets containing soybean genetically modified for CP4 EPSPS or wild type soybean (Malatesta *et al.*, 2002a, b; 2003; 2005; Vecchio *et al.*, 2004). In particular, these studies used staining techniques for various indicators of transcriptional activity, such as chromatin-associated elements in the cell nuclei. In short, these studies indicate that the feeding of GM soybean is associated with changes in nucleic transcriptional activity, which the authors relate to the presence of glyphosate in the GM soybean. However, the studies do not provide a detailed account of the origin and characteristics of the soybeans used. In addition, it is noted that in these studies very specific endpoints were examined but not those parameters which are normally

regarded as indicative for specific organ toxicity. Therefore the toxicological relevance of the findings is not clear.

SAFOTEST

Animal feeding studies

Ninety-day rat feeding studies have been carried out with GM rice (EU project SAFOTEST; Poulsen *et al.*, 2006a, 2006b; Schrøder *et al.*, 2006)), in order to develop and validate the scientific methodology, which is necessary for assessing the safety of foods from GM plants.

In the GM rice, the kidney bean (*Phaseolus vulgaris*) lectin agglutinin E-form (PHA-E lectin) was expressed. The core study was a 90-day rat feeding study with (i) control diet containing parental rice as 60% of the basic purified diet, (ii) test diet containing GM rice with PHA-E lectin expressed as 60% of the nutritionally adjusted, purified diet, and (iii) test diet containing GM rice with PHA-E lectin expressed as 60% in the nutritionally adjusted, purified diet, and spiked with 0.1% PHA-E lectin [a level corresponding approximately to the Lowest Observed Adverse Effect Level (LOAEL) observed in a 28-days rat study]. The spiking level of 0.1% corresponded to a daily intake of approximately 70 mg PHA-E lectin/kg body weight. The contribution of PHA-E lectin from the PHA-E rice corresponded to approximately 30 mg PHA-E lectin/kg body weight/day.

Major differences in macro- and micronutrients between the parental rice and the corresponding GM rice were adjusted and balanced in the overall diet to prevent the study outcome from being disturbed artifactually by foreseeable nutritional imbalance. Adjustment was done if the level of nutrients between diets differs by more than 5% in the total diet.

Prior to the 90-day study, a 28-day rat study was performed with recombinant PHA-E lectin mixed with a purified diet containing 60% conventional rice in concentrations of 0, 0.01, 0.02 and 0.08%. Observed effects in this study were weak at the dosage levels tested. At necropsy, a statistically significant increase in absolute and relative weights of the small intestine of female rats and relative small intestine weight of male rats was seen in the highest dose group. In addition, the absolute and relative pancreas weights were significantly increased in nearly all female groups given PHA-E lectin. A dose level of 0.08% PHA-E lectin was considered to be the lowest-observed-effect-level (LOEL), based on the increased weight of the small intestine. It is questionable whether this parameter reflects a genuine adverse effect or simply a response to exposure.

An overview of the results from the 90-day feeding study in rats is given in Table 3.

Table 3 Results of the 90-day feeding study with PHA-E rice

Plasma biochemistry/relative organ wt/length	PHA-E rice (30 mg/kg bw/day PHA) vs. control	PHA-E rice (30 mg/kg bw/day PHA) and spiked with PHA (70 mg/kg bw/day) vs. control
Sodium	↔	↓
Protein	↔	↓
Albumin	↔	↓
Creatinine	↔	↓
Plasma ALAT	↔	↑
Urea	↓	↓↓
Mesenterial lymph nodes	↔	↑↑
Small intestine	↑↑↑	↑↑↑
Stomach	↑↑↑	↑↑↑
Pancreas	↑	↑
Small intestine (length)	↔	↑↑↑

Arrows reflect statistically significant differences from the control group, with ↑/↓: $p<0.05$, ↑↑: $p<0.01$, ↑↑↑: $p<0.001$. ↔ reflect no significant differences from the control group.

Measurements of blood biochemistry turned out to be sensitive parameters for effects of the PHA-E lectin in the 90-day study. Significant changes occurred in most parameters for the group given rice with pure PHA-E lectin added compared to the group given control or PHA-E rice alone. The increased alanine-aminotransferase (ALAT) activity in the group given PHA-E rice spiked with PHA-E lectin could be indicative of liver damage, but histopathology revealed no such findings.

The significantly higher relative weight of the small intestine and the stomach as well as the increased length of the small intestine of groups fed GM rice may reflect the crypt cell hyperplasia and increased epithelial cell size observed histopathologically. These effects were more pronounced in the group fed GM rice spiked with PHA-E lectin than with the GM rice alone.

In this 90-day study only the relative pancreas weight of the groups fed GM rice was statistically significantly different from the control group. Absolute and relative weights of the mesenteric lymph nodes were 35 and 42% respectively higher in the rats receiving a diet containing PHA-E rice and this rice spiked with PHA-E lectin than in the control.

The observed effects were consistent with the known toxicity of the expressed gene product, the PHA-E lectin. For most of the changes seen in the two groups given GM rice, the effects are either statistically significant or more prominent in the group fed PHA-E rice spiked with PHA-E lectin. This supports the evidence that the effects identified in the 90-day study are caused by the presence of the gene product rather than by the genetic modification as such.

Microarray experiments

Within SAFOTEST, gene expression profiling was performed on small intestinal scrapings from a 28-day and a 90-day feeding experiment using rat oligo microarrays. The 90-day experiment was the same as the one described by Poulsen *et al.* (2006). The 28-day study had a similar design to the one described by Poulsen *et al.* (2006).

From the 28-day rat study four groups of 6 animals each were chosen for microarray analysis, *i.e.* two treatment groups (male and female) that were fed a basic purified diet with 60% rice and 0.08% recombinant PHA-E and two control groups (male and female) that were fed the same diet except for the PHA-E spike. Microarray data analysis showed that PHA-E had an effect on gene expression in the small intestinal lining, in particular on the expression of genes involved in cholesterol biosynthesis. Although this effect was found both in female and male rats, it was more pronounced in the females. The study also revealed some minor gender-specific effects of PHA-E, such as effects on arachidonic acid metabolism (*i.e.* synthesis of eicosanoids and leukotrienes) in female but not in male rats.

In the 90-day study, microarray hybridisations were performed with RNA from intestinal scrapings of seven female rats (two from the control group, two from the group fed with PHA-E rice, and three from the group fed with PHA-E rice supplemented with 0.1% purified PHA-E). In the PHA-E rice group the pathways most affected were those related to the metabolism of eicosanoids and leukotrienes. Although in the spiked PHA-E rice group these pathways were also found to be affected, the pathway most prominently affected in this latter group appeared to be cholesterol biosynthesis. Taken together, the results showed that microarray technology allowed to identify similarities as well as differences in gene expression in the small intestinal lining of rats fed with PHA-E rice and/or recombinant PHA-E.

2.1.5 Safety testing of extracts from (GM) foods using other tests

Allergenicity testing using food extracts

Several examples of screening whole food (extracts) for potential allergenicity have been described. Protein extracts from GM soybeans expressing the 2S albumin of Brazil nut have been demonstrated by a radioallergosorbent test (RAST), SDS-PAGE and skin-prick testing (SPT) to bind to IgE in serum from subjects allergic to Brazil nuts (Nordlee *et al.*, 1996).

By using RAST, *in vitro* cell-based histamine release assays and SPT, Sten *et al.* (2004) detected no significant difference in the allergenic potency between extracts of GM (glyphosate-tolerant) and non-GM soybeans.

Lee *et al.* (2006) compared the allergenicity of GM potatoes with that of non-GM potatoes by SPT, ELISA, and SDS-PAGE followed by immunoblotting. From this study, in which sera from 1886 patients with various allergic diseases were used, it was concluded that genetic modification did not result in increased allergenicity.

In an *in vivo* murine model for oral allergen-specific sensitization it was shown that protein extracts of GM (Roundup Ready) soybeans induced an immunological response comparable with that induced by non-GM soybean extracts (Gizzarelli *et al.*, 2006).

Cytotoxicity testing of food extracts

Assays that measure lactate dehydrogenase (LDH) release/neutral red (NR) uptake, and the conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide (MTT) assay have been used to assess the cytotoxicity of tomato extracts (Noteborn *et al.*, 1997). Aqueous and chloroform /methanol extracts of red-ripe tomato fruits up to a maximum of 10% suspensions (w/v) were found to be non-cytotoxic to intestinal epithelial cell lines. Furthermore it was shown that extracts from green tomato fruits were cytotoxic and that GM (antisense RNA exogalactanase) extracts and non-GM extracts were not different with respect to their activities in the cytotoxicity assays.

Mutagenicity testing of extracts

Using the COMET assay, aqueous and chloroform /methanol extracts of red-ripe tomato fruits did not exhibit DNA-damaging effects in various rat and human intestinal epithelial cell lines. Genetic modification (antisense RNA exogalactanase tomato) did not result in increased genotoxicity, but tomato fruit extracts, irrespective of whether they were GM or non-GM were able to suppress the DNA-damaging effects of known genotoxins H_2O_2 and MNNG (Noteborn *et al.*, 1997).

In order to assess the potential carcinogenicity of GM foods, *in vitro* mutagenicity testing is an option analogous to the use of mutagenicity tests as a tool for prescreening single substances for their carcinogenic potential.

However, the testing of whole foods *in vitro* poses specific problems:

- Contrary to single compounds whole foods cannot be assessed at high concentrations;
- Whole foods are usually incompatible with the test system, since they are generally not soluble and have to be applied in a matrix that is appropriate for *in vitro* test systems. For the latter, freeze-drying and homogenization is often needed;
- Certain constituents may interfere with the test system used (e.g. histidine in the Ames test);
- Poor bioavailability of bioactive compounds due to the matrix structure, e.g. binding to insoluble carbohydrates or proteins;
- Interaction between different bioactive compounds.

In order to solve some of these problems and to concentrate any mutagenic components present, extraction procedures are needed. In cases in which the chemical nature (e.g. polarity) of the possible mutagenic compound or whether the mutagenic component(s) are polar or apolar is not known, an extraction scheme has to be applied differentiating between hydrophilic and lipophilic fractions. If further separation is needed in order to identify the mutagenic compounds, additional fractionation in acid, neutral and basic fractions, or high and low molecular weight fractions may follow. Furthermore for a targeted approach to extract specific groups of compounds additional techniques can be applied such as solid phase extraction, supercritical fluid extraction (e.g. for antioxidants), immune-precipitation, HPLC and GC, etc. If the compounds are bound to the matrix, cleavage reactions through hydrolysis such as saponification are needed before extraction can take place.

Assessment of gene expression upon exposure of cells to extracts

Noteborn *et al.* (1998) have used the eukaryotic stress gene assay, also referred to as the CAT-Tox(L) assay, to screen tomato extracts for possible toxicity. This assay consisted of human liver cells (HepG2 cell line) stably transfected with chloramphenicol acetyltransferase (CAT) reporter constructs in which CAT expression is driven by promoters of stress-related and/or toxicologically relevant genes (Todd *et al.*, 1995). Non-cytotoxic amounts of aqueous extracts of red-ripe GM antisense RNA exogalactanase tomato fruits and non-GM tomato fruits did not result in any molecular responses related to cellular stress and toxicity. However, extracts of green GM and non-GM fruits induced the construct containing the xenobiotic response element (Noteborn *et al.*, 1998).

A relatively small number of studies have used DNA microarrays to analyse potential effects of food extracts on gene expression profiles in cell lines derived from various organs and tissues. After exposure to garlic extracts, colon carcinoma cells showed induction of apoptosis and cell cycle arrest (Frantz *et al.*, 2000; Su *et al.*, 2006). Li *et al.* (2002) have investigated the effect of *Ginkgo biloba* leaf extract on the transcriptomes of human breast cancer, glioma and hepatoma cells and were able to identify common gene targets.

2.1.6 Conclusions

- Extensive experience has been built up with the safety and nutritional testing of irradiated foods. The safety of high-dose irradiated foods has been evaluated in many feeding studies involving a variety of laboratory diets and food components given to a broad selection of animal species. These investigations have included subacute, chronic, reproductive, multigeneration and carcinogenicity studies. The extensive animal data set demonstrates that irradiated foods using a variety of radiation sources under a variety of radiation conditions are toxicologically safe;

- Long term testing of vegetables and fruits using laboratory animals regarding their potential influence on carcinogenesis is feasible when proper balanced animal diets can be prepared; these studies indicated only a low incidence of adverse effects; moreover, in many cases a preventive effect of vegetables of fruit items on the appearance of cancer in test animals was observed;

- Many feeding trials have been reported testing GM maize, potatoes, rice, soybeans and tomatoes fed to rats or mice for prolonged periods, and parameters such as body weight, feed consumption, blood chemistry, organ weights, histopathology etc have been measured. The foods/feed under investigation were derived from GM plants with improved agronomic characteristics like herbicide tolerance and/or insect resistance. The majority of these experiments did not indicate clinical effects or histopathological abnormalities in organs or tissues of exposed animals. These studies can be used to assist in the safety evaluation of GM plant derived foods/feed and to reach conclusions on whether they can be considered as safe as their conventional counterpart. In some cases adverse effects were noted, which are difficult to interprete due to shortcomings in the studies;

- Testing of GM foods/feed and extracts in *in vitro* assays may yield relevant information on the potential toxicity and/or allergenicity, which will further guide the safety assessment of the GM plant derived foods/feed.

2.2 Nutritional assessment of GM foods/feed

Nutrition and nutritional value of foods/feed are major determinants of human and animal well-being. Thus ensuring the nutritional quality and equivalence of GM foods and feed is of critical importance to man and livestock. Additionally, the potential for antinutrients to adversely affect health either directly or indirectly is well known. In consequence it is important to demonstrate that a food derived from GM plants is not only as safe but also as nutritious as that derived from the conventional variety.

Compositional analysis

There are now numerous papers published comparing the compositional analyses of GM plants modified for herbicide tolerance (HT) and insect resistance (Bt) which show compositional equivalence between the GM plant and its near isogenic counterpart, except for the inserted traits. These studies have been reviewed and summarized by Clarke and Ipharraguerre (2004) and Flachowsky *et al.* (2005). The work conducted by Ridley *et al.* (2002) provides an excellent example of the extensive compositional analyses conducted when comparing the grain and forage component of HT maize (NK603) with its near isogenic counterpart and a number of commercially grown varieties. Compositional equivalence between the GM and non-GM plants was clearly demonstrated. Even though some differences between the GM material and its near isogenic counterpart were statistically significant, the values fell within the range of the currently available varieties and those noted in literature (OECD, 2002a; ILSI, 2003a).

2.2.1 Nutritional testing of GM foods in laboratory animals

The design of studies to test the nutritional properties of GM foods in laboratory animals is in most cases identical to the design of the safety studies discussed in Chapter 2.1.4. A number of nutritional studies, including performance and balance studies, have been performed (Table 4). In addition to the general parameters of body weight and feed intake, each of the studies focused on specific parameters that are linked with the physiological target of the particular trait of the plant. In some of these cases, changes in the nutritional performance have been observed, which may confirm the intended effect of the genetic modification.

Table 4. Nutritional studies on laboratory animals dosed orally with GM plants[1]

Plant	*Trait*	*Species*	*Duration*	*Parameters*	*Reference*
Canola (oil)	High γ-linolenic acid (Δ^6- and Δ^{12}-desaturases from *Mortierella alpina*)	Rat	84 days	Feed consumption, body weight, organ weights, blood cell count, blood chemistry Lipid chemistry of plasma, liver, muscle, and adipose tissue Histopathology	Liu et al., 2004

Plant	*Trait*	*Species*	*Duration*	*Parameters*	*Reference*
Canola (oil)	High γ-linolenic acid (Δ^6- and Δ^{12}-desaturases from *Mortierella alpina*)	Rat	42 days	Feed consumption Body weight Organ weights Plasma and organ lipid chemistry	Palombo et al., 2000
Canola (oil)	High γ-linolenic acid (Δ^6- and Δ^{12}-desaturases from *Mortierella alpina*)	Rat (lymph fistulated)	1 day	Lymph flow Lymphatic lipid chemistry	Tso et al., 2002
Maize	CP4 EPSPS (*Agrobacterium*)	Rat	21 days (balance 7 days)	Feed consumption Body weight Protein conversion	Chrenkova et al., 2002
Pea	α-amylase inhibitor (Kidney bean [*Phaseolus vulgaris*])	Rat	10 days	Feed consumption Dry matter and N digestibility Body and organ composition	Pusztai et al., 1999
Soybean	CP4 EPSPS (*Agrobacterium*)	Rat	30 days	Feed consumption, body weight, organ weights, histopathology of pancreas	Hammond et al., 1996
Sweet potato	ASP-1 protein (Synthetic)	Hamster	28 days	Feed consumption, body weight, organ weights Protein conversion Serum and liver chemistry	Shireen and Pace, 2002
Sweet potato	ASP-1 protein (Synthetic)	Hamster	28 days	Feed consumption, body weight, serum and bone calcium Bone weight	Shireen et al., 2002

1 [1] Data from publicly available reports, collected by Dr. G.A. Kleter, RIKILT.

A number of recently published rodent studies focused on the potential toxicological and nutritional properties of purified GM canola oil that has elevated levels of γ-linolenic acid (GLA), a polyunsaturated fatty acid. For example, Liu *et al.* (2004) fed diets containing 5, 10, or 15% GM canola oil, which itself contains 36% GLA, or a diet containing 15% borage oil, which contains 22% GLA, to rats for 84 days. Several differences were noted between groups fed GM canola oil and borage oil, most of which the authors relate to a different intake and bioavailability of GLA. Examples of differences included decreased body weight, higher liver fatty infiltration, lower plasma cholesterol, higher liver cholesterol, altered levels of various polyunsaturated fatty acids in plasma, liver, muscle, and adipose of GM-canola-oil *versus* borage-oil-fed groups.

In a study of 42 days' duration, performance and lipid composition of plasma and liver was studied in rats fed GM canola oil and borage oil, both at levels of 23% GLA in dietary triglycerides (Palombo *et al.*, 2000). No differences in body weight and feed intake were observed. In the lipid composition of liver and plasma, there was a consistently higher level of docosapentanoic acid (22:5n-3) in animals fed GM canola oil. The authors considered that balancing the GLA content of diets would not exactly balance the contents in other n-3 fatty acids. In addition, Tso *et al.* (2002) found that after intake of feed containing either GM canola oil or borage oil by lymph-fistulated rats, lymph flow and output of lymph triglycerides and cholesterol were similar between groups, whereas the lipid composition of the lymph differed. Lymph triglycerides in animals fed GM canola oil showed lower levels of linoleic acid (18:2n-3), and higher levels of oleic acid (18:1n-9) and GLA (18:3n-6).

Hammond *et al.* (1996) measured the performance, including body weight gain and feed consumption, of rats fed diets containing GM and control soybean meals for four weeks. This experiment was part of a larger study in which also the performance of target domestic animals was studied. Overall, the authors concluded that performance of animals fed GM and control diets was similar.

In addition to performance, also the bioavailability and utilization of particular nutrients has been measured. For example, Shireen and Pace (2002) measured the effect of feeding hamsters with GM sweet potato for four weeks. This GM sweet potato had been modified with the newly introduced synthetic ASP-1 protein, which is rich in essential amino acids. The new sweet potato also contains higher levels of protein than the conventional sweet potato, which has comparatively low levels of protein. The experiments showed, among other things, that the protein quality of GM sweet potato had improved, measured as greater protein conversion (Shireen and Pace, 2002). No effect was observed on calcium bioavailability from the GM sweet potato (Shireen *et al.*, 2002).

Chrenkova *et al.* (2002) studied the utilization of protein by rats from GM maize resistant towards the herbicide glyphosate. Rats received a diet containing approximately 94% maize, either GM or control. The composition of the diet was measured, as was the nitrogen content of fecal matter and urine. Various parameters related to protein conversion were thus calculated, and showed no difference between GM maize and its control.

Pusztai *et al.* (1999) carried out an experiment on rats fed GM peas containing a transgenic α-amylase inhibitor originating from beans at 30% and 65% dietary inclusion rates. In addition, control diets contained lactalbumin protein with or without added recombinant bean α-amylase inhibitor. The authors concluded that at 30% inclusion rate, some minor differences were noted between groups fed GM and control peas. For example, decreased values for body water content and dry matter digestibility, and increased values for fecal excretion and DNA content of caecal tissue were noted. At 65% inclusion, the GM groups also showed lower body water content, in addition to higher excretion of nitrogen. A conspicuous feature was the apparently unaffected starch digestibility in animals fed GM pea, despite the modification with α-amylase inhibitor. By contrast, addition of purified bean α-amylase inhibitor to diets

did decrease starch digestibility. In a short experiment, it was confirmed that α-amylase inhibitor purified from GM pea did not impair starch digestion in animals. According to the authors, this might have been due to increased sensitivity of the transgenic α-amylase inhibitor in pea to intestinal proteases compared to the bean analog (Pusztai *et al.*, 1999).

2.2.2 Nutritional testing of GM feeds with agronomic input traits in target animal species

Recently many studies with GM plants with agronomic input traits were carried out in target species to assess the nutritive value of the feeds and their performance potential. The studies are summarized by CAST (2006), Clark and Ipharraguere (2001), Flachowsky *et al.* (2005a) and others.

a) Nutrient availability

Although compositional analysis is the cornerstone of nutritional assessment it does not result in a complete picture as it does not provide information on nutrient digestibility, which is an important parameter in the nutritional assessment of feed resources. Both i*n sito* and *in vivo* methodologies can be used to assess bioavailability of nutrients. Comparisons between *in sito* and *in vivo* methodologies in assessing the nutrient availability of GM and conventional plants should be undertaken.

A number of livestock feeding studies have now compared the *in vivo* bioavailability of nutrients from a range of plants with their near isogenic counterpart and commercial varieties (Hammond *et al.*, 1996 (broilers, lactating dairy cows, catfish); Maertens *et al.*, 1996 (rabbits); Daenicke *et al.*, 1999 (sheep); Böhme *et al.*, 2001 (pigs and sheep); Aulrich *et al.*, 2001 (broilers); Barriere *et al.*, 2001 (sheep); Gaines *et al.*, 2001b (pigs); Bertrand *et al.*, 2002 (*in vitro* digestibility); Reuter *et al.*, 2002 a, b (pigs); Stanford *et al.*, 2003 (sheep); Hartnell *et al.*, 2005 (sheep**)**. An overview of experiments performed with food producing animals is given in Table 5. The results all showed that the bioavailability of a wide range of nutrients from a range of GM plants modified for agronomic input traits was comparable with those for near isogenic non-GM and conventional varieties. While some statistically significant differences were noted these were generally small, inconsistent and not considered to be biologically meaningful.

Table 5: Published feeding studies with food producing animals fed with feedstuffs from GM plants with input traits in comparison with near isogenic plants (summarized by Flachowsky *et al.*, 2005a).

Animal species/ Categories	**No of experiments**	**Nutritional assessment**
Ruminants Dairy cows Beef cattle Others	 23 14 10	No significant differences in composition (except lower concentration of mycotoxins in Bt-maize) No significant differences in digestibility of nutrients, animal health, animal performances, composition and quality of foods of animal origin between feeds from near isogenic or GM plants
Pigs	21	
Poultry Laying hens Broilers	 3 28	
Others (fish, rabbits etc.)	 5	

b) Production studies with monogastric livestock

i) Poultry

The animal test model, using broiler chicks to compare the nutritional equivalence of conventional and GM plants is described below. Numerous feeding studies with one-day old broiler chicks have now been reported (Brake and Vlachos, 1998; Halle *et al.*, 1999; Mireles *et al.*, 2000; Sidhu *et al.*, 2000; Aeschbacher *et al.*, 2001; Gaines *et al.*, 2001a; Taylor *et al.*, 2001a, b, 2002, 2003 a, b, c, 2004 a, b, c; Stanisiewski *et al.*, 2002; Brake *et al.*, 2003; Tony *et al.*, 2003; Elangovan *et al.*, 2003; Querubin *et al.*, 2004; Kan and Hartnell, 2004a,b). These authors included varieties of Bt and HT maize, soybean, canola and wheat and appropriate counterparts. Only some experiments are available with laying hens (Aulrich *et al.*, 2001, Halle *et al.*, 2006) where Bt maize hybrids were compared with near isogenic counterparts. The diets were all formulated to contain a high proportion of the test material. In each study the composition of the feed ingredient produced from the GM varieties, the near isogenic non-GM varieties, and the commercial hybrids was determined and found to be comparable, while at the same time the results established nutritional equivalence and showed no biologically meaningful differences in the production parameters measured.

Ten and four generation experiments with growing and laying quails were carried out to test diets with 40% (starter) or 50% (grower layer) isogenic or GM maize (Bt 176, Flachowsky *et al.*, 2005b; Halle *et al.*, 2006). Feeding of diets containing Bt maize did not significantly influence health, hatchability and performances of quails nor did it affect the quality of meat and eggs of quails compared with the near isogenic counterpart.

One noticeable exception is the study reported by Piva *et al.* (2001a, b) which compared diets containing Bt or conventional maize grain. The authors noted that significantly improved animal performance was associated with the diet containing the Bt maize. This improved animal performance was supposed to be linked to the fact that the use of Bt varieties reduced secondary fungal infection and, as a consequence, reduced mycotoxin contamination.

A number of GM maize varieties modified with single or stacked genes (CP4 EPSPS and Cry proteins) showed compositional and nutritional equivalence (Taylor *et al.*, 2003a, b, c; 2004a, b, c; 2005).

Thus based on recent studies with poultry the conclusions may be drawn that once compositional equivalence has been established then nutritional equivalence of GM feeds modified for agronomic input traits can be assumed. Further animal feeding studies will add little to their nutritional assessment, and that this is equally applicable to plants that have been genetically modified though the insertion of one or more genes.

ii) Pigs

Numerous comparative feeding studies have now been conducted with growing and finishing pigs (Böhme *et al.*, 2001; Gaines *et al.*, 2001b; Stanisiewski *et al.*, 2001; Weber and Richert, 2001; Bressner *et al.*, 2002; Cromwell *et al.*, 2002; Fisher *et al.*, 2002; Reuter *et al.*, 2002 a,b; Aalhus *et al.*, 2003; Bressner *et al.*, 2003; Fisher *et al.*, 2003; Peterson *et al.*, 2003; Hyun *et al.*, 2004; Cromwell *et al.*, 2004; Custodio *et al.*, 2004; Stein *et al.*, 2004). In these studies a range of feeds including maize grain, sugar beet, soybean meal, canola meal, rice and wheat, modified for agronomic input traits, such as HT and Bt, were compared with near isogenic non-GM and commercial varieties. With few exceptions these studies contained data on both the compositional analysis of the feeds and the results of nutritional assessment using a range of endpoints for the feeding study. These trials have also shown that when compositional analyses of GM

varieties and the near isogenic non-GM and commercial varieties were comparable then nutritional equivalence was also established.

c) Production studies with ruminants

Ruminants may consume both forages, which form 20-100% of the diet and consist of fresh (e.g. grass and lucerne) or ensiled forage (e.g. grass, lucerne or maize silage) or plant residues (e.g. maize stover or cereal straw) and supplements to provide additional energy (e.g. cereal grain) and protein (oil seed meals such as soybean meal and canola meal). Many of these feed resources are now obtained from GM plants. Sheep, beef cattle and dairy cows have all been used in studies to compare feed resources derived from a range of plants which have been genetically modified for agronomic input traits such as HT and Bt with their near isogenic counterpart and commercial hybrids.

i) Beef cattle

Studies with beef cattle including those reported by Daenicke *et al.*, (1999); Kerley *et al.*, (2001); Petty *et al.*, (2001 a,b); Berger *et al.*, (2002, 2003) and Folmer *et al.*, (2002) are amongst those reviewed by Flachowsky *et al.*, (2005a) who reported that the performance of beef cattle fed maize grain, maize silage or stover from GM plants was comparable to those recorded for conventional plants. In addition they noted that when the authors also presented data on plant composition, the nutritional equivalence was a consequence of compositional comparability.

ii) Dairy cows

Between 1996 and 2004, over 20 studies in which the performance of lactating dairy cows which received feed ingredients derived from plants genetically modified for agronomic input traits have been compared with their near isogenic non-GM control and commercial hybrids. An extensive range of GM feed ingredients were used in these studies and included Bt maize silage and maize grain, derived from plants, which were modified to be protected against European Corn Borer (Barriere *et al.*, 2001, Donkin *et al.*, 2003) and Corn Root Worm (Grant *et al.*, 2003), Bt cotton seed (Castillo *et al.*, 2004) and HT soybeans (Hammond *et al.*, (1996), HT maize silage (Ipharraguerre *et al.*, 2003) and/or HT maize grain (Donkin *et al.*, 2000), HT fodder beet (Weisbjerg *et al.*, 2001), HT cotton seed (Castillo *et al.*, 2004).

These studies demonstrated that the important end points of feed intake, milk yield and composition of lactating dairy cows was unaffected by the inclusion of feed ingredients derived from a wide range of GM plants. Milk quality is generally measured as the fat, protein and lactose concentration and as such there is no evidence to suggest that the inclusion of GM feed ingredients affects milk quality.

As with other livestock species, studies with lactating dairy cows also showed that once compositional equivalence was demonstrated then nutritional equivalence occurred.

d) Fish, rabbits and other animals

Apart from poultry, pigs and ruminants some production studies were also done with fish (cat-fish – Hammond *et al.*, 1996, rainbow trout – Brown *et al.*, 2003, salmon – Sanden *et al.*, 2004, 2005, 2006) and rabbits (Maertens *et al.*, 1996, Chrastinova *et al.*, 2002).

2.2.3 Nutritional testing of GM feed with improved nutritional characteristics in target animal species

There is only a small number of published studies on the nutritional assessment of GM plants modified for enhanced nutritional characteristics. Examples of livestock feeding studies to demonstrate the expected nutritional characteristics are presented below.

i) Nutritional assessment of GM plants modified with traits to enhance animal performance through an increased level of a specific nutrient

A recent publication by Taylor *et al.* (2005) provides an example in which maize specifically modified to contain an increased level of lysine has been assessed using broiler chickens. The study showed that the performance parameters for the GM variety were similar to those of a variety with similar genetic background and commercially relevant varieties, when supplemented with synthetic lysine.

ii) Nutritional assessment of GM plants modified with traits to increase bioavailability of nutrients

A number of studies have been reported in which comparisons have been made between conventional feeds and those that have been genetically modified for improved nutritional characteristics. Working with lupins which had been modified by the insertion of albumin gene from sunflowers Molvig *et al*. (1997) reported an increased concentration of both lysine and methionine and also reported that protein digestibility as measured in rats was significantly increased from 89.4 to 95.7 %. Similar results are also reported by Ravindran *et al.* (2002) after nutritional assessment of transgenic high-methionine lupins with broilers. It is also interesting to note that when feeding Merino sheep the transgenic lupin seed containing sunflower albumin, White *et al.* (2001) reported increased efficiency of wool growth and live weight gain.

iii) Nutritional assessment of GM plants modified to decrease anti-nutritional factors such as phytate

There are many examples of anti-nutritional factors present in a wide range of feeds. These include alkaloids, glucosides, glucosinolates, lectins and phenol derivatives, such as tannins and gossypol, and protease inhibitors and phytate (ILSI, 2003a; Jeroch *et al.*, 1993; Kling and Wöhlbier, 1983; OECD, 2001a,b; 2002a,b,c). GM varieties exist in which the concentration of these undesirable substances has been substantially reduced, low-phytate maize being one example. Compositional analysis is not sufficient to provide a robust and comprehensive nutritional assessment of such feeds, and livestock feeding studies with appropriate target species are required.

In studies with fattening pigs Spencer *et al.* (2000 a,b) compared diets containing maize grain derived from either a commercial variety or one which had been modified to have a low phytate content. The authors showed that while similar feed intake and live weights were recorded for conventional and GM diet with the low-phytate maize, its use removed the need for phosphorus supplementation and significantly reduced phosphorus excretion, with potentially important effects on reducing the environmental footprint of monogastric livestock production systems.

2.2.4 Conclusions

- Targeted compositional analysis is the cornerstone of the nutritional as well as the safety assessment of GM plants modified for agronomic input traits;

- once compositional equivalence has been established for such plants, feeding studies with livestock species have added little to their safety and nutritional assessment, other than to provide further reassurance that they are as safe and nutritious as their traditional counterparts;

- while broiler feeding studies are considered a sensitive model for nutritional assessment, it is noted that until now no unintended effects have been detected in GM plants with agronomic input traits;

- whilst many plants are in the process of being genetically modified for a wide range of improved nutritional characteristics, few plants have yet reached field trials and even fewer have reached commercial production;

- as with GM plants modified for agronomic input traits, targeted compositional analysis is the cornerstone of the nutritional assessment of GM plants with enhanced nutritional characteristics;

- however, unlike GM plants modified for agronomic input traits, livestock feeding studies are required to demonstrate that the expected nutritional effect or benefit is achieved when feeding the new variety;

- as well as providing a nutritional assessment of the new varieties, livestock feeding studies with typical nutritionally sensitive target animals may help detect unintended effects that could have arisen from potential alterations of physiological and metabolic pathways. In consequence the results from such studies, when conducted, should be taken into account together with the safety assessment data;

- all studies should be conducted according to internationally accepted protocols;

- other types of studies are necessary with feeds from GM plants with output traits (see 3.5.2.).

2.3 Human studies of GM foods

Empirical observations on the safety of genetically modified foods in the human diet are few. One example of a specific concern recently addressed regarding survival of transgenic DNA in the human gut is discussed below.

The risk of trans-species/kingdom DNA transfer has generally been considered very low. The particular concerns relevant to human safety aside from direct harmful effects of the gene or its product are a) dissemination of antibiotic resistance through transfer of antibiotic resistance marker (ARM) sequences into gut bacteria (EFSA, 2004b), and b) gene transfer into mammalian cells, particularly those of the gut mucosa (primarily epithelial cells and lymphoid tissue).

2.3.1 *In vivo* studies

Avian studies have suggested that maize transgenes are completely degraded in the gizzard before entering the small intestine. In mammalian studies stability in saliva, simulated gastric and intestinal fluid, rumen fluid, and silage effluent have been investigated. Although plasmid material may be detected after 30 minutes in rumen fluid or silage effluent it appears that transformation potential is lost within 30 seconds, suggesting rapid degradation. On the other hand both survival and retention of transforming

potential were retained after 24 hour exposure to saliva. These studies have recently been reviewed (Goldstein *et al.*, 2005).

The issue of gene transfer has also been directly studied *in vivo* in humans (Netherwood *et al.*, 2004). Twelve subjects with intact gastrointestinal tracts and 7 with ileostomies were given a meal of GM soy containing the *epsps* transgene, a construct of 2,266 base pairs. Polyethylene glycol (PEG) 400 markers were simultaneously administered so that recovery of digesta could be ascertained. The survival of both the transgene and the endogeneous soybean lectin gene *Le1* in stools or stoma effluent were measured. Since the *epsps* transgene (but not *Le1*) was recovered from microbes cultured from intestinal digesta prior to the administration of the test meal, the results of this study were inconclusive.

Amongst subjects with an intact gut 90-98% of the PEG marker was recovered without detection of any transgene in the faeces. A smaller proportion of marker was recovered (20-100%; over 80% in 5 subjects) from the ileostomists, but a variable number of transgene copies, ranging up to 3.7% of the estimated total administered in one subject. Similar quantities of the native lectin gene *Le1* were recovered. It was not possible to isolate or identify directly bacteria harbouring the transgene either by colony blotting or antibiotic selection. Moreover it was not conclusively proven that the transgene remained intact; as the digesta were subjected to PCR the possibility of PCR-mediated recombination of *epsps* fragments could not be ruled out (Netherwood *et al.*, 2004).

2.3.2 *In vitro* studies

Alongside these *in vivo* experiments the possibility of transgene transfer from gut bacteria into mammalian intestinal cells was separately investigated *in vitro* using the model intestinal cell line Caco-2. *Lactobacillus plantarum* and *Salmonella* Typhimurium were respectively transformed with the plasmids pBK-CMV and pLN1 which confer neomycin resistance. Incubation of Caco-2 cells with a 1000-fold excess of either the recombinant *Lactobacillus plantarum* or *Salmonella* Typhimurium failed to demonstrate any transfer of resistance to the neomycin analogue G418 from prokaryotic to eukaryotic cells. This was nevertheless demonstrable (with a frequency of 1 in 3,000) following direct transfection of Caco-2 cells with the plasmids pBK-CMV or pLPN (positive control) (Netherwood *et al.*, 2004).

2.4 Post market monitoring of GM and/or novel foods

Where appropriate a post market monitoring (PMM) programme should be performed for GM foods. PMM does not substitute for a thorough pre-marketing toxicological testing programme but complements it in order to confirm the pre-market risk assessment. It may increase the probability of detecting rare unintended effects. Therefore the PMM for GM foods should be designed to generate reliable and validated flow of information between the different stakeholders which may relate GM foods consumption to any (adverse) effect on health.

A PMM should therefore address the following questions: i) is the product use as predicted/recommended? ii) are known effects and side-effects as predicted? and iii) does the product induce unexpected side effects? (Wal *et al.*, 2003).

2.4.1 A feasibility study for assessing population variation in consumption after marketing

The feasibility of using current commercial food databases in attempting to quantify exposure to novel foods has been systematically studied in the United Kingdom (Robertson *et al.*, 2004; Elliott *et al.*, 2003). The study was based upon a market-research company (Taylor Nelson Sofres, TNS) database providing information about the food purchases of 33,177 households (105,667 individuals) over ten years (1991-2000). Nutritional information of about 39,530 foods required coding. The demographic structure of the sample was broadly comparable to that of the UK as a whole.

Estimated energy intake was used to assess external validity of the purchasing patterns observed. It was approximately 33% below the estimated energy requirement of adult males, probably reflecting a) a known tendency to under-report energy intakes when these are measured against more reliable methods, such as doubly-labelled water, b) the exclusion of "impulse purchases" and foods consumed outside the home. There did not, however, appear to be distortion of the dietary macronutrient balance, suggesting that there was no systematic bias attributable to exclusion of particular foods or food groups. The methodology was capable of detecting some statistically significant differences in purchasing attributable to region, social class and deprivation group. However, it should be noted that these may not be indicative of consumption; for example, it is feasible that persons in higher income groups have higher wastage. Consumption is not directly measured by such methods, nor is it possible to give any indication of variation attributable to age or gender since only household data are available.

The purchasing of four novel "marker products" introduced to the market after 1991 was mapped. Only 4% of households ever bought any, but there was sufficient statistical power to demonstrate significant variation attributed to region of residence (2.2-5.8%) or deprivation group (most affluent 5.1%, least affluent 2.9%). Only in the case of one product were data sufficient to map temporal trends.

2.4.2 Systems for detecting suspected adverse events: the example of Starlink maize.

Starlink is a variety of maize genetically modified to express the insecticidal protein Cry9c derived from *Bacillus thuringiensis*. Whilst Cry9c is not known to cause allergic manifestations in humans it shares certain physicochemical characteristics with known allergenic compounds; for this reason the US Environmental Protection Agency (EPA) granted approval in 1998 for use in animal feed only. In view of this restriction the US Food Drug Administration (FDA) put no measures in place to monitor the human food supply for presence of Starlink. However, two years later, in September 2000, presence of Starlink in the human food supply (taco shells) was reported. Very large quantities of food were withdrawn from the market. It is important to note that withdrawal was the consequence of admixture of maize intended for animal feeding with that intended for human consumption, rather than the verification of adverse effects.

Exposure assessment was based upon patterns of maize product consumption known from previous dietary surveys. It also required assumptions about the extent of planting (production) and admixing with conventional maize. The extent of cross-pollination was uncertain. A "worst case" estimate suggested that the highest consumers were likely to be Hispanic American children 7-12 years of age, the 95th percentile average daily intake being approximately 17 micrograms Cry9c protein per day.

The FDA, through its EPI-AID mechanism, received reports of adverse events related to ingestion of maize from 51 individuals. When case definition was refined by applying temporal and clinical criteria (to distinguish, for example, allergic reactions from other types of intolerance) this fell to 28, 24 of whom cooperated with further investigation. An ELISA test was developed for the detection of Cry9c specific IgE and applied to sera obtained from these individuals, as well as positive and negative controls

(individuals with known food allergies and pre-Cry9c release samples from the general population; Sutton *et al.*, 2003). No positive samples were identified and it was concluded that IgE-mediated adverse reactions to Cry9c had not occurred (Centres for Disease Control and Prevention, 2001).

A number of observations have been made (Bucchini and Goldman, 2002) about this sequence of events which illustrates well the difficulties associated with post-market monitoring when an adverse event is suspected.

2.4.3 Monitoring of a novel food within the EU: Post-launch monitoring of phytosterol consumption

In 2000, after a safety evaluation of the Scientific Committee on Food (SCF, 1999), the European Commission authorised the placing on the market of yellow fat spreads containing specific amounts of phytosterols as a novel food or novel food ingredient (EC, 2000a). The applicant (Unilever) was required to establish a surveillance programme in order to provide data on individual intakes of the product. In particular, it should be examined whether patterns of consumption fell within those estimated in the application and whether the target group (consumers wishing to reduce plasma cholesterol concentrations) was being reached.

As no study design was stipulated by SCF or prescribed by the Novel Foods Regulation (EC, 1997b) the manufacturer used two avenues of enquiry: a) monitoring of calls to a product "care line", and b) market research conducted on a total of 2000 households in the EU (Belgium, France, Germany, Netherlands and the UK) up to 2001. Despite the spread over five countries it should be noted that this sample is quite small and the study duration short-term by comparison with that described by Robertson *et al.* (2004) above. The specific findings of this survey are summarised elsewhere, together with an opinion of the SCF (SCF, 2002).

From a risk assessment perspective the findings of this study accord broadly with those of both studies cited above: the market research methods employed had insufficient resolution to identify patterns of consumption below household level. Although certain inferences could be made from household structure, it was not possible to confirm directly that potential vulnerable groups such as children or pregnant women did not consume the product. Nor was it possible to confirm directly upper bounds of consumption by individuals, as opposed to households. It should also be emphasised that the task of monitoring a single product introduced to the market ought to be simpler: today there is a variety of products containing phytosterols (e.g. milk products, including milk-based beverages and cheese products, as well as spreads) provided by different manufacturers, which would make characterisation of consumption much more difficult. Moreover trends in consumption remain unknown: these could only be established by continuing dietary surveillance rather than "one-off" post-launch surveys. Some 227 of 84,000 "care line" contacts related to "health issues" but none of the adverse effects mentioned could be explained by the known properties of sterols. This further emphasises the likelihood of considerable false positive reporting with surveillance systems.

SCF, in its conclusions, drew attention to the general difficulties of risk assessment in the absence of an agreed system for the post-market monitoring of novel foods.

2.4.4 Conclusions

- For the three cases described above, only population exposure to foods could be measured. Monitoring of a single product new to the market ought to be simpler than for commodity foodstuffs such as maize, soy etc. where different mixtures commonly occur;

- the three studies have shown that reliable information on potential adverse effects can hardly be obtained at the individual level, but at best at the level of regional or socio-economic groups. The absence of any dose-response relationship makes risk characterisation difficult;

- these studies confirm that post market monitoring cannot be a substitute for a thorough pre-market risk assessment.

3. CONSIDERATIONS FOR SAFETY AND NUTRITIONAL ASSESSMENT OF GM FOODS AND FEED

3.1 Introduction

The overall safety and nutritional testing strategy for GM plant derived foods/feed takes its starting point from the available knowledge about the parental plant, the gene insert, its source and its intended role in the GM plant as well as its possible toxic or allergenic potential. The toxicity and allergenicity of the product of the insert may be pursued in *in silico, in vitro* and *in vivo* tests according to appropriate OECD guideline studies for single chemicals.

Any unintended effect(s) resulting from the genetic modification would result in a compositional change in the matrix of the whole GM food or feed and, therefore, thorough compositional analyses of the GM and parental foods/feed for essential nutrients and toxicants are performed. Changes may be qualitative or quantitative and should be assessed regarding their possible impact on human and/or animal health. This can best be determined both analytically and/or using toxicological/nutritional screening models.

The current performance of the safety assessment of whole foods is mainly based on the protocols (e.g. OECD, 1995) developed over the last 50 years or more for low-molecular-weight chemicals such as pharmaceuticals, industrial chemicals, pesticides, food additives and contaminants. However without adaptation these protocols have limitations for testing of whole foods/feed. This primarily results from the fact that defined single substances can be dosed to laboratory animals at very large multiples of the expected human exposure, often at several orders of magnitude greater than 100-fold thus giving a large margin of safety which helps to compensate for other uncertainties such as interspecies and interindividual differences, etc. In contrast foodstuffs are bulky, lead to satiation and can only be included in the diet at much lower multiples of expected human intakes, giving around 100-fold margins of safety. Added to this, the GM foods developed to date have all been modified from traditional crops having a history of use and thus the logical comparator is the non-GM crop from which they are derived. In consequence, testing protocols for GM plants are designed on a comparative basis, non-GM versus GM, where similarities and differences become the focus of the safety assessment. Most of the protocols below cannot be used without customisation and adaptation for the evaluation of food safety although they do provide a helpful collation of the toxicological endpoints that may be studied according to need when triggered.

The solution to the above limitations has been to develop new testing strategies and protocols for the evaluation of whole food safety. This utilises a concept where semi-synthetic diets are prepared where the whole food to be tested is incorporated in the diet at the expense of corresponding nutrients in order to maintain a satisfactory nutritional balance. Using this methodology and subject to palatability it is possible to achieve the incorporation of whole foods as an integral and not additional part of the diet at levels as high as 60% or more (OECD, 1995; Huggett *et al.*, 1996). The whole topic of dietary incorporation is discussed further in Chapter 4.

The scientific tools available for studies on the safety of GM foods/feed include *in silico, in vitro*, and *in vivo* methods. Any programme for the safety assessment of GM feed/food should first consider what safety aspects need to be investigated and whether initial studies using *in silico* and *in vitro* approaches may answer some of the safety questions and enable subsequent *in vivo* studies, and hence the use of animals, to be reduced. All studies should be preceded by a detailed chemical characterisation of the whole food, *i.e.* a comprehensive compositional analysis. A suggested strategy for selecting the appropriate *in vitro*, *in vivo* and *in silico* tests for assessing the safety of GM foods/feed is set out in Chapter 6.

In vitro methods have clear advantages with respect to savings in terms of time, costs and animal use. *In vitro* methods, where rigorously validated, are best suited to the study of defined substances or extracts of whole foods, rather than whole foods *per se*. During the last two decades significant progress has been made in reducing pain and distress of animals in regulatory testing without reducing the stringency of the safety assessment of chemicals, finished products or food. However, few *in vitro* tests have so far met the necessary criteria of validation and reproducibility required to gain regulatory acceptance, the exceptions being short-term tests for eye irritation and genotoxicity. Little progress has so far been made in reducing or replacing the use of animals in repeated dose studies, such as 28-day or 90-day studies. Thus, at present, *in vitro* tests should be considered as complementary to current *in vivo* testing methods and as early warning systems which may provide a quick and inexpensive way for gaining additional insights into potential toxicity endpoints.

To ensure current best practice and a standardised approach to testing, internationally agreed protocols are used. Most have evolved and have been refined over the last 50 or more years for the safety assessment of chemicals. In the case of laboratory animal safety tests, the methods described by the OECD or in the most up-to-date European Commission Directive on dangerous substances are recommended (OECD, 1995; EC, 2002). Use of any methods that differ from such protocols should be justified. Studies should be carried out according to the principles of Good Laboratory Practice (GLP) described in Council Directive 2004/10/EC (EC, 2004) and must be accompanied by a statement of GLP compliance.

3.2 *In silico* and *in vitro* methods

Evaluation by in silico methods and digestibility testing

An *in silico* search for sequence and structural homology of the novel protein or its degradation products to known toxic or allergenic proteins, peptides or short amino acid sequences in databases is normally undertaken in order to provide additional information to guide the safety testing procedure. Such a search may be done using protein structure databases such as GenBank, SwissProt and PIR and alignment programs such as the FASTA and BLAST algorithms (Stadler and Stadler, 2003; Brusic *et al.*, 2003; Pearson and Lipman, 1988; Altschul *et al.*, 1990).

The possible toxicological consequences of intended changes in GM plants is normally studied in part by subjecting individual compounds (e.g. proteins, metabolites) to *in vitro* testing protocols. In the case that genetic modification, through the insertion of a particular gene, results in the expression of a novel or modified protein, the toxicological analysis will be guided by two aspects that should be considered in advance. First, as protein toxicity could arise from the function or properties of the intact protein or its breakdown products, *in vitro* degradation experiments should be performed. The digestive stability of the protein can be analysed *in vitro* in simulated gastric and intestinal fluids (Astwood *et al.*, 1996). Dynamic multi-compartmental gastrointestinal models are available that simulate conditions in the human gastrointestinal tract and are validated for the digestibility of proteins (Minekus *et al.*, 1995). The information obtained from these *in vitro* biodegradation analyses is considered helpful to guide the case-by-case design of the further safety assessment programme, *in vitro* and *in vivo*. Analysis of the stability of the novel protein under heat or other processing conditions (followed by analysis of digestive stability) might also be an important aspect of the safety testing as it can affect the safety of the gene product. For example latent epitopes may be exposed by heat treatment of proteins which could alter the allergenic status. Equally proteins may be denatured thus reducing any potential for immune sensitization.

Genetic modification also may result, intentionally or unintentionally, in altered levels of secondary plant metabolites. Metabolites, whether known or unknown, could be toxic, depending on the nature and the

amount of the metabolites present. The hazard(s) arising from the presence of qualitatively and quantitatively defined metabolites having known toxicological properties can often be assessed using existing published knowledge of the compounds and does not require any further characterisation. However, this is not the case for metabolites that are unknown or are known but are insufficiently characterised particularly with respect to their possible toxic properties. With respect to the latter metabolites, *in silico* studies and *in vitro* biodegradation and bioavailability experiments might help direct further toxicity testing.

Genotoxicity testing

A number of *in vitro* genotoxicity test methods that screen for point mutations, chromosomal aberrations and DNA damage/repair have been validated and incorporated in OECD guidelines (OECD, 1995) (see Table 6). These tests, designed for defined single chemical substances rather than whole foods, are all accepted by regulatory authorities.

Table 6

OECD 471	Reverse mutation assay (*Salmonella* Typhimurium)	In these two assays bacteria are exposed to the test substance both in the presence and absence of an appropriate metabolic activation system (microsomes)
OECD 472	Reverse mutation assay (*Escherichia coli*)	
OECD 473	*In vitro* mammalian cytogenetic (chromosome aberration) test	
OECD 476	*In vitro* mammalian cell gene mutation test	This test uses mouse lymphoma cells (L5178), Chinese hamster cells (lines CHO, AS52, V79) or human lymphoblastoid cells (TK6) with mutations in TK, HPRT or XPRT
OECD 479	*In vitro* sister chromatid exchange (SCE) assay in mammalian cells	
OECD 480	*Saccharomyces cerevisiae*, gene mutation assay	
OECD 481	*Saccharomyces cerevisiae*, mitotic recombination assay	
OECD 482	DNA damage and repair, unscheduled DNA synthesis in mammalian cells *in vitro* (primary mammalian hepatocytes/estabished cell lines)	

Allergenicity testing

With very few exceptions, most allergens are proteins. Thus, the potential allergenicity of newly introduced proteins is one of the major safety considerations in the assessment of foods. For a description of an approach for the prediction of potential allergenicity of foods the reader is referred to the EFSA Guidance Document (EFSA, 2006a). The document outlines an integrated, stepwise approach for the assessment of possible allergenicity of newly expressed proteins as has been put forward by the Codex *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology (Codex Alimentarius, 2003).

Criteria in the approach, and the i*n silico/in vitro* and *in vivo* tests which have been recommended to address these criteria include:

- the sequence homology of the transgene product to known allergens; *in silico* search for sequence similarity using protein databases such as TrEMBL, PIR, and SwissProt and alignment programs such as FASTA;

- the immunochemical (cross)reactivity of the protein with IgE from serum of individuals known to be allergic to the source of the protein (or to the source of a protein with a defined extent of similarity to the protein in question); *in vitro* IgE-binding tests such as radioallergosorbent test (RAST) and enzyme-linked immunosorbent assay (ELISA);

- the stability of protein under gastro-intestinal conditions; *in vitro* stability tests such as pepsin resistance tests.

It should be mentioned that no single one of these criteria can provide absolute proof of the (absence) of allergenic potential of the protein in question. For instance, the amino acid sequences are not known for all allergens. Moreover, tertiary structures of proteins, which are important determinants of allergenicity, are not well predicted from amino acid sequences. The absence of positive identification of allergenicity in tests may not rule out that the food in effect is allergenic. For example, a causal relationship between stability of a protein to proteolysis by gut enzymes and allergenicity has not been established. Similarly, with *in vitro* testing using human sera, the methods described depend on the allergen specificities of the sera of the allergic individuals used. *In vivo* human studies, especially those involving provocation, are often considered unethical and cannot be easily performed. Alternative testing may therefore involve animal testing (see below) although it should be reinforced that so far no validated animal tests to detect potential allergenicity of foods for humans are available (see later). Full assessment of allergenicity of foods should be based on a case-by-case, weight of evidence approach based on all the available information. Moreover most proteins, including those already in the plants, have the potential to evoke food allergenicity (IgE antibodies) in one or more persons indicating the near impossibility of confirming an absolute lack of allergenic potential for most proteins.

There are no internationally harmonised guidelines for testing for potential allergenicity of food proteins in laboratory animals but it can be done on an experimental basis. A review of the use of animal models in the assessment of studies of potential food allergenic activity has been published recently (Prescott and Hogan, 2005).

The animal models all have in common the production of specific IgE antibodies to the specific proteins. Some models (including different strains of rats and mice) comprise intraperitoneal injection, and analysis of specific IgG and IgE responses. Those proteins that readily produce food allergy in humans are claimed to produce more pronounced IgE responses relative to IgG responses whereas proteins that do not readily cause food allergy are claimed to induce poor IgE responses relative to IgG responses. Adjuvants are often used to induce the immune response.

An animal model has been developed to test the potential allergenicity of food components, in which Brown Norway rats (high IgE responders) are sensitised with or without an adjuvant prior to intraperitoneal and oral exposure to test the compound (Atkinson *et al.*, 1996). In order to avoid the induction of tolerance, these rats are reared for at least two generations on an allergen-free or test protein diet prior to challenge with the test compound. The outcome of such experiments should be carefully evaluated. It should be re-called, for example, that a rat experiment has failed to demonstrate the allergenicity of the 2S albumin from Brazil nut transferred into soybean, whereas individuals allergic to Brazil nut reacted positively to the novel product (Melo *et al.*, 1994).

Other models use rats or mice that are orally exposed to the proteins, in which the IgE response and mast cell mediator release upon challenge after a period of sensitisation to the protein is analysed. These latter models have the benefit of a more relevant route of exposure and a clinical outcome (De Jonge *et al.*, 2006). It should, however, be mentioned that in these animal models, induction of specific IgE is not always associated with clear clinical signs of food allergy in a way they occur in human food allergic patients. Essentially, most of these animal models so far are only able to indicate sensitization (induction of IgE), although other phenomena associated with allergy (delayed-type hypersensitivity, eosinophilia, mucous secretion) have also been noted. It has yet to be established whether induction of specific IgE and related immune responses in these models correlate with the ability of the food proteins to induce food allergy in humans.

Application and potential of profiling technologies

Recent developments in molecular biology and analytical chemistry have provided new opportunities to evaluate the effect of chemicals in food and diet on mammalian cells at various integration levels (e.g. RNA, protein, metabolite). Transcriptomics (transcript profiling), proteomics (protein profiling using among others 2D-gel electrophoresis and MS) and metabolomics (metabolite profiling using techniques such as LC-MS, GC-MS, NMR) are technologies, which facilitate a non-targeted approach and permit the measurement of thousands of variables simultaneously. These "omics" technologies applied to toxicology, also referred to as toxicogenomics, are currently in their infancy, but provide an opportunity to better understand the mechanism of action of chemicals and contribute to the development of alternatives to animal testing (Kuiper *et al.*, 2003). However, further validation of these technologies and better knowledge of how to interprete the complex results is needed before they can be applied in routine safety assessment of foods/feed.

Conclusions

Various *in silico* and *in vitro* methods can contribute to the safety assessment of GM plant derived foods/feed and components thereof:

- *in silico* searches for sequence and structural homology of novel proteins or their degradation products to known toxic or allergenic proteins, peptides or short amino acid sequences in databases may provide relevant information for the characterization of these compounds;

- the digestive stability of newly expressed proteins can be analyzed *in vitro* in simulated gastric and intestinal fluids. Furthermore analysis of the stability of the novel protein under heat or other processing conditions (followed by analysis of digestive stability) might also be an important aspect of the safety testing;

- a number of *in vitro* genotoxicity test methods that screen for point mutations, chromosomal aberrations and DNA damage/repair have been validated. These tests, designed for defined single chemical substances rather than whole foods, may be applied if considered relevant, and it should be realized that testing of whole foods *in vitro* poses specific problems, as discussed under Chapter 2.1.5;

- for a prediction of the potential allergenicity of newly expressed proteins and of whole GM foods, an integrated, stepwise approach has been put forward by the Codex Alimentarius and by EFSA

(EFSA, 2006a). It is emphasized that no single one of the identified assessment criteria can provide absolute proof of the (absence) of allergenic potential of any protein.

3.3 Laboratory animal models for toxicity testing of single substances

Laboratory animal toxicity models have recently been reviewed in an EU funded research programme and have been considered, with certain qualifications, good models for predicting toxic outcomes in humans (FOSIE, 2002).

Single dose toxicity testing

Single dose toxicity testing, also known as acute toxicity testing, is normally conducted in rats or mice and is of principal importance to confirm the lack of any acute toxic potential. In a non-food safety context the purpose of acute toxicity testing is to identify a clearly toxic but sublethal dose, to try to identify major target organs for toxicity, and to provide a rough guide for the selection of doses for subsequent, repeated-dose range-finding toxicity tests. However acute toxicity testing has very little to contribute to the risk assessment of dietary exposure to the whole food because of the low amounts of chemicals that are generally encountered in foods.

Repeated-dose toxicity testing

The primary objective of repeated-dose toxicity testing in laboratory animal species is to determine any adverse effects of repeated daily exposure to chemicals/pharmaceuticals, food chemicals or food components over period of 1 month or longer using large multiples of the anticipated human exposure. Such studies, using animals treated from a relatively young age, are designed to reveal any targets for toxicity, ranging from organs or tissues to cells, and resulting either from direct effects of the test substance on the gastrointestinal tract or from systemic exposure to the test substance or its metabolites. Not only should the design of the test enable potential toxic hazards to be identified but it should also permit identification of dose-response relationships for any targets of toxicity, thereby allowing the nature and severity of toxic effects to be ascertained and the doses without any effects to be established. The aim of subchronic studies is to provide information after administration for a period sufficient to reveal most major toxic effects without any confounding age-associated change in tissue morphology or function. Long-term studies, extending over most of the lifetime of the test species, are typically used to assess the potential chronic toxicity and/or carcinogenicity for single defined substances. The species used are usually rats and mice for both subchronic and chronic studies, and sometimes a second non-rodent species is employed such as the dog for subchronic studies. Relevant OECD Guidelines for subchronic studies with chemical substances are Test Guidelines (TG) Nos. 407 (28-day oral toxicity study in rodents), 408 (90-day oral toxicity study in rodents) and 409 (90-day oral toxicity study in non-rodents). For chronic studies the relevant Guidelines are TG Nos. 451 (carcinogenicity studies), 452 (chronic toxicity studies) and 453 (combined chronic toxicity/carcinogenicity studies).

Repeated-dose toxicity studies conducted to standard protocols generate very large amounts of data, and not only those relevant to potential tissue and organ damage, but also measurements of more subtle changes in physiological functions and the functioning of organ systems. They are often sufficient to allow risk assessment to proceed to a conclusion but, in some instances, effects on particular tissues or target organ may need to be further investigated in specially designed mechanistic studies.

Reproductive and developmental toxicity testing

The primary objective of reproductive toxicity testing is to detect any effects of a test substance or its metabolites on adult mammalian reproductive function, or on growth, development and reproductive capacity of offspring. Tests are normally conducted in rats. The relevant OECD Guideline is TG No. 416 (two-generation reproduction toxicity study).

The purpose of developmental toxicity studies (teratology studies) is to identify any lethal, teratogenic or other toxic effects on the embryo and foetus, by counting of embryonic and foetal resorptions or deaths, measurement of foetal weight and sex ratio, and examination of the external, visceral and skeletal morphology. Tests are normally conducted in two laboratory species, a rodent such as rat or mouse, and a non-rodent such as rabbit. The relevant OECD Guideline is TG No. 414 (prenatal developmental toxicity study).

In cases where it is decided that investigation of both reproduction and developmental toxicity potential is appropriate, one species for developmental toxicity may suffice (e.g. rabbit), if the reproduction study is conducted on another species (such as rat or mouse) (Hurtt *et al.*, 2003; Cooper *et al.*, 2006).

Immunotoxicity testing

Immunotoxicity may take several forms. One form is direct toxicity on the components of the immune system, resulting in malfunction, eventually leading to decreased host resistance or dysregulation that may have consequences for allergic or autoimmune processes. Other forms of immunotoxicity are the induction of allergy or autoimmunity, in cases where the specific compound is recognised by the immune system as an allergen, or in cases where the compound alters components of the host in such a way that they are no longer recognised by the immune system as being self.

Testing for direct immunotoxicity can be undertaken as one aspect of an initial apical test, for instance based on OECD guideline 407, describing the 28-day oral toxicity test, or 408, describing the 90-day toxicity test, followed by further in depth investigations at lower tier levels, if necessary. Testing for direct immunotoxicity includes assessment of a number of non-functional parameters of the immune system, such as routine hematology, including differential cell counting, and weight and histology of lymphoid organs and tissues. For studies on effects of food on the gut, relevant lymphoid organs are the Peyer's patches and mesenteric lymph nodes. To assess systemic effects, also weights and histopathology of the thymus, spleen, and distant lymph nodes are investigated. Also bone marrow cellularity and serum immunoglobulin levels are analysed.

Refinements of standard histopathology that may be applied are immunocytochemistry or the use of flow cytometry. These may give additional information on specific cell types, such as macrophages, T cells, B cells, Natural Killer cells, in their histological context. When experiments yield information on changes in these immune parameters that cannot be attributed to indirect effects, further functional testing is warranted. A prime functional analysis that can be performed is investigating the immune response, usually antibody response, to a T-cell dependent antigen. Other functional assays can probe effects on the capacity of macrophages to phagocytose, Natural Killer cells to lyse tumor target cells, cytotoxic T-cells to lyse specific target cells, and T lymphocytes or B lymphocytes to proliferate in response to specific mitogens.

Conclusion

In principle the OECD test guidelines for chemical toxicity work well for the safety testing of defined new products from the inserted gene. The detailed testing strategy should be selected on a case-by-case basis based on the prior knowledge regarding the biology of the gene product, so that the relevant endpoints are measured in the individual test.

3.4 Laboratory animal models for the safety and nutritional assessment of GM foods/feed

3.4.1 Purpose and limitations of 90-day rodent feeding trials for the safety assessment of foods/feed

When indicated by molecular, compositional, phenotypic, agronomic or other analysis (e.g. metabolic pathway considerations) there may be cause to check in a sentinel study whether the GM plant or derived food or feed is as safe and nutritious as the traditional near isogenic non-GM parental variety. Typically a 90-day rodent feeding study is employed for this purpose being widely regarded as the single most appropriate test for the detection of a wide range of toxicological endpoints, when suitably conducted.

The design of the 90-day rodent feeding study for assessment of the safety and nutritional properties of the GM foods/feed is adapted from the OECD 90-day rodent toxicity study, Guideline 408 (OECD, 1995). The aim of the study is to establish that the GM food/feed is as safe and nutritious as its traditional comparator. There is no intention to establish a formal dose-response curve, since potential effects of the equal intake of the GM foods/feed and its comparator are compared in order to confirm equal safety of the GM and non-GM food/feed. Therefore normally only two dosages of the GM crop are tested against the traditional crop. In order to be able to incorporate high levels (33-60 % or even higher on a case-by case basis) of both the GM crop and the traditional crop in the animal feed without nutritional distortion of their diet, recipes for purified diets with interchangeable elements are used as the basis for the compound feed. The precise study design has to take into account the nature of the foods/feed and the characteristics of the new trait(s) and their intended role in the GM foods/feed. These considerations also include whether or not to use spiking with defined compounds to separate intended and unintended effects and to measure nutritional or health promoting efficacy of the new traits.

There has been considerable discussion over the relevance and sensitivity of rodent 90-day repeat dose dietary testing for the detection of potential intended and unintended effects of whole foods/feed. Animal feeding trials with whole foods/feed are conducted to establish whether the novel food derived from a GM plant is “as safe and nutritious as” its near isogenic counterpart. In essence one is conducting a bioassay to demonstrate the absence of unintended effects of toxicological concern. This is clearly a different rationale and use of the 90-day study compared with its traditional application to hazard identification for low-molecular-weight substances. It is therefore appropriate to consider the strengths and weaknesses of the 90-day rodent feeding study advocated according to the need identified in the GMO Guidance Document (EFSA, 2006a).

3.4.2 Capacity of the 90-day feeding study to detect unintended effects

The capacity of the subchronic toxicity study to detect potential toxicological effects can be deduced from its efficacy in the evaluation of a range of chemical compounds of divergent structure, function and potency (Munro *et al.*, 1996). The database of Munro *et al.* (1996) covers the toxicology of over 600 compounds, representing a range of industrial chemicals, pharmaceuticals, food substances, and

environmental, agricultural and consumer chemicals. The LOELs of 121 chemicals administered by the oral route to rats in subchronic studies were taken from the data tables. The LOELs ranged from 0.2 to 5000 mg/kg bw/day with a median LOEL of 100 mg/kg bw/day. Using the LOEL and knowing the amount of whole food in the diet from which the putative toxicant derives, it is then possible to calculate the detectable concentration of toxicants in the diet. By retro-fitting these data to various plant substances it is possible to model the sensitivity of the rat subchronic feeding study for the detection of hypothetically increased amount of compounds such as anti-nutrients, toxicants or secondary metabolites.

If a theoretical assumption is made that a potentially toxic substance with a LOEL of 100 mg/kg bw/day (the median value from the database) was produced (over-expressed/up-regulated) in a GM plant such as maize, then the chemical would theoretically need to be present in the plant at the level of 0.4% in order to be detected in a 90-day rat feeding study.

This is demonstrated as follows: consider that a rat consumes 25 g of maize/kg bw/day in a 90-day study when averaged over the entire 90 days at a 33% dietary incorporation rate of maize in the diet. Hence, 25 g maize must contain 4 mg/g maize (25 g x 4 mg/g = 100 mg) of the potential toxic substance to expose the rat at the LOEL of 100 mg/kg bw/day. Thus, the concentration of the potentially toxic substance in maize equivalent to this LOEL is 4 mg/g maize, or 4000 mg/kg, (4000 ppm or 0.4%) in the maize grain.

If the LOEL was 2 mg/kg bw/day in the case of a more toxic substance, then the substance would need to be present at 80 mg/kg in the maize grain (80 ppm or 0.008%). For substances that had higher LOELs, and were therefore not very toxic (LOEL = 1000 mg/kg), they would have to be present at 4% or higher levels in grain for detection of adverse effects.

In conclusion, a 90-day study shows a relatively large capacity to detect unintended changes. However, it is unlikely that substances present in small amounts and with a low toxic potential will result in any observable unintended effects in the 90-day study.

3.4.3 Predictivity of subchronic animal tests

For more than four decades, since Weil and McCollister in 1963, the optimum duration of rodent testing for non-tumorigenic effects has been debated. Initially it was assumed that study durations just below the lifespan of the laboratory species would be the most stringent test and would overcome the perceived potential inadequacy of shorter subchronic studies. To this end a pilot study was undertaken to show whether toxicological effects are adequately identified in 3-month subchronic studies in rodents by comparing the non-tumor findings at 3 and 24 months for a range of more than 40 different substances tested by the United States National Toxicology Program (NTP), (Betton *et al.*, 1994). For 70% (57 of 81) of the studies evaluated, all toxicological findings in the 2-year tests were seen in or predicted by the 3-month subchronic tests. New, unpredicted findings were identified in the 2-year test for 24 studies, 12 in mice and 12 in rats. For 5 of these, the new findings were either very mild (e.g. cystic follicles in the thyroids of mice treated with ziram), not clearly treatment-related (thyroid follicular hyperplasia in mice treated with TRIS), seen in controls but at a higher instance in treated animals (adrenal medullary hyperplasia in rats treated with benzoin) or possibly secondary to a finding observed within 3 months (adrenal cortical focal hyperplasia in rats treated with C1 acid red 14, which was secondary to kidney injury seen within 13 weeks). In the remaining l9 studies a range of new target organs were identified after two years of treatment, a significant proportion of which included organs commonly showing acute toxic effects such as the liver, kidney and thyroid. Additionally it should be borne in mind that the 13-week and 2-year studies subject to review were carried out at different times, and sometimes in different institutions rendering them not strictly comparable. In addition there was no access to the pathologists and other

experts who conducted or reported the studies in order to discuss the significance of the new findings, or to ascertain whether the original study was rechecked for subsequently observed subtle changes, such as weak thyroid stimulation. Even with these limitations, all general toxicological findings were identified within three months for more than two thirds of the studies. It is possible that more of those effects observed in the longer-term studies would have shown up at three months by utilising current OECD type protocols rather than the range-finding experiments undertaken by NTP. The review concluded that it was unclear whether any of the new findings would have contributed materially to the conclusions drawn from the 3-month studies.

Munro *et al.* (1996) reviewed four data sources covering different substances and utilised, in addition to NTP, the toxicological monographs prepared by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), the Integrated Risk Information System (IRIS) database and the Developmental and Reproductive Toxicology (DART) database. Although the intent was to develop a database consisting mainly of no-observed-effect levels (NOELs) from long-term studies, having looked at 613 substances they noted that "in many cases, the lowest and most conservative NOEL for a substance came from a subchronic study".

It is noteworthy that in the review of the substances referred to above, NOELs were more frequently based on body weight changes than on clinical endpoints measured (Munro *et al.*, 1996). This observation is supported by Borzelleca (1996), who determined in regard to macronutrient safety assessment that "the single most effective way to evaluate the overall health status of an animal is to observe the effects of treatment on body weight, food consumption and food efficiency".

As regards study duration, also for non-rodents it has been shown in dogs that 90 days are sufficient for the identification of toxicological effects (Gerbracht and Spielmann, 1998; Spielmann and Gerbracht, 2001; Box and Spielmann, 2005; Baetcke et al., 2005).

It is worth noting that subchronic 90-day studies in rodents (in combination with studies on genotoxicity) are also normally required in the EU for confirming the safety of enzyme preparations produced by fermentation using microorganisms (SCF, 1991). The study is not needed to confirm the safety of the enzymes *per se*, but to confirm that there are no uncharacterised mycotoxin or bacterial toxin contaminants from the fermentation medium present at levels that would produce toxicity.

Although the 90-day subchronic toxicity study is not designed to detect effects on reproduction or development other than effects on adult reproductive organ weights and histopathology, analyses of NOELs and LOELs in databases covering subchronic and reproductive effects have addressed the question of whether reproduction/development might be particularly sensitive endpoints. In a further extension of the work published by Munro *et al.* (1996), Kroes *et al.* (2004) have explored NOELs for the toxicological endpoints of embryotoxicity and teratogenicity in tests conducted in rodents and rabbits, to see if such effects might occur at lower doses than are needed for the types of toxicity detected in subchronic and chronic toxicity studies. The NOELs for embryotoxicity and teratogenicity from oral studies on 35 substances ranged from 1 up to 500 mg/kg bw/day, with the exception of ochratoxin A and dioxins, which have high carcinogenic potency. Thus, the majority of substances had NOELs for embryotoxicity and teratogenicity that were higher than the NOELs from subchronic studies. Similarly, in an analysis by Cheeseman *et al.* (1999) of data from 3306 substances showing reproductive toxicity, none of the LOELs for reproductive effects were below 0.5 mg/kg bw/day, with only 5% of the substances having LOELs between 0.5 and 5 mg/kg bw/day. These analyses indicate that, for a wide range of substances, reproductive and developmental effects are not potentially more sensitive endpoints than those examined in subchronic toxicity tests. However, it should be borne in mind that this is a generalisation across a range of substances but for individual substances it cannot be predicted whether the most

sensitive effect will be a reproductive/developmental effect or an effect from the subchronic endpoints. Should there be structural alerts for reproductive/developmental effects or other indications of the need for such tests from the data available on a GM foods/feed, then these tests should be considered, either for the identified substance of concern, the whole food or, exceptionally, for both.

3.4.4 Margins of safety between animal and human intake

By relating the amount of whole test food/food ingredient consumed on average per rat per day in the subchronic 90-day feeding study, to the estimated daily intake (EDI) or theoretical maximum daily intake (TMDI) per consumer for that given whole food/ingredient (or the sum of its individual commercial constituents), it is possible to establish the margin of safety for consumers.

Margins of safety are calculated from a 90-day feeding study by dividing a NOEL or no-observed-adverse-effect level (NOAEL) by the anticipated mean *per capita* daily dietary intake by adults or sensitive groups such as toddlers, pregnant women etc. For GM foods that have been tested in such studies to date, margins of safety have been found to be typically greater than 100-fold (ENTRANSFOOD, 2004).

Examples are:

Maize

A number of 90-day rat subchronic studies have been undertaken by different laboratories where maize has been included in the diet at 33% (w/w) or more and where this dietary inclusion level has been established as the NOAEL. A young adult male rat weighing 250 g eats typically 25 g rodent diet/day, ***i.e.*** 100 g diet/kg body weight/day. At 33% (w/w) dietary incorporation this represents 33 g maize/kg body weight/day. Averaged over the whole study a rat typically consumes 25 g maize/kg/day, which provides a conservative NOAEL. Human daily intake of maize varies since maize enters the food chain both processed and unprocessed from a multiplicity of routes. However, key sources would include maize flour, maize oil, sweet maize, bran and popcorn. Typically oils would be highly processed and unlikely to contain any remaining GM derived proteins and, in particular, toxicants or antinutrients. A typical EU TMDI for maize as described above would be 17 g/person/day. For a 70 kg human this equates to 0.24 g maize/kg body weight/day. This again provides an exposure margin of over 100-fold when the NOAEL is divided by the EU TMDI for maize and its derivatives.

Soybean

Subchronic feeding studies in rodents have been undertaken with 15% (w/w) or more soy. As 15% (w/w) is a typical inclusion level of soy in commercial laboratory rodent diets, and has been found to be a NOAEL this figure is useful for demonstration purposes. On the basis of a young adult rat eating circa 100 g diet/kg body weight/day this represents 15 g soy meal/kg body weight/day or 7.5 g/kg soy protein. Based on average EU soy consumption figures of less than 1 g soy protein/person/day (van Erp-Baart *et al.*, 2003; Clarke and Lloyd, 2004; van der Schouw *et al.*, 2005) and assuming a body weight of 70 kg, this equates to 0.014 g soy/kg body weight/person/day. Thus, there is a margin of safety of over 500-fold between the NOAEL in rats and the TMDI for man in the case of soy protein.

Tomatoes

In a 90-day feeding trial, rats fed a commercial semi-synthetic diet with 10% of lyophilised tomatoes containing the insecticidal protein CRY1A(b) did not show adverse effects compared to animals receiving a diet containing unmodified tomatoes. The average daily intake of tomato powder corresponded to 200 g/kg body weight/day, equivalent to a human consumption of 13 kg tomatoes/person per day (Noteborn *et al.*, 1995). The typical mean male daily intake of raw and cooked tomatoes (National Diet and Nutrition Survey, 2002) is 20 g person/day. Assuming a body weight of 70 kg, a mean daily intake of 0.286 g/kg body weight/day can be obtained. This gives a margin of exposure for man of 200/ 0.286 = 700 fold.

The above margins of safety can be refined by utilising, for example, 97.5th percentile calculations, or looking at a range of demographic population groups and age groups (e.g. because of their smaller body size, toddlers and infants often have a higher intake per kg body weight than adults).

In the case of GM plants, precise dietary intake assessment can be complicated: (i) the GM plant may only be a small fraction of the comingled seed/food, (ii) food ingredients from commodity crops such as maize and soybean enter a very wide variety of products in the food chain, requiring aggregate assessments through various food products, and (iii) food products are often processed into ingredients and/or incorporated in formulated processed food products, where the new protein and/or the novel secondary gene product attrition will occur. This may result in significant reduction in the theoretical maximum daily intake (TMDI) of the novel gene product, resulting in over-estimated exposure levels and even larger margins of safety for man.

3.4.5 Conclusions

- In the context of the safety and nutritional assessment of GM plant derived foods/feed, the adapted 90-day rodent feeding study, if triggered, is a sentinel study designed to detect potential unintended effects of toxicological and/or nutritional relevance;

- the purpose of subchronic feeding trials with whole GM plant derived foods/feed is to determine whether they are *as safe and nutritious as* the traditional counterpart rather than determining qualitative and quantitative intrinsic toxicity as is the case for single chemicals;

- 90-day rodent feeding studies, when adequately controlled both in terms of nutritional balance and traditional reference plants/whole foods, form a sensitive comparative platform with which toxicologically significant differences as well as nutritional deficiencies/improvements can be detected between the whole GM plant derived food/feed and the comparator;

- it is possible to substitute the typical content of an ingredient derived from, e.g. traditional or non-GM maize in commercial rodent diets without causing significant nutritional changes, especially where the ingredient represents a normal certified dietary constituent;

- if deemed necessary other types of rodent feeding studies covering additional endpoints like reproduction and chronic toxicity may be used in the safety assessment of GM plant derived foods; these too require adaptation, e.g. the same principles for nutritional adjustments of rodent diet as described for the 90-day studies above should be taken into account;

- results obtained from the comparative testing of foods derived from GM plants and their traditional counterparts in rodents indicate that large (at least 100 fold) 'safety' margins exist

between animal exposures without observed adverse effects and estimated human daily intake. This applies to the generation of GM plant derived foods/feed with improved agronomic characteristics tested so far, but may not be valid for nutritionally improved GM foods/feed due to the upper safe levels identified for many nutritional elements.

3.5 Target animal models for the nutritional and safety assessment of GM feed

3.5.1 Assessment of GM feed with agronomic input traits

Introduction

For at least 50 years, yield and composition of livestock feeds have been important parameters in the nutritional assessment of conventionally bred varieties. Compositional data have formed the basis of feeding standards and diet formulation for both monogastric and ruminant livestock.

Soybean, maize, canola and cotton have all been genetically modified for agronomic input traits such as herbicide tolerance (HT) and/or insect resistance (Bt). These plants are all used in both monogastric and ruminant diets as energy and/or protein sources. They are included either in the form of fresh or ensiled whole crop forage (e.g. lucerne and maize), as a specific crop component (e.g. maize grain), or as co-products (e.g. oilseed meals or maize stover). As with conventionally bred varieties, GM plants have been subjected to detailed compositional analysis that is still the cornerstone of nutritional assessment of livestock feeds. However, feeding studies using GM plants modified for agronomic input traits have also been conducted with a range of target animal species (see Chapter 2.2.2).

Livestock Feeding Models

The livestock feeding studies for the nutritional assessment of feed ingredients, whether derived from conventional or GM plants, should be carried out according to robust and internationally accepted protocols. The International Life Sciences Institute (ILSI, 2003b) has addressed this issue in "Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits". Some recommendations are given in Table 7. Greater emphasis should be given to the use of confidence intervals in classical and bioequivalence testing frameworks (Tempelman, 2004).

Table 7: Recommendations from the "Best practices for the conduct of animal studies to evaluate crops genetically modified for input traits (GM plants of the first generation)"; adapted from ILSI (2003b).

Animal (species/categories)	Number of animals (coefficient of variation 4 to 5%)	Duration of experiments	Composition of diets[1]	Measurements
Poultry for meat production	10 to 12 pens per treatment with 9 to 12 birds per pen	5 weeks or more	Balanced diets	Feed intake, weight gain, feed conversion
Poultry for egg production	12 to 15 replicates per treatment with 3 to 9 layers per pen	18 to 40 weeks of age, at least three 28-day phases	Balanced diets	Feed intake, egg production, feed conversion, egg quality

Pigs	6 to 9 replicates per treatment with 4 or more pigs per replicate	Piglets (7-12 kg), 4-6 weeks Growers (15-25 kg), 6-8 weeks	Balanced diets	Feed intake, weight gain, feed conversion, carcass quality
Growing and finishing ruminants	6 to 10 replicates per treatment with 6 or more cattles per replicate	90-120 days	Balanced diets	Feed intake, gain, feed conversion, carcass data
Lactating cows	12-16 cows per treatment	Latin square: 28 day periods	Balanced diets	Feed intake, milk production and composition
	28 cows per treatment	Randomized block		body weight, body condition score (BCS), cell counts in milk, animal health composition

[1] Feed from GM plants should be included in high portions in diets and compared with isogenic counterparts.

The use of rapidly growing animals has been proposed as a useful model for the nutritional assessment of GM feed ingredients. The use of monogastric livestock is clearly appropriate for cereal grains such as maize and protein supplements like soybean meal. Growing or lactating ruminants should be used to test forages.

3.5.2 Assessment of GM feed with enhanced nutritional characteristics

Background

Animal production is often restricted by the fact that feed resources are deficient in a specific nutrient or the bioavailability of a nutrient is low or it is constrained by the presence of an anti-nutritional factor. A number of plants with genetic modifications aimed at improving nutritional characteristics have been developed (Table 1) and are currently in trials.

The role of compositional analyses

Compositional analysis is the cornerstone for the nutritional assessment of plants modified for improved nutritional characteristics. The composition of these plants is compared with their nearest isogenic counterpart and commercial varieties to determine if, with the exception of the intended changes, the plants may still be considered as comparable. This is particularly relevant for GM plants modified for enhanced nutritional characteristics as metabolic and physiological pathways may be altered, which may have unexpected effects on plant composition and accumulation of secondary metabolites.

Models for livestock feeding

In the case of GM plants with improved nutritional characteristics, various types of livestock feeding studies with target species should be conducted on a case-by-case basis to confirm the expected nutritional benefits. Some examples are described in the previous section. These studies should be conducted according to internationally agreed standard protocols. While the "Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits" (ILSI, 2003b) provides a sound basis (section 2.2.2 and Table 7) for many of the details required for the conduct of such experiments it does not cover some key aspects such as the appropriate comparator or experimental design. Animal

health and behaviour and quality of foods of animal origin are further parameters that have to be considered.

Appropriate comparator and experimental design

The exact experimental and statistical design of animal experiments to test the safety and nutritional value of GM plants with enhanced nutritional characteristics will depend on a number of factors and will include animal species, plant trait(s), the size of the expected effect and the amount of GM feed available (which will in turn affect the number of animals per treatment group). Endpoint measurements will vary with the target species used in the study but will include feed intake, animal performance, bioavailability of nutrients, environmental impact, and animal health and welfare (see Flachowsky and Böhme 2005 for more details).

Feeds including co-products from industrial use with intended beneficial physiological properties like amino acids, fatty acids, minerals, vitamins and other substances or reduced content of undesirable substances may contribute to higher feed intake of animals and/or improved conversion of feed/nutrients into food of animal origin and lower excretion of nitrogen, phosphorus and other nutrients. The experiment should be designed to demonstrate the claimed effects. Various experimental designs are necessary to demonstrate the efficiency of changes or of expressed nutrients/constituents:

- bioavailability or conversion of nutrient precursors into nutrients (e.g. ß-carotene);
- digestibility/bioavailability of nutrients (e.g. amino acids, fatty acids, vitamins);
- efficiency of substances which may improve nutrient digestibility/availability (e.g. enzymes);
- utilization of substances with surplus effects (e.g. prebiotics);
- improvement of sensoric properties/palatability of feed (e.g. essential oils, aromas);
- lower concentration of inhibiting substances (e.g. phytate, lignin);
- lower concentration of toxic substances (e.g. alkaloids, glucosinolates, lectines, saponins, mycotoxins).

Example models for livestock feeding studies with GM varieties with increased concentration of desirable nutrients.

a) To provide a nutritional assessment of a GM feed ingredient in which a nutrient precursor such as ß-carotene has been increased

Treatment structure	**Added supplement/comment**
T1 Near isogenic parental line	No supplement
T2 Near isogenic parental line	ß-carotene supplement provides ß-carotene comparable with T3.
T3 GM variety, enhanced ß-carotene	No ß-carotene supplement needed, ß-carotene content is comparable with T2.

Balance studies with target animal species/categories are necessary to assess the conversion of nutrient precursors (e.g. ß-carotene) into nutrients. At least two groups (T2 and T3) of animals are necessary to

assess the conversion of the precursors into the nutrient. Dose-response studies with the supplemented precursor and the GM feed with enhanced nutritional characteristics could improve the assessment, but are more expensive in time, money and feeding materials. Specific markers or target organs (e.g. vitamin A in the liver in the case of ß-carotene as the best indicator of vitamin A status, Goodman *et al.*, 1984) should be used to assess the bioavailability of the nutrient precursor. Various models to determine the bioavailability of micronutrients have been discussed by House (1999), Van Campen and Glahn (1999) and Welch and Graham (2004). Howe and Tanumihardjo (2006) tested the carotene conversion into vitamin A of carotenoid-biofortified maize. In addition to the model proposed above they used a fourth group (T4) with vitamin A supplement to the near isogenic parental line matched to the high ß-carotene maize. Such a design allows to assess the conversion of ß-carotene from the GM plant and to compare it with added vitamin A and ß-carotene.

b) *To provide a nutritional assessment of a GM feed ingredient in which the concentration of a specific nutrient such as an amino acid or fatty acids has been increased.*

Treatment structure	**Added supplement/comment**
T1 Near isogenic parental line	No amino acid supplement
T2 Near isogenic parental line	Amino acid supplement provides balanced diet.
T3 GM variety: enhanced amino acid content	No amino acid supplement needed. Balanced diet comparable with T2.
T4 Commercial variety	Diet composition comparable with T2

This treatment structure is appropriate for the nutritional assessment of a range of GM varieties in which the nutrient content of a specific nutrient has been enhanced and the need for providing a synthetic supplement has been removed. Comparison between T1 (negative control) and T2 (positive control) will show the benefit of synthetic amino acid supplementation while the comparison between T2 and T3 will demonstrate the efficacy of the GM variety while a comparison between T3 and T4 will provide further comparisons between the use of a nutritionally enhanced GM variety and a commercial variety. Such studies would be conducted on target species and diets would be offered *ad libitum* and a range of animal performance endpoints would be measured. If the endpoint measurements comparing T2 with T3 are similar then this would indicate that the bioavailability of the nutrient enhanced in the GM variety is similar to that of the synthetic supplement and a digestibility study per se is not required. However, if the endpoint measurement were markedly different then this could indicate the presence of decreased bioavailability and/or the presence of an unintended effect.

c) *To provide a nutritional assessment of a GM feed ingredient when the digestibility of a specific nutrient such as nitrogen or fibre has been increased*

Treatment structure	**Level of feeding**
T1 Near isogenic parental line	Fixed
T2 GM variety: enhanced digestibility	Fixed
T3 Near isogenic parental line	*Ad libitum*
T4 GM variety: enhanced digestibility	*Ad libitum*

There is continuing debate about the level of feeding that should be imposed during a digestibility study. In many cases a fixed level of intake or pair feeding models are recommended as this will provide a clear comparison between the digestibilities of the nutrients under investigation. Such data would be obtained from a comparison of T1 and T2. Nevertheless, there is a case for using an *ad libitum* level of feeding as this will provide evidence as to the effect on feed intake and animal performance. However, under these conditions it is not easy to distinguish between the effects of increased digestibility and increased intake. A possible compromise is that the level of feeding is restricted but is still 90% of *ad libitum* intake. The

endpoint measurements are those recorded in standard total tract digestibility studies. While the decision on level of feeding should be made on a case-by-case basis it is important that the target species is selected carefully and is appropriate to the test product.

d) *To provide a nutritional assessment of a GM feed ingredient in which the content of a nutrient enhancer such as enzymes has been increased*.

Treatment structure	Added supplement/comment
T1 Near isogenic parental line	No supplement
T2 Near isogenic parental line	Enzyme supplement (e.g. phytate) provides the enzyme comparable with T3
T3 GM variety, enhanced enzyme	No enzyme supplement is needed; enzyme content is comparable with T2

Expression of substances which improve nutrient utilization is one of the objectives of output traits. Enzymes like phytase or non starch polysaccharides degrading enzymes are examples for such compounds. Efficacy of such substances should be demonstrated using specific experimental designs. If any influence on the level of feed intake is expected, the experimental design has been dramatically extended (T4, T5 and more) to measure the influence of the GM expressed feed additive on the feed intake. Dose response studies would be needed to determine the optimum inclusion rate of the phytate containing feed ingredient.

e) *To provide a nutritional assessment of a GM feed ingredient in which the content of substances with surplus effects such as prebiotics (e.g. inuline) or essential oils has been increased*

Treatment structure	Added supplement/comment
T1 Near isogenic parental line	No supplement/Fixed
T2 Near isogenic parental line	Prebiotics supplement (e.g. inuline) or substances which improve sensoric properties or palatability (e.g. essential oils) provides the enzyme comparable with T3/Fixed
T3 GM variety, enhanced prebiotics or essential oils	No prebiotics or other supplement is needed; content of such substances is comparable with T2/Fixed.
T4 Near isogenic parental line	T2/ A*d libitum*
T5 GM variety, enhanced prebiotics or essential oils like T3	T 3/ *Ad libitum*

Prebiotics (e.g. inuline), herbs, essential oils or other substances may influence the palatability of feeds, processes in the digestive tract or the immune response. Some of those properties can be introduced into GM plants with output traits. There efficiency must be demonstrated in specific experiments with target animal species or categories. Restricted, pair feeding models or *ad libitum* feeding (T4, T5) is recommended to assess the influence of the substances on feed intake.

f) To provide a nutritional assessment when the concentration of an anti-nutritional factor such as phytate is decreased in a GM variety

Treatment structure	Added supplement
T1 Near isogenic parental line	No supplement
T2 Near isogenic parental line	Phosphorus supplement added.
T3 GM variety: reduced phytate content	No phosphorus supplement added, but dietary phosphorus content comparable with T2

When an effect of a decrease in an anti-nutritional factor such as phytate, which reduces phosphorus availability, is being evaluated then a relatively simple treatment structure is appropriate. A comparison between T1 and T2 shows the benefit of a phosphorus supplement to provide the monogastric target species with a balanced diet while the comparison between T2 and T3 provide the nutritional assessment of the GM variety with decreased phytate content when compared with the current practise of using a traditional variety and a phosphorus supplement. Spencer *et al.* (2000a,b) used such a design and tested a fourth group (T4), where low phytate maize was also supplemented with inorganic phosphorus. Such a design is possible, but not urgently necessary to demonstrate the higher phosphorus bioavailability of low phytate-crops. While feed intake and animal performance are clear endpoints to be measured, nutrient digestibility and environmental measurements such as phosphorus excretion could also be measured. Furthermore metabolic studies as strength of the bones or ash content of indicator bones (e.g. 4th metacarpal bone) may help to assess the consequences of reduced phytate content of GM feed on animal health.

g) To provide a nutritional assessment of a GM feed ingredient in which the content of toxic substances as mycotoxins is decreased in GM variety

Treatment structure	**Level of feeding**
T1 Near isogenic parental line	*Ad libitum*
T2 GM variety	Pair fed to T1
T3 GM variety	*Ad libitum*

Genetic modification may directly or indirectly contribute to lower concentrations of toxic substances. A direct decrease means a reduction by genetic modification such as a lower concentration of glucosinolates, allergenic substances, etc. An indirect decrease could be a secondary effect of genetic modification as a lower contamination of Bt-maize with Fusarium toxins in consequence of reduced infection with the European corn borer. Animal studies to demonstrate the effects of lower concentrations of toxic substances in GM plants in comparison with the isogenic counterparts seem to be necessary. The effects of lower mycotoxin-concentration in Bt-Maize on feed intake and animal growth was shown by Piva et al. (2001a,b) for piglets and broilers.

3.5.3 Conclusions

- Compositional analysis is the cornerstone for the nutritional assessment of any new plant variety whether produced by traditional breeding or by biotechnology. The analyses required for the assessment of feed derived from GM plants modified for agronomic input traits should be determined on a case-by-case basis;

- the need for livestock feeding studies with target animal species/categories should be determined on a case-by-case basis and if conducted should be carried out according to internationally recognised protocols such as those described in “Best Practices for the Conduct of Animal Studies to Evaluate Crops Genetically Modified for Input Traits” (ILSI, 2003b);

- livestock feeding studies with target species should be conducted on a case-by-case basis to establish the nutritional benefits that might be expected. These studies should span either the finishing period to slaughter for chickens, pigs, fish and beef cattle or a major part of a lactation cycle, lasting at least 100 days, for dairy cattle and should be conducted according to internationally agreed standard protocols and should use carefully selected comparators with similar genetic background and commercial varieties;

- in cases where GM plants have been fed to livestock with the intention of modifying the nutritional components to be deposited in the consumed tissue of the animal, specific tests for content should be conducted;

- studies with target animal species to assess the nutritive value of feed derived from GM plants with output traits should be combined with safety studies.

3.6 Human studies with GM foods

3.6.1 Safety

Safety is the over-riding concern where food for human consumption is concerned. This outweighs any potential economic or nutritional benefit associated with the product. Thus pre-market testing and risk assessment must assure negligible risk of unintended effects at expected levels of consumption. Demonstrating this poses a range of problems, over and above those associated with testing in animal studies: these include the length of the human lifespan, possible variation in susceptibility at different phases of development and senescence, greater dietary diversity (both between and within individuals), and the prevalence of morbidity.

3.6.2 Exposure assessment

Typically population diet and nutrition databases are used to estimate likely consumption for the purpose of assessing risk. Assumptions are made about substitution of the novel food /ingredient for conventional ingredients and age/gender characteristics of high consumers identified (using the 90^{th} or 97^{th} centile). Such estimates are prone to a number of potential errors. Firstly, full data are not available for all countries. Even where they do exist (e.g. UK National Diet and Nutrition Survey; NDNS, 2002) certain population groups who may differ in eating behaviour are either excluded (e.g. pregnant women) or are present in numbers too low for meaningful analysis (e.g. ethnic minorities). Foods consumed infrequently may not be captured in 4 or 7-day surveys, so the accuracy of predicted consumption may be low and subject to systematic bias requiring adjustment (e.g. liver and retinol) (Scientific Advisory Committee on Nutrition, 2005). Secondly, it is feasible that dietary patterns could be susceptible to commercial influences (e.g. price /marketing) associated with the novel ingredient and therefore change. Thirdly, an ingredient may be approved and later incorporated in a broader range of foods (e.g. phytosterols) than initially assumed. Note in this context that the current European process requires the novel ingredient (NI) manufacturer, not the distributors of food end-products, to apply for regulatory approval. Post-market monitoring (PMM) has a role in the validation of estimated exposure assessment, particularly to quantify the precision and accuracy of such assessments and refine the process (see below, 3.6.6).

3.6.3 Susceptibility and identification of vulnerable population groups

The primary purpose of identifying high consumers is to establish margins-of-safety based upon safety data derived from controlled studies in humans or animals. High consumption constitutes only one source of vulnerability. Usually, for ethical reasons, tolerance studies are conducted on healthy adult volunteers. However digestibility and bioavailability change during growth and development, pregnancy, lactation and senescence. Judgements about the safety of novel foods and ingredients in these groups therefore depend upon extrapolating what is known about changes in absorption, distribution, metabolism and excretion (ADME), something not always understood with certainty. Additionally individuals in the

population with chronic disease may show increased susceptibility, either as the result of alteration in ADME or through increased susceptibility to symptoms (e.g. low digestibility carbohydrates; laxatives; irritable bowel syndrome; toddler diarrhoea).

3.6.4 Dietary diversity needs to be considered in designing studies

The variety of foods consumed in the diet may create difficulties in both estimating the risk of unintended effects before approval and conducting exposure assessment after marketing. Although risk assessments are generally conducted on a product-by-product basis, different GM food products may have similar characteristics with respect to both physiological function and unintended effects (e.g. low digestibility carbohydrates). Although the intake of each GM food product individually may be assessed as safe, the resultant cumulative dietary intake may not be. Whilst it is conceivable that this might be modelled in a pre-marketing assessment, the summation of errors associated with modelling exposure to individual products (see above) would suggest it is unlikely to be helpful. Moreover regulatory approval of any individual product does not currently require risk assessment of cumulative consumption of novel ingredients. Specific hypothesis-based research may be needed to explore such possibilities.

It could alternatively be argued that the dietary diversity of humans constitutes a safeguard, effectively diluting individual intake of novel ingredients. In this context it is important to emphasise the specific risk applying to young children, particularly infants, as the result of decreased dietary diversity. This is most striking in the case of the artificially fed infant, who may consume the same product exclusively for 6-months at a critical stage of development. These particular circumstances support the view that the safety and efficacy of any change to the composition of artificial feeds for infants, including the use of novel ingredients, should be assessed in adequately powered, randomised controlled trials (RCT) (COMA, 1996). This exemplifies a currently rare situation in which methodology normally associated with pharmacological innovation is applied to food.

In view of the constraints on generalisability outlined in preceding paragraphs the RCT more generally has limited value for safety assessment in humans, though intervention studies (including RCTs) are required to confirm the veracity of any health claim (Aggett *et al.*, 2005). Under these conditions it is possible that meta-analysis of data may prove of value in safety assessment. Standardisation of outcomes/endpoints may facilitate this.

3.6.5 Products to be assessed

Application is generally made for the approval of a GM plant or its derived food or feed and is sought by the originator of the process. However the product may appear in the human diet in many different final forms as prepared food. Preparation involving a range of manipulations such as preparing, processing and cooking may modify further its digestibility and bioavailability. The consumer may moreover be unaware of its presence in the diet.

3.6.6 Post market monitoring (PMM)

Where appropriate a PMM programme should be performed for GM foods. PMM does not substitute for a thorough pre-marketing toxicological testing programme but complements it in order to confirm the pre-market risk assessment. It may increase the probability of detecting rare unintended effects. Therefore the

PMM for GM foods should be designed to generate reliable and validated flow of information between the different stakeholders which may relate GM foods consumption to any (adverse) effect on health.

A number of possible functions for PMM need to be critically evaluated. Examples are given below.

Is the use of the product as expected?

Conceptually this would seem the easiest application of PMM, though it is vulnerable to several sources of error described above, particularly those associated with consumer's awareness, diversity of diet and frequency of consumption. Problems associated with tracking throughout the food chain are also of relevance, particularly where an ingredient rather than a branded product are concerned.

Are know effects and side-effects as expected?

The prospect of using PMM is to confirm the pre-market assessment of large populations including specific segments of the population at risk in the every day life conditions.

Detection of unintended adverse effects, such as allergic reactions

The concept of using PMM to detect unintended effects arises from the pharmaceutical industry. However, unlike medicines, foods and food ingredients are not ingested in fixed doses for fixed periods. Moreover the consumer may be unaware of ingestion. Therefore the relationship of any unintended effect to a food may be obscure.

IgE mediated hypersensitivity may be an exception as characteristic manifestations are immediate and therefore recognisable, particularly if the occurrence is repeated. The signs may also be witnessed by a physician. However non-IgE mediated allergic phenomena and other food intolerances are unlikely to be recognised by a case reporting mechanism and arguably are of greater population significance than immediate hypersensitivity reactions in a quantitative sense. Qualitatively it should be mentioned that food intolerance does not result in lethality. Specific hypothesis-based monitoring studies demanding some concept of the specific "unintended" effect sought would be required. This could be problematic and is more accurately described as epidemiological research.

Documenting effects on chronic disease processes

It is difficult to appreciate how PMM might detect any risk of exacerbating chronic disease without any specific investigative hypothesis. The variable interval between novel ingredient consumption and health outcome poses significant problems. However it is feasible that any benefit to health (associated with a health claim) identified in controlled studies might be studied epidemiologically at the population level, given a clear hypothesis. Indeed this evidence could be required to confirm generalisability of benefit demonstrated in the narrow context of a pre-marketing intervention study designed to support any functional claim. In large population-based studies of this nature it might then be possible to identify clusters or gradation of risk apparent at quintiles of intake. Again this should be described as epidemiological research, rather than monitoring: a clear hypothesis would be tested using detailed information about consumption and health collected at the individual level. Data would preferably be collected prospectively.

Limitations of PMM and its place in the risk assessment

As post-marketing monitoring by definition follows risk assessment, it is a separate process. Characteristics of a PMM system which need to be considered include:

- *Demographic validity:* is the system geographically and socially representative, or are certain groups under-represented?
- *External validity* of estimated intakes: do the data appear representative of key nutrient intakes, e.g. energy intake, or is there under-reporting? How is wastage estimated?
- *Do the data capture all sources of the novel ingredient?* Are sufficiently complex data available to relate to recorded intake, and how will foods eaten outside the home be captured?
- *Resolution of exposure:* can data be related to individual intake, or just household or some higher level?
- *Time course of effect sought:* is the effect sought immediately associated with consumption, medium-term or long-term?
- *Linkage to health data:* could consumption data be related to health data? Clearly this poses significant ethical problems. Currently ethical and information technology constraints appear to make this not feasible.

When tested against these criteria the uses of PMM are limited. Prospective nutritional monitoring utilising large, market-research food consumption databases combined with sufficiently comprehensive food composition data could be capable of describing patterns of novel ingredient or food exposure at household level. It could also be used to monitor temporal changes in consumption (Robertson *et al.*, 2004). However, both ethical and information technology constraints suggest linkage to health data is not feasible. Knowledge gained through PMM might therefore at best describe only broad patterns of human nutritional exposure. It does not have the sensitivity to estimate individual intakes, nor intakes of particular age groups. It should not be considered a feature of the risk assessment but a later step which may additionally inform risk management. It should not be relied upon as a technique for monitoring adverse events or other health outcomes related to novel food consumption.

3.6.7 Conclusions

- Post marketing monitoring (PMM) should not be viewed as an activity intrinsic to the risk assessment, but a part of the subsequent risk management process;
- many factors contribute to uncertainty in the estimation of human exposure prior to marketing. PMM has some value in confirming both the veracity of assumptions made during risk assessment and compliance with any stipulations made upon approval;
- the complexity of food markets presents challenges for the tracing of novel products or ingredients. Existing food purchase databases are not capable of tracing consumption beneath, at best, household level. This means that estimates of consumption by particular age or gender groups usually depends upon inference about household composition;

- food composition databases need to be improved and maintained to keep abreast of change so that the purchasing of novel ingredients can be traced;

- PMM may have value in detecting unexpected adverse effects, though surveillance systems solely dependent on positive reporting will not capture population incidence since the number of individuals with unreported symptoms (false negative) is unknown. Intuitively this seems likely to be lower in the case of severe effects (for example anaphylaxis) than milder ones (for example mild gastrointestinal disturbance). Hypothesis-based population surveys are required to measure true incidence;

- systems of PMM need to be considered on a case-by-case basis, preferably prior to marketing in order to facilitate prospective observation;

- currently a number of major information technology and ethical barriers preclude the linkage of healthcare and food consumption databases.

4. STANDARDS FOR TEST SAMPLE PREPARATION, TEST MATERIALS, DIET FORMULATION AND ANALYSIS

To meet the requirements of Good Laboratory Practice and sound science the test article must be checked for identity and the formulated diets checked for achieved concentration, homogeneity and storage stability.

4.1 Identity, specification, sampling and analysis of the test material (GM plant or derived foods/feed)

Test materials should be quality assured with respect to geographical origin, genetic modification, chemical and microbiological analysis. Additionally samples must be tested for homogeneity and identity in terms of event specific PCR in the case of GMO containing diets. The test material analysed should be an aliquot of the material to be incorporated into the animal diet. Analyses should be carried out according to appropriate standard analytical methods on recommended analytes (OECD & ILSI) to agreed quality standards. It is important to ensure that fully representative samples of the test material are analysed and that fractions of the test samples are retained under appropriate storage conditions for possible future revalidation. In order to conduct an analysis a small number of random samples should be taken from the batch(es) of test material provided from the field trials, mixed thoroughly and then analysed. This procedure should be used on every individual batch provided and utilised for dietary formulation.

4.2 Formulation of test and control diets

4.2.1 Types of diets used in laboratory animal studies

In the preparation of laboratory animal diets for the safety testing of novel foods including GM and macronutrients, three different types of animal diets are considered:

- natural-ingredient diet;
- purified diet;
- human-type diets.

Natural-ingredient diets are made with agricultural products and by-products, and have often been used for rodent feeding studies testing GM plants. These diets are nutritionally acceptable to most animals.

Purified diets, sometimes referred to as semi-synthetic diets are often used when testing macronutrients and whole food because it is easy to manipulate ingredients in this type of diet. They are made of a restricted number of ingredients, which are well-characterised.

Human-type diets should represent a balanced human meal, but at the same time fulfill the nutritional requirements of the experimental animal.

When performing a 90-day safety study with a GM foods/feed, all three types of diet can be recommended. However, it must be emphasised that adjusting a natural-ingredient diet and a human-type diet can be complex.

4.2.2 Dietary Incorporation levels/homogeneity of whole foods in laboratory animal diets

When testing whole foods it is desirable to obtain the highest concentration possible of the GM food in the animals' diet. The maximum test level depends on the type and nutritional composition of the food. It is possible to substitute the typical content of whole foods, e.g. maize or soy in commercial rodent diets, without causing significant nutritional changes, especially where the whole food represents a normal certified dietary constituent.

For GM plants like wheat, maize and rice very high inclusion levels, up to 80%, can be used without significant impacts on dietary balance. GM foods such as potatoes and tomatoes and novel fruits and vegetables with a relatively high water content can be freeze-dried before incorporation in the animal diets, which permits high inclusion levels as well. In this case the limiting factor will be inherent toxicants and/or inherent levels of minerals in the food. Another limiting factor could be the expression level of the inserted trait, such as β-carotene in "golden rice".

Maize may be added to commercial animal diets at levels of 33% (w/w) based on nutritional formulas developed over years by laboratory diet manufacturers. So it is relatively straightforward to request the commercial diet manufacturer to reformulate the same commercial diet by incorporating, for example, up to 33% GM maize instead.

For soybean, maximum incorporation rates of 15% (w/w) are normally used based on past experience with tried and tested commercial formulas for rodent diets. However, there are literature reports where higher levels of soybeans have been fed to rodents.

While higher levels of maize and soybean meal can be fed to rodents and still maintain relative nutritional balance, the potential problem is that there are no historical data to rely on to resolve the biological meaningfulness of random statistical differences which can occur in these kind of studies.

Normal practice is to use a minimum of 2 test dose levels and negative control and reference (near isogenic) control formulations with which to create nutritionally equivalent balanced diets in a comparative protocol. Where a lower level is employed such as 11% (w/w) in the case of maize, 22% (w/w) conventional maize is added to bring the total grain content back to 33% (w/w) in order to maintain nutritional balance. Specialist diet formulators can undertake such work resulting in formulations that are nutritionally and compositionally comparable to their standard certified laboratory diets. This should be GLP compliant.

When the diets have been formulated to these standards it is important to undertake analytical studies in order to confirm that the mixing process has indeed produced diets of the intended concentration and by sampling at different levels in the kegs of diet produced that the mixes are homogeneous throughout.

4.2.3 Dietary stability

It is essential to check the stability of the diet that is formulated with the GM plant or derived foods/feed at the inclusion levels prepared. This is because endogenous dietary fat and other substances can interact with the test material leading to reductions in concentration resulting in the potential for test animals to be under-dosed. In consequence it is normal practice to establish formulated dietary stability tests to determine whether any special storage conditions such as refrigeration, protection from UV light etc are required in order to maintain dietary concentrations of the test substance between different periodic mixes.

4.2.4 Processing of the GM food for inclusion in the test diet

Normally, foods undergo some kind of preparation before consumed by humans. For instance potatoes can be cooked, boiled, steamed, fried or baked. It is not feasible to make an animal study for each of these preparation methods although a number of changes may happen both with the target chemical(s) and with many other chemical entities inherent in the food. The role of the animal study is to deliver data from the basic, universal, presumably worst case situation for use in the hazard characterization. In practice, worst case will be decided on a case-by-case basis, but will most often be to test the GM food in its original raw form. However, the nutritional and physiological needs of the experimental animals need to be taken into account before feeding experimental animals high amounts of raw foods, like for instance potatoes. The influence of food preparation should ideally be covered in the exposure assessment, where the chemical consequences of different preparation methods on the target chemicals, the so-called reduction factors, should be assessed.

4.2.5 Choice of control diet / comparator

The use of a proper control diet in the control group is of great importance in the design of the animal studies. A high inclusion level of a food in a diet may, due to matrix effects, affect the animals compared to a normal control diet solely based on well-defined feed ingredients. Before embarking on feeding studies it is therefore important to test the effect of the food *per se* in a preliminary study. Alternatively an additional control group without the test food can be included the study design. For GM foods an obvious comparator can be found in the parental line and for modified macronutrients as for example starches a comparator is the unmodified form of the macronutrient. For investigating GM foods/feed with enhanced nutritional properties (e.g. increased levels of ß-carotene, amino acids, fatty acids), choices for control diets should be made on a case-by-case basis (see Chapter 3.5.2). For instance in case of a nutritionally improved GM food/feed with an excess of a nutrient, the safety impact of potential unintended changes, which may be masked by the effects of a nutrient, could be assessed by using a control diet without the specific nutrient.

When testing new fruits and vegetables for which a natural comparator does not exist, the task is much more complicated. Closely related types of fruits and vegetables could be included as a comparator, but is not recommended, as these comparators themselves can contain anti-nutritional and toxic compounds not present in the GM food to be tested. The best approach would be to ensure that the test diet with the GM fruit or vegetable would have the same overall composition regarding macro- and micronutrients as the diet for the control group.

Spiking

The purpose of spiking a diet with the compound that is expressed in the GM plant is twofold, namely to test the sensitivity of the test system, *i.e.* to discriminate between adverse effects possibly induced by the newly expressed compound(s) and those induced through unintentional events as result of the genetic modification. In the SAFOTEST 90-day study, the test diet containing the GM rice was spiked with PHA-E lectin at a dose level comparable to that which induced effects in a 28-day feeding study, with the expectation that this test group would exhibit the same types and at least the same degrees of adverse effects as registered in the preceding 28-day study with the pure compound, and possibly unintended toxic effects caused by secondary changes (see Chapters 2.1.4 and 6.7). Discrimination between effects induced by the inserted lectin and/or by other (unknown and unintentional) factors may not be easy following this approach.

Spiking of a control group diet which contains the non-GM plant derived foods/feed with the compound expressed in the GM plant derived foods/feed is an alternative way in order to discriminate between intentional and unintentional effects.

It is obvious that spiking will only contribute meaningfully to the safety assessment of GM foods/feed if the novel gene product possesses a significant toxic or nutritional potential at the typical level of expression in the plant. For gene products with low or no toxicity or nutritional value it would be almost impossible to establish a LOAEL and thereby a spiking level that can be used in the 90-day study. The Bt-toxin is an example of a gene product for which spiking will not be useful.

4.2.6 Criteria for balancing the diet

When incorporating the test food in either a natural-ingredient, human-type or a purified diet, it is not advisable to add the food directly into a diet of standard composition as this will result in dilution or overload of essential nutrient in the diets. Therefore, ingredients from the diet need to be adjusted in order to avoid nutritional imbalance of the diet. When the diet is balanced, potential "noise" arising from the difference in composition of one or several nutrients should be removed, which is a prerequisite for the detection of unintended effects. Information needed to formulate the diet for the experimental animals includes a detailed compositional analysis of the test food (see Chapter 4.2), but also, if available, information about the bioavailability of the nutrients in the GM food and the control counterpart of the same food.

For GM foods with a comparator, it can be discussed how large the differences between the non-GM food (control) and the GM food should be in order to require balancing of the diet. It is recommended that there should be a significant difference observed in the compositional analysis between the control and GM food for the particular nutrient that would lead to a difference in the total diet of at least 5%. It is not recommended to balance out the level of the new gene product(s) expressed in the GM food, but the presence of the gene product(s) in the diet must be taken into account in the evaluation of the study.

Not only the concentration of ingredients in the diets need to be adjusted, but also the energy content of the diet as difference in energy content can cause different food and water intake and further elicit different physiological responses in the animals. If such a difference in energy content exists, adjustment of the control diet should take into consideration the difference in food intake.

4.2.7 Restricted feeding vs. *ad libitum* feeding

Experimental animals are often fed diet *ad libitum* in the feeding studies although it is acknowledged that restricted feeding *per se* in long-term studies has a positive effect on the health of the animals. A chronic 30-40% restriction of energy intake without essential nutrient deficiency reduces the severity and/or onset of most spontaneous degenerative diseases and extends the average and maximal life span of rodents (Keenan *et al.*, 1994), and even a moderate dietary restriction regimen of 70-80% of the maximum unrestricted *ad libitum* food intake level will improve the laboratory animals' long-term health (Keenan, 1996). However, the practice of restricted feeding is quite laborious and has not yet been incorporated in any international study guideline. It is therefore commonly acceptable to still use the *ad libitum* feeding in the safety studies.

When the purpose of the animal feeding study is to evaluate a nutritional or beneficial effect of the GM food the use of pair- feeding is recommended. For example the testing of a GM food with altered levels of

either toxins or beneficial compounds can only be evaluated if the animals in each group are offered the same amount of diet. *Ad libitum* feeding should be used, if an influence of feed intake could be expected by the genetic modification (see Chapter 3.5.2.).

4.2.8 The value of a de-minimis diet

Contrary to a *de minimis* diet which is defined as a diet that just maintains normal growth, development and well-being in the young growing animals, most commercial animal diets used for conventional toxicological studies of defined chemical substances contain a surplus of proteins and essential amino acids, fats and unsaturated fatty acids, vitamins and minerals. Normally, this excess of nutrients does not disturb the outcome of traditional toxicity testing, because there is normally no interference between the mechanisms and endpoints of toxicity for the xenobiotic chemical substance and the mechanisms and endpoints for action of the nutrients.

In cases where a competition or interference between the toxic mechanism of a chemical and the function of a nutrient can be expected, the diet poor in that nutrient may enhance the toxicity of the chemical, whilst a diet rich in that nutrient may actually mask the toxicity of the chemical.

For use in animal studies, a *de minimis* diet must be used in combination with restricted feeding to ensure a similar overall feed intake between the experimental groups. The composition of the *de minimis* diet must be determined case-by-case, as it is dependent on the nutritional needs of the particular animal species, the composition of the GM food to be tested and the endpoints to be included.

To ensure a sufficient sensitivity of animal studies testing GM foods/feed, the use of a specially designed *de minimis* diet should be considered. If for example the effect of phytic acid on the uptake of minerals is to be tested, special attention should be given to the amount of minerals in the diet. The purpose is to end up with a level of minerals that will just fulfil the nutritional needs of the animals and whose bioavailability is known to be affected by the phytic acid content of the diet. It will be almost impossible to show any difference in mineral uptake if the animals are given an excess of minerals.

4.2.9 Preliminary palatability/tolerance studies

To investigate whether the animal “likes” the taste of the test food (palatability) and whether it is tolerated in high amounts a short feeding trial, 14 to 28 days, may be conducted. If the diet with the food is not tolerated by the animals, indicated by lower feed conversion, feed intake and/or body weight, the concentration of the GM food in the diet should be lowered. If there is a taste difference influencing the food intake in a moderate way, the nutrient composition of the control diet can be balanced.

4.2.10 Analyses of the processed diets/quality assurance and storage

It is necessary to have appropriate protocols and procedures according to GLP and other quality assurance systems that cover the whole diet formulation process. Before embarking on the safety study, it is advisable to analyse the concentration of a number of key nutrients in the final feed to ensure that the feed has been properly prepared and mixed. In case of use of outside cultured crops or vegetables and fruits also the presence of contaminants and pesticides should be analyzed.

Batches of feed for the safety study should be kept as cold as possible, depending on the storage time needed. All diets should be sampled and analysed in order to check against nutritional, mycotoxin and pesticide residue specifications.

4.3 Types of diets used for target animal studies

It is noted that, while depending on the GM feed ingredient and class of livestock, the inclusion rate of the test feed can form a major part (20-100%) of the total diet. For example, if conducting a nutritional assessment on herbicide tolerance (HT) and/or insect resistance (Bt) maize silage with ruminant livestock, the test ingredient could form between 60 and 100% of total dietary material, while monogastrics' diets may contain 20-70% of feed ingredients such as maize grain and the co-product soybean meal.

The ILSI publication (2003b) describes in detail recommendations for the production, handling, storage and processing of feed to be evaluated and the sampling and analysis of harvested and processed plant material.

The document also covers the issue of the use of appropriate comparators for target animal studies of GM feed and suggests that a GM feed ingredient should be compared with material from the near isogenic line and a number of non-GM commercial varieties, typically produced in the region where the GM variety is likely to be grown. It suggests for instance that in the case of broiler chickens, between 2 and 4 non-GM varieties are included and that with dairy cows one or more non-GM varieties are included. The reason for including these varieties is to allow comparison to be made within the range of expected endpoints achieved from traditional non-GM varieties.

4.4 Conclusions

- When testing whole foods, it is desirable to obtain the highest concentration possible of the GM food/feed in the animal diet without causing nutritional imbalance. Normal practice is to use a minimum of two test dose levels and negative control and reference (near isogenic) control formulations with which to create nutritionally equivalent balanced diets in a comparative protocol;

- for GM foods/feed the comparator can be found in the parental (isogenic) line and for modified macronutrients as for example starches a comparator is the unmodified form of the macronutrient. For investigating GM foods/feed with enhanced nutritional properties, choices for control diets should be made on a case-by-case basis. In case of GM foods/feed for which no natural comparator does exist, the test diet with the GM food/feed should have the same overall composition regarding macro- and micronutrients as the diet for the control group;

- the purpose of spiking a diet with the compound that is expressed in the GM plant is twofold, namely to test the sensitivity of the test system, i.e. to discriminate between adverse effects possibly induced by the newly expressed compound(s) and those induced through unintentional events as result of the genetic modification. Spiking will only contribute meaningfully to the safety assessment of GM foods/feed if the novel gene product possesses a significant toxic or nutritional potential at the typical level of expression in the plant.

5. DATA COLLECTION, ANALYSIS AND INTERPRETATION IN THE HAZARD CHARACTERISATION PROCEDURE

The aim of this chapter is to explain in general terms how the data and findings from animal studies are derived and evaluated in order to draw conclusions on any potential impacts that might be predicted for human and animal health, safety and nutrition.

5.1 Data generation, collation and quality assurance

The purpose of conducting livestock feeding trials or laboratory animal (toxicity) tests may be summed up as a prospective means for generating data with which to predict safety and nutrition for target food producing animals and for man. Their value depends upon a range of critical determinants in their conduct which includes clear objective(s), study design, dose level selection, sensitivity, statistical validity, protocol, compliance, data analysis and science-based interpretation.

Experimentalists who conduct this work are normally members of multi-disciplinary teams comprising qualified experts who have undergone professional training in fields such as toxicology, animal physiology, animal nutrition, dairy science, animal husbandry, haematology, clinical biochemistry, DNA detection, pathology, statistics, data analysis and risk assessment. Under the requirements of Good Laboratory Practice (GLP) (EC, 2004), studies are normally checked randomly for compliance by Quality Assurance (QA) auditors both during the life phase of the study and *post mortem*. Each specialist contributing to the study normally completes their activity by compiling the raw or individual data by group, by sex, by sampling point into tables with group mean or median values. As much of the individual data acquired during a study is entered directly into a computer from the animal room or laboratory, the tables are normally prepared automatically using commercially available software packages. Statistical analysis is normally undertaken in parallel and a short written summary of the findings prepared by the relevant expert, e.g. a pathology report by the pathologist for inclusion in the report. The draft final report is then built up by collating the individual "results" sections of the report.

In the case of safety tests, such as a 90-day feeding study, they might include, but not be limited to, dietary intake (dosage), clinical signs, body weight, food intake, water intake, food conversion efficiency (FCE), haematology, clinical chemistry, gross macroscopic findings, organ weights, macroscopic findings and histopathology. This compilation is normally undertaken by a qualified professional, in this case a toxicologist who is responsible for the study from start to finish. This role is a formal requirement under GLP and he/she is known as the Study Director.

On completion of the draft final report this is passed to the QA personnel, who are independent from the reporting line of the Study Director and other experimentalists, for final audit. Recognising that a typical 90-day rat feeding study may generate over 100,000 bits of data, this is a major exercise and takes considerable time. It is an interactive process with a QA report being raised on any potential discrepancies or points requiring clarification. This report goes to the Study Director and laboratory concerned for formal response and resolution. The final report of the study cannot be issued until a formal QA Certificate of approval is issued. The final report must also be signed-off as a faithful reflection of the experiment by the Study Director and key experts involved in the study.

It is worth noting that many studies are not conducted in the facilities of the organisations, public or private, sponsoring the work. Instead the investigation testing is often done by third party organisations known as Contract Research Organisations (CROs). Thus a final report of a typical safety test is not based

on one person's views but is based on its multidisciplinary content, the totality of all the analysis and measurements performed by different expert teams, during the course of the experiment. As such most safety reports will have received repeated iterative Peer Review before finalisation.

5.2 Data evaluation and analysis

5.2.1 Framework for data analysis and evaluation

The purpose of this section is to present a very general guidance framework for evaluation and analysis of data from laboratory animal and livestock feeding studies. It is not intended to take the place of the many excellent texts on the subject of toxicology and animal nutrition, nor does it attempt to consider all possible effects and patterns that may be encountered.

The studies may be on the gene product(s) in which case they are likely to involve laboratory animals. If the composition of the new GM plant or derived food or feed is modified substantially or if there are any indications of untoward effects, animal studies should also be conducted on the relevant food or feed matrix (EFSA, 2006a). These experiments, which are described more fully in Chapter 3, may involve both laboratory animal safety (toxicology) studies such as a 90-day subchronic study in rats as well as a livestock feeding study(s) designed to investigate nutritional performance in food producing animals. Both classes of study complement each other. Testing methodologies are basically the same and the same level of data quality is required.

5.2.2 Data presentation

The quality, integrity and completeness of reporting are essential to the proper analysis and evaluation of the submitted studies. There are three important considerations when it comes to the pre-screening of reports for acceptability.

- The adequacy of the experimental design, e.g. it needs to be considered whether the study meets the prescribed regulatory guidance concerning suitable protocols, such as OECD and follow Good Laboratory Practices regulations (EC, 2004);

- the proficiency and adequacy of the study conduct and reporting;

- the effects of modifying factors that may result in inequalities between control and treated animals (Paynter *et al.*, l985). These may result from the location of cages in the racking in animal rooms, more or less light, heat, humidity, exposure to test substance in the air, idiosyncratic disease, circadian rhythms, cycling synchrony etc. The modifying factors which can influence responses can be problematic when their effects are confused with or misinterpreted as toxic or adverse.

The above general points concerning data presentation and acceptability are not intended to be prescriptive. The fundamental question is how well does the study *in toto* identify potential responses, or lack thereof.

5.2.3 Data analysis – common principles

The objective of data analysis is to determine whether any association exists between exposure and outcome. This is the first step in determining whether a meaningful hazard or potential benefit exists following treatment.

Dose-response relationship

The term dose or exposure level refers to a stated dosage concentration, often expressed as mg/kg animal body weight/day, or dietary inclusion level, as parts per million (ppm) equivalent to mg/kg in the diet. Dietary levels may also be shown in terms of percentage inclusion. Dose-response relationship means the correlative association existing between the dose administered and the response (effect) or profile of responses that is obtained. The concept and philosophy referred to by the dose-response relationship is fundamental to the identification, evaluation and interpretation of responses seen in an animal study and their association or otherwise with the experimental treatment. The primary assumption is that the response (effect) observed is a result of exposure to a known substance. Correlative assumptions are that (a) the observed response is a function of the concentration at a site, (b) the concentration at a site is a function of the dose, and (c) response and dose are causally related.

The essential purpose for animal studies is to maximise the opportunity for the detection of valid biological evidence of an effect (response). Dose levels and dietary inclusion levels play a key role as they have the potential to alter or interfere with nutritional equivalence between groups with the potential to cause artefacts. Thus protocols must maximise the sensitivity of the test without significantly altering the accuracy and interpretability of the data obtained (see Chapter 4).

Response in toxicity studies

It is very important for the scientist analysing the data to distinguish between 3 major response types physiological/nutritional, adaptive and toxic.

Physiological or nutritional responses are those which vary within the ‘normal’ day to day limits of living beings. These might include altered blood gas levels associated with exercise or seasonal variations in weight due to small changes in fat storage. Such variations are usually referred to as within the established “normal range” providing such “normal range” data has been documented and can be demonstrated. Generally changes in clinical indices within the “normal range” are not considered to be of toxicological significance and hence adverse unless they exceed these normal limits. They may however be either statistically or biologically significant or both. If such minor alternations are observed they should be checked for any correlation with other toxicity end points which may be present. Equally, a lack of any such correlation helps to reduce possible concern that they may reflect an adverse effect.

Adaptive responses are reversible and of limited duration and may be distinguished from toxic (adverse) effects by generally not causing injury. An increase in pulse rate associated with exercise or an induction of liver enzymes accompanied by a small increase in liver weight, would be considered adaptive, non-toxic events.

Toxic responses may be reversible or irreversible but differ from the above by being injurious to the experimental animal. This may simply reflect frank tissue toxicity at the intentionally exaggerated dose levels that are normally used experimentally.

Threshold dose level and No-observed-adverse-effect level (NOAEL)

One of the benchmarks for extrapolating data to man is to establish the NOAEL in a sensitive animal species. It is a professional opinion based on the design and integrity of the study (Dorato and Engelhardt 2005). This is the highest experimental level at which no adverse effects are seen.

The NOAEL has been criticised for not considering all of the dose-response data generated in a given study. In consequence an alternative to the NOAEL approach is referred to as the *benchmark dose* (BMD) approach. This uses all of the experimental data to fit one or more dose-response curves (Crump, l984). These curves are then used to estimate a benchmark dose that is defined as "the statistical lower bound on a dose corresponding to a specified level of risk" (Allen *et al.*, l994).

The potential advantages of the BMD approach are (1) the ability to take into account the full dose response curve as opposed to the single dose level utilised in the NOAEL approach, (2) the inclusion of a measure of variability (confidence limit, which can be varied), (3) the use of actual responses encountered within the experimental treatment range as opposed to low dose extrapolation, and (4) the use of a consistent BMD response level for Acceptable Daily Intake (ADI) calculations across studies.

Whereas ADI values are typically calculated from NOAEL values by dividing by uncertainty factors (UF) such that ADI = NOAEL/UF, they may also be calculated using the BMD where ADI = BMD/UF. It is helpful to attempt to define the threshold dose, that is the borderline level above which a very weak effect at first occurs and below which an adverse response is not elicited.

A minimum of two dose exposure levels are normally employed, the intention being to maximise the potential to detect any dose-response-relationship in order to facilitate the extrapolation of any potential hazards or benefits, for example nutritional enhancements to man. In consequence the largest dose or exposure should be established normally via dose range-finding and palatability studies, which produces minimal effects that do not compromise the biological interpretability of the observed responses.

In contrast the lowest dose should be planned to be just below the threshold dose described above. Depending on the grade of the findings at the highest dose or exposure level the slope of the dose-response curve may either be very shallow, very steep or intermediate. Establishment of the dose-response is very important in understanding the quantitative nature of the hazard and its potential impact for man and animals. As the dose level increases one expects to find more animals affected with a greater degree of response. Sporadic findings that follow no obvious dose response should be carefully examined but in the absence of a clear dose-related pattern or other correlative findings may be unrelated to treatment. Sporadic changes may be within or outside normal limits the latter for example in the case of an animal that may have naturally developed an intercurrent disease.

Statistical evaluation

The increasing complexity of both the theory and practice of toxicology over the last 25 years has led to increasing options and controversy in the interpretation of study findings. In consequence, as data analysis has become more complicated, the use of appropriate statistics and statistical techniques as additional interpretive tools can be of considerable importance (Gad and Weil, 1994).

Today, statistical analysis is often conducted in parallel with automated data collection using computers. It is essential that any analysis of study results is both planned and interpreted by professionals who clearly understand both the difference between biological and statistical significance and the nature (e.g.

discrete, continuous, ranked, quantal) of different types of data. To this end statistical techniques must take account of the effects of potential or known confounding factors as well as estimating the significance of the responses under investigation.

Statistical methods normally carry out one of three possible activities:

- hypothesis testing – determining if two (or more) groups of data differ from each other at a pre-determined level of confidence;
- model construction – e.g. dose-response prediction using linear regression or correlation testing;
- cluster analysis – used to reduce the number of variables in a system in order to visualise "central tendency".

To maximise the confidence that can be drawn from a statistical analysis, key considerations with continuously distributed data are:

- the probability of committing a Type 1 error, e.g. saying a novel protein affects a liver parameter when in truth it does not; false positive;
- the probability of committing a Type II error, e.g. saying a novel protein does not affect a liver parameter when it does; false negative;
- the desired sensitivity in an assay to detect a given, e.g. 10% increase in a liver parameter, in a test group population;
- the inevitable variability of biological systems and the effects of chance errors;
- the necessary sample or group size to achieve 1 to 4 above.

For quantitative data such as body weights, food and water consumption, the mean values for treated and control groups are calculated. The variation of the individual data about the mean is usually represented by the standard deviation (SD). In the case of physical signs or histopathological data the number of animals affected as a proportion of the total is normally recorded together with the grade of the finding, e.g. not observed, mild, moderate or severe. A written description on an individual animal basis is also prepared by the experimentalist.

There is a need for a more uniform approach in the set-up of animal feeding trials having a comparative design as well as field trials, and analysis of data using appropriate statistical models. The EFSA GMO Panel has recently initiated a self-tasking activity in this area. Univariate data analysis methods will be explored with respect to reliability of conclusions, *i.e.* the probabilities of the occurrence of false positives or false negatives, and an initial assessment will be made of the potential contribution of multivariate methods. An important aspect to be considered is the incorporation of background variability of test parameters due to genetic and environmental causes. The suitability and possible application will be assessed of both the bioequivalence and the difference testing approaches for the safety assessment of GM plants and derived foods/feed.

5.3 Data Interpretation

Data from treated groups are compared with data from the control group(s) to determine if any treatment related effects have taken place.

The process of data interpretation requires extensive professional experience of the field, be it toxicology, allergenicity or animal nutrition, and a thorough understanding of the concept of causality. The stronger the association between an exposure and an outcome, the greater the likelihood that is causal.

To evaluate the results and whether a relationship with treatment is causal or not, a number of criteria are typically employed, which include, but are not limited to:

- dose-related trends or relationships;
- findings in both sexes;
- consistency of findings (within study and with other studies) related to findings in other parameters;
- plausibility in terms of test substance and putative mechanism of action (MOA);
- reversibility on cessation of treatment;
- temporal relationships (could the observed response have occurred in the period of exposure?);
- reproducibility (e.g. at the other sampling occasions during the study or observed in the same or other test species in independent studies);
- intensity or magnitude of findings and presence of intercurrent disease.

In practice, in nearly all animal studies where data from treatment groups are compared with data from the concurrent control group, differences will be seen. Thus the pivotal requirement is to distinguish those effects which are potentially treatment-related from those that can be differentiated as spurious occurrences or result from normal individual biological variation.

Two approaches are followed. The first involves looking over the data by eye, looking at individual values, group means, the magnitude of changes, trends and patterns to detect differences worthy of further consideration. This is based on the experience of the toxicologist, specialist e.g. pathologist, reviewer or Peer Reviewer who also has a sound understanding of historical control data for the age, sex, strain, species, laboratory and dietary background of the animals under test. The second approach utilises interpretation of the statistical findings which highlight differences between treated and control animals, where the probability that the difference(s) occurred by chance, is low. Manual and statistical evaluation of the data should always be used in combination. In terms of hierarchy it is important to depend on manual examination of the data first and foremost. While statistics is an extremely powerful tool, it should not be used alone to detect treatment-related effects, as "statistical" outliers are not always biological outliers and a 'significant' statistical test ($p \leq 0.05$) does not always indicate biological significance, (FDA/FDCA, 1993). Conversely it is possible for an effect to be of potential biological or toxicological significance even if it is not statistically significant.

Following interpretation of the data, it is helpful for the report author to prepare a narrative discussion of the findings. This is a transparent way by which to explain how his/her judgement and rationale have led

to the overall report conclusion(s), that may refer to the NOEL or NOAEL. Explanation for the interpretation of findings as treatment- or dose-related, or as toxicologically or biologically significant, should be included here, as well as reasons for considering results to be borderline, non-adverse, or not toxicologically relevant. In both cases supporting observational and statistical evidence including historical, published, concurrent or laboratory control data should be referred to. Moreover uncertainties inherent to the experimental set up and obtained results should be discussed.

Various considerations are taken into account according to the data undergoing interpretation. While not exhaustive, some of these are listed below for a range of toxicological and nutritional parameters and should also be considered in parallel with the general points discussed for data interpretation.

Mortality

Survival/mortality can be directly related to administration of the substance being tested, but equally can be influenced by many other factors. Every effort must be made to determine the cause of individual deaths and where animals are moribund they should be killed *in extremis* in order to prevent the potential loss of evidence due to *post mortem* autolysis. Signs, behavioural changes, haematology, clinical chemistry, macroscopic necropsy findings, organ weights and histopathology should all be evaluated as far as possible in order to complete the case history of each mortality.

Mortality as an endpoint is a key example of a parameter that requires more than statistical treatment for analysis and interpretation. Each death should be reviewed individually and classified as treatment or non-treatment related.

The separation of non-treatment related mortalities from those considered due to treatment requires meticulous attention to the whole case history, *pre-* and *post mortem* as well as to the patterns of any other deaths, clusters, dose relationships, sex relationship etc. Factors that might help in this distinction also include the presence of intercurrent infection, non-infectious disease, degenerative processes, anatomical abnormalities and trauma. Historical data for the species, animal room and different testing laboratories may also be helpful.

Physical signs

Clinical signs observed during the exposure period, if treatment related, should correlate with other observations such as alteration in weight gain, physiological and toxic effects. Some may not be judged as adverse even though they may be related to treatment, again this depends on the precise situation. Signs are normally categorised, counted, scored for severity (intensity) and tabulated as incidence. Statistical analysis is normally of very limited value for the purpose of interpretation of the parameter.

Body weight, food and water consumption

Body weight change is often a very sensitive indicator of animal well being. It integrates many other parameters and often, in particular, food consumption. A reduction in weight gain compared with control may not be due to an adverse effect *per se,* but due to poor dietary palatability or a nutritionally poorly balanced diet due to incautious incorporation of the test material in the animal feed.

Interpretation of body weight changes can be aided by graphing group values over time, while food and water intake values are generally represented as weekly group mean values ± SD using bar charts.

Statistical analysis of changes compared with control to determine any significant differences is normally performed routinely.

Clinical chemistry, haematology and urine analysis

Careful interpretation of these data can help to provide insights into the nature of treatment-related effects and possible mechanisms of action in the case of adverse effects. However, it must again be borne in mind that stress, restraint, exercise and intercurrent disease as well as normal hormonal changes can each create potential false positive findings. In consequence there is often much "noise" in the findings. The data often presents with scattered, statistically significant effects in the absence of any evidence for correlative clinically significant or other dose related relationships.

"Normal values" generally depend on the precise methods and type of equipment and manufacturer used for the determinations. In consequence concurrent control values from the same laboratory are of prime importance and literature values which do not specify methods used for the generation of data should be used with caution. When findings that appear to be statistically significant appear randomly or sporadically across dose and time in the absence of any other toxicological correlates, the interpreter of the data should explain his/her reasoning for considering the findings unlikely to be related to treatment.

Organ weights and organ body and or brain weight ratios

Organ weight is normally reported in absolute terms and in relation to body or sometimes brain weight, hence the terms, absolute and relative organ weights. The consideration of organ weight in the context of body weight is designed to 'normalise' organ weight in the event of heavier or lighter animals. However, considerable experience is required in the interpretation of such data as some organ weights are largely independent of body weight (and loss or gain) such as the brain whereas others remain dependent.

Other factors, involve variables that can be experimentally controlled and those which cannot. In the former category critical *post mortem* procedures to be controlled include, time, sequence, cross-group randomisation, method of anaesthetisation, exsanguination, speed of dissection, order (receptacle for organs (dehydration or not), organ weighing (trimmed or untrimmed), fixed or unfixed (e.g. pituitary) and having a pathologist on standby. In the latter category there is, *inter alia*, individual animal response to treatment and or disease, non-treatment related variations in body weight, failure of randomisation procedures, absence of potential correlates, absence of or abnormal data due to mortality or *post mortem* autolysis/change.

Ultimately the interpretation of organ weight must not be made solely on the basis of statistical significance compared with concurrent control group values. Correlations between other organ weights, between sexes, dose response, macro- and micro-pathology, body weight and laboratory indices must all be taken into account. Explanations for interpretive comments should be shown in the report as discussed in earlier sections, to help the evaluator to understand the logical steps and hence the justification used by the report author in order to draw his/her conclusions.

Macroscopic and microscopic (histopathological) findings

Histopathology can be decisive in identifying treatment related effects. It can be a relatively black and white or subtle endpoint but is often critical to the establishment of the presence or absence of dose

response relationships. As in human medical diagnosis, the pathologist uses a great deal of expert experience in reading slides and interpreting the observations. The pathologist will normally have all the other data from the study made available before 'reading' the study. The slides may be 'blinded' so that the pathologist is unable to tell which group the sections under review came from. The code is then broken when the examination is complete. Alternatively the pathologist is aware of the treatment group the slides derive from. The pathologist will normally check the macroscopic findings recorded at the *post mortem* examination to establish if there are any particular morphological, colour or other changes to be taken into account when the slides are read, and also that the section(s) presented include any structural abnormality (ies) observed at the *post mortem* examination.

The pathologist's role is to state very clearly and precisely what he/she sees through the microscope without in the first instance forming a diagnosis. In this way each significant lesion type can be discussed with other competent pathologists in terms of its appearance, nature, severity and potential relevance. This is particularly important as the nomenclature for pathological lesions does vary despite valiant attempts at international harmonisation. Confirmation of the possibility of findings being treatment related or of a particular severity or stage is often undertaken by a formal QA controlled process known as pathology Peer Reviewer. Additional individual expert opinions are generated which are then re-discussed with all parties present to obtain a consensus interpretation of the data.

The overall weight-of-evidence for a potential treatment related effect

Recognising that a typical 90-day rat subchronic study might have a minimum of 80 to 120 rats divided evenly between males and females, that many of the parameters are recorded daily on an individual animal basis and that multiple analyses are conducted on blood (some 20 or so parameters on haematology and 20 or 30 also on clinical chemistry), it is easy to see how false positive and false negative findings could occur looking at statistical interpretation or individual findings in isolation. In consequence, the overall interpretation about the potential of a substance to cause adverse effects is stronger if multiple lines of evidence, namely cumulative observational and experimental data, are utilised to come to a conclusion. This is described as a weight-of-evidence approach.

It is emphasised that because protocols and methods are still evolving and the animal study(s) may not be fully conclusive, there is always a level of *uncertainty*. Ancillary data from the literature, *in silico*, *in vitro* and *in vivo* should always be considered where appropriate together with the universal aim to reduce animal studies as far as possible that do not serve a clear experimental purpose. Replicate studies may be utilised if absolutely necessary although variation not only occurs between individual animals, but between animal cohorts, hence individual studies as well.

5.4 Conclusions

- Hypothesis testing – determining if two (or more) groups of data differ from each other at a pre-determined level of confidence;

- data generation for the prediction of safety and nutrition value of GM plant derived foods/feed must be of high quality in order to perform a proper hazard identification and risk assessment. This is normally based upon the use of standardised study designs conducted to the principles of Good Laboratory Practise, incorporating random quality assurance audits of all phases of the study;

- critical determinants of a well designed study include clear objective(s), study design, protocol, dose level selection, sensitivity, statistical validity, compliance, data analysis and science-based interpretation;

- expert data evaluation and analysis are critical for establishing any association between exposure and outcome. This not only involves specialists such as toxicologists, haematologists, clinical biochemists, pathologists, nutritionists etc but also biostatisticians who can help with the detection of trends and toxicological/nutritional significance as opposed to background variation;

- the final phase is the process of data interpretation which requires extensive professional experience of the field be it for toxicology, allergenicity, nutrition, biochemistry or statistics, and a thorough understanding of the concept of dose response and causality. One of the pivotal requirements is to distinguish those effects which are potentially treatment-related from those that can be differentiated as spurious occurrences or the result of normal individual biological variation. In these circumstances it is some times appropriate to look at relevant associated information in order to develop a conclusion based on a weight of evidence.

6. STRATEGIES FOR THE SAFETY AND NUTRITIONAL ASSESSMENT OF GM PLANT DERIVED FOODS/FEED

6.1 Introduction

In this Chapter the various elements of the safety and nutritional assessment procedure for GM plant derived foods/feed are discussed and brought together in a strategic framework to be used for the assessment of these products.

The European Union (European Commission, 2003a), the United Nations Food and Agriculture Organisation (FAO) and World Health Organisation (WHO) (FAO/WHO, 1996; 2000; 2001), the Codex Alimentarius (2003), the Organisation for Economic Cooperation and Development (OECD, 1993, 1997 and 2002d) have established a broadly harmonised safety assessment framework to ensure the safety of foods and feeds derived from GM plants.

The risk assessment of GM plants and derived foods/feed follows a *comparative* approach, *i.e.* the derived foods/feed are compared with their near isogenic counterparts in order to identify differences which subsequently are assessed with respect to their potential impact on the environment, safety for humans and animals, and nutritional quality (this is the Concept of Substantial Equivalence or Comparative Safety Assessment; OECD, 1993; Kok and Kuiper, 2003).

The rationale for the comparison of the GM plant derived foods/feed with non-GM traditional varieties is that traditional varieties, because of their history of use, are generally regarded as safe to eat. Due to the complexity of whole foods, the goal of the assessment is to provide the same level of safety as accepted for traditional foods. The criterion is therefore not *absolute* but *relative* safety.

The risk assessment approach for GM plant derived foods/feed, is a *stepwise* procedure and considers two main categories of hazards, *i.e.* those related to the intended intrinsic properties and function of the introduced trait(s), and those resulting from insertion of the introduced gene(s) into the plant genome that might cause unintended effects (ENTRANSFOOD 2004, EFSA Guidance Document, 2006a). Key elements of the assessment procedure are the molecular, compositional, phenotypic and agronomic analysis in order to identify similarities and differences between the GM plant and its near isogenic counterpart which need further evaluation.

The first category of hazards is taken into account by detailed evaluation and if appropriate, by *in silico*, *in vitro* and *in vivo* safety studies of newly expressed protein(s), newly formed metabolites, and of natural substances whose levels may have been altered as result of gene insertion. This assessment should be done using on a case-by-case basis standardised toxicological methodology designed for assessment of simple chemically defined substances as described in Chapter 2 and in the EFSA Guidance document for the risk assessment of GM plants and derived food and feed (EFSA, 2006a).

The second category of potential risks (*i.e.* the occurrence of unintended effects) may be covered, where appropriate by employing rodent feeding studies on *whole* GM foods/feed. Thus the safety assessment paradigm developed for GM plants derived foods/feed is essentially a combination of the safety evaluation procedure for *single* defined dietary chemical substances, e.g. the introduced trait, and of the *whole* food containing the new trait(s).

Two classes of GM plant derived foods/feed have been considered in this report (see also Chapter 1.4):

- products derived from GM plants, with improved agronomic characteristics. In these plants, in general little or no changes are observed in phenotypic and compositional characteristics;

- products derived from GM plants with enhanced nutritional values and/or health benefits, having in some cases new metabolic pathways not previously present in the parent plant species. A small number of these plants are presently on the market (see Table 1).

Each of these above classes requires a proper general strategy for safety and nutritional testing. While for the first category of GM plant derived foods/feed, assessment of the *safety* is the major objective, for the second category, it is essential to assess not only the *safety* but at the same time to test the *nutritional value* for human consumers or target animal species.

6.2 Molecular, compositional, phenotypic and agronomic analysis

A detailed description of the requirements for molecular data of GM plants and derived foods/feed is given in part C of the Directive 2001/18/EC (EC, 2001) and in the EFSA Guidance Document for risk assessment of GM Plants and derived foods/feed (EFSA, 2006a). Comparative molecular, compositional, phenotypic, agronomic or other analyses (e.g. metabolic pathway considerations) are key elements in the safety assessment and should provide evidence for possible differences between the GM plant and its appropriate near isogenic counterpart. Molecular characterisation covers the characterisation of intentional insertion and expression of new traits and on the occurrence of possible unintentional effects such as gene disruption/silencing/deletion or the occurrence of open reading frames (ORFs) which may have adverse safety impacts.

The choice of the appropriate comparator is important and should include preferably the non-genetically modified isogenic variety used to produce the GM line. Since many plants are produced by back-crossing the most appropriate control lines should include parental lines used during back-crossing. In the case of GM plants containing new molecular events obtained by conventional crossing (hybrids), the genetic backgrounds of the controls should be as close as possible to the GM hybrid plants (EFSA, 2006a).

The performance of field trials, selection of traits and compounds for analysis and the use of statistical models for analysis should follow procedures as described by OECD (OECD, 2001a,b; 2002, a,b,c) and EFSA (ongoing self-tasking activity on statistics of the EFSA GMO Panel). Identified statistically significant differences in parameters measured during the comparative analysis should be evaluated regarding their biological relevance and potential safety and/or nutritional impact. To this end the availability of normal ranges of variation of the measured parameters is essential. Identified consistent differences that fall outside normal ranges of variation may be indicative for the occurrence of unintended effects and need further toxicological and nutritional assessment. Approaches for statistical analysis of data obtained from field trials are further elaborated by the EFSA GMO Panel (see Chapter 5).

It is emphasised that results of the molecular characterisation, the comparative, phenotypic and agronomic analyses should be evaluated *comprehensively* in order to decide on the further steps to be carried out during the risk assessment.

6.3 Safety assessment of the newly expressed protein(s), other new constituents and natural occurring constituents whose levels may have been altered in the GM plant or derived foods/feed

Recommendations for the safety testing and evaluation of newly expressed protein(s), other new constituents and natural occurring constituents whose levels may have been altered are outlined in the Guidance document for the risk assessment of GM plants and derived food and feed (EFSA, 2006a). Framing of the safety/nutritional assessment procedure for GM plant derived foods/feed should first consider what safety aspects need to be investigated and whether initial studies using *in silico* and *in vitro* methods may generate relevant information. This will further focus subsequent *in vivo* studies in laboratory animals, and possibly help to refine, reduce or replace their use.

In case of newly expressed proteins, natural occurrence, physiological function/activity, structural homology with other known toxic/allergenic proteins, degree and type of glycosylation, degradability in the digestive tracts of humans and animals or simulated fluids or systems, history of use etc are important aspects to be considered. For newly expressed non-protein constituents, information on structure/functional relationships with other chemicals and their overall toxicological database, including indications for genotoxic potential, are primary knowledge needs.

Various laboratory animal models are available to evaluate the toxicity of defined single substances (chemicals) and in case of GM plant derived foods/feed, newly expressed proteins and other constituents. Guidelines have been developed by OECD describing detailed protocols for the performance of such studies (see Chapter 3 for further details).

It is emphasised that the above mentioned tests, in essence developed for the safety assessment of *chemicals*, should only be applied for newly expressed constituents in GM plants and derived foods/feed according to need, that is *selectively* and on a *case-by-case* basis, depending on the class, novelty and type of substance, data available on structural relationships and toxicity, occurrence and history of use.

6.4 Safety testing of GM plant derived foods/feed using 90-days rodent feeding trials

Testing of the safety and nutritional value of the *whole* GM plant or derived foods/feed should be considered where the composition of the GM plant is modified substantially, or if there are any indications for the potential occurrence of *unintended* effects as a result of the genetic modification based on the preceding molecular, compositional, phenotypic or agronomic analysis. In such a case, the testing program should include at least a 90-day toxicity study in rodents (EFSA, 2006a).

Rodents are, with certain qualifications, good models for predicting toxic outcomes in humans. The importance of animal model selection, taking into account species differences in toxicity and potential differences in absorption, distribution, metabolism and excretion (ADME) of compounds, is well recognised (FOSIE, 2002).

Based on published findings together with theoretical calculations (see Chapter 3), animal feeding trials in which whole foods/feed are fed to rodents, are generally sensitive and specific to detect toxicologically relevant effects of newly expressed compounds in whole foods/feed, and also relevant unintentional events which may have taken place as result of the genetic modification. Their sensitivity depends on the intrinsic toxic potency of the expressed compound and also the inclusion levels of the whole food in the test diet. The SAFOTEST project has yielded valuable information in this respect showing that biological effects of PHA-E lectin can be traced in a 90-day comparative animal feeding trial (see Chapter 2).

By reviewing the toxicological literature it has been shown that 90-day studies are of sufficient duration for the identification of the principal general toxicological effects of compounds that may also be seen after chronic exposure. Moreover from meticulous studies with irradiated food and other work, it appears in general that long term, chronic toxicity testing of foods/feed does not seem to generate information additional to what is already known from *in silico/in vitro* testing in combination with well performed subchronic testing. However bioassays are by definition at best only predictive and there may be exceptions. In the presence of structural alerts or other relevant information about new food components, the performance of chronic or other appropriate testing should be considered.

By relating the estimated daily intake (EDI) or theoretical maximum daily intake (TMDI) *per capita* for a given whole food (or the sum of its individual commercial constituents) to that consumed on average per rat per day in the subchronic 90-day feeding study, it is possible to establish the margin of exposure (safety margin) for consumers (see Chapter 3). Results obtained from testing GM foods/feed in rodents indicate that large (at least 100-fold) 'safety' margins exist between animal exposure levels without observed adverse effects and estimated human daily intake. Actual safety margins are in fact higher, since in the absence of any obvious treatment-related effects, NOAELs could be higher than the highest doses used in the experiments. These considerations are valid for the generation of GM plant derived foods/feed with improved agronomic characteristics, but still have to be confirmed for the next generation of nutritionally improved GM foods/feed.

The 90-day subchronic toxicity study is a *sentinel* study intended to show whether the considerations that triggered its use are of toxicological relevance or not. The study is a general toxicity study and as such is *not* specifically designed to detect effects on reproduction, development, or other toxicological endpoints for which individual tests have been developed in their own right. However, in the event of toxicologically relevant findings, these should then be followed-up, case-by-case in specific studies or investigative programmes.

A well-designed 90-day rat study may also give an indication of a relevant unintended *nutritional* effect, since such studies are required to start with juvenile animals in rapid growth phase that are sensitive to effects on weight gain. Reduced weight gain may be due either to toxicity or to nutritional influences.

Ninety-day studies in rodents fed a diet containing GM plant derived foods/feed are *not* appropriate to demonstrate food or protein IgE mediated allergenic potential. Specific *in vitro*, bioinformatic and specially designed animal studies should, where needed, be performed to address this issue. However 90-day studies do contain the necessary parameters with which to determine at the first Tier level, potential effects on the immune system, both direct and indirect.

If at high multiples of human daily intake the whole food shows no significant qualitative or quantitative differences to the traditional counterpart, when fed in the 90-day rat study, it is then reasonable to conclude that none of the individual constituents of the whole food is sufficiently toxic to lead to unintended toxicity. The absence of any adverse findings in the treated compared with the control groups is reassurance that any minor variations in compositional analysis which can be seen from plant to plant are of no safety significance to man. Alternatively, if under the same experimental conditions an adverse effect(s) is seen, the study would have fulfilled its sentinel role and warned of a difference requiring further toxicological and analytical investigation. At the same time the absence of adverse findings also points at the presence of nutritional balance.

In the situation where molecular, compositional, phenotypic and agronomic analysis have demonstrated *equivalence* between the GM plant derived foods/feed and their near isogenic counterpart, except for the inserted trait(s), and do not indicate the occurrence of unintended effects, the performance of 90-day

feeding trials with rodents or with target animal species would be considered to add little if anything to the overall safety assessment. This is demonstrated by the results of safety testing in laboratory and livestock animals of foods/feed derived from GM plants modified through the introduction of one or a few genes coding for herbicide tolerance, insect resistance or a combination of these traits. These studies did not show any indication for the occurrence of unintended effects (see Chapter 2).

The use of ninety-days studies in rodents is the most likely route for the detection of relevant unintended effects in foods/feed derived from GM plants which have been more extensively modified in order to coop with environmental stress conditions like drought or high salt conditions, or GM plants with quality or output traits with the purpose to improve human or animal nutrition and/or health. In these plants the internal metabolism may have changed significantly, leading to compositional alterations which to a limited degree may be picked up by compositional analyses of major toxicants and nutrients. The impact of these undetected changes on toxic and nutritional responses is not foreseeable in a conclusive manner, and therefore a 90-day rat feeding study is a useful biological instrument for assuring the wholesomeness of these GM foods/feed. Supplemental information on the possible occurrence of unintended effects may be obtained from comparative growth performance studies conducted with young rapidly growing target species, e.g. broiler chicks lambs, calves, fish and other rapidly growing species.

6.5 Nutritional assessment of GM plant derived feed using target animals

The need for conducting animal feeding studies using target animals in order to evaluate the nutritional characteristics of GM plants, modified for agronomic input traits such as herbicide tolerance and insect resistance, should be carefully assessed. Compositional analysis is the cornerstone for the nutritional assessment, as it is for safety assessment, and consensus documents prepared by the OECD (OECD, 2001a,b; 2002a,b,c) and an ILSI database with compositional data of crops (ILSI, 2003a) provide an excellent guide for the specific analyses needed for this initial part of the nutritional assessment, crop by crop.

Numerous feeding studies with feed derived from GM plants with improved agronomic properties, carried out in a wide range of livestock species, did not show any biologically relevant differences in the parameters tested between control and test animals (see Chapter 2). Thus it can be concluded that in the presence of a satisfactory molecular analysis, once *compositional, phenotypic and agronomic equivalence* has been established, then *nutritional equivalence* may also be assumed and that feeding trials with target animals add little to the nutritional assessment of the feed.

Livestock feeding studies with target animal species should be conducted on a case-by-case basis to establish the nutritional benefits that might be expected from GM plants with claimed nutritional/health benefits. Possible effects of the new feed resource on animal performance, animal health and welfare, efficacy, and acceptability of the new feed ingredient should be investigated, and studies should span either the finishing period to slaughter for chickens, pigs, and beef cattle or a major part of a lactation cycle for dairy cattle.

6.6 Need for long term testing of GM plants derived foods/feed?

The issue of potential long term adverse effects induced by the consumption of GM plants derived foods/feed is an important one, and has been addressed previously by among others the FAO/WHO Expert Consultation on safety aspects of GM foods of plant origin (FAO/WHO 2000). In general very little is known about potential long term effects of *any* foods, and confounding problems, resulting from the wide

genetic variability in the human population, variations in dietary habits, and changes in food compositions over time, have been noted.

The pre-market assessment of safety and nutritional properties based on extensive molecular, compositional, phenotypic, agronomic and other analysis (e.g. metabolic pathway considerations), and on *in silico, in vitro* and *in vivo* testing with newly expressed proteins and metabolites, and if needed with the whole foods/feed, provides sufficient assurance in order to decide whether the new foods/feed is as safe as its conventional counterpart (see Chapter 3).

Rodent feeding studies of 90 days duration appear to be sufficient to pick up adverse effects of diverse compounds that would also be seen after chronic exposure, and therefore in general, chronic toxicity testing of GM foods/feed does not seem to generate additional valuable information to what is already known from earlier analyses (see Chapter 3.4.3). Moreover reproductive and or developmental endpoints are not normally more sensitive, *i.e.* do not normally occur at lower dose levels than those detected in general toxicity studies. In this regard the 90-day study appears satisfactory in this sentinel role. In cases where structural alerts or other information is available about new food components, the performance of specific toxicological testing, e.g. chronic, reproductive etc., should be considered case-by-case, but normally only for the single substance of concern.

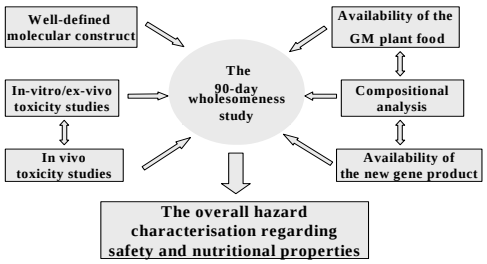
Long-term, livestock feeding studies with target animal species should be conducted on a case-by-case basis to establish either the equivalence in growth performance or the nutritional benefits that might be expected from GM plant derived feed with claimed nutritional/health benefits. Such studies should span either the finishing period to slaughter for chickens, pigs, and beef cattle or a major part of a lactation cycle for dairy cattle. Such data should be factored into the remainder of the safety assessment programme to add to the overall body of evidence concerning the wholesomeness of the new foods/feed.

6.7 SAFOTEST Approach

An example of a design for a 90-day rat feeding study which is specific and of sufficient sensitivity to characterise the safety and nutritional properties of GM foods, is the EU-sponsored SAFOTEST project (see section 2.1.4). The project was focussed on the safety testing of an experimental genetically modified (GM) rice variety expressing the kidney bean *Phaseolus vulgaris* lectin agglutinin E-form (PHA-E lectin).

The SAFOTEST approach is drawing both on preceding knowledge about the parental plant, identity of the genetic change, characteristics of the gene constructs and insertional site(s), data from the initial toxicity studies *in vivo and in vitro* on the new gene product, compositional data of the GM food and on the results from the 90-day feeding study with the GM food with and without the spiked material (see Figure 1).

Figure 1 The SAFOTEST approach with hierarchical use of knowledge



The dose level is selected to be as high as possible without distorting the dietary composition in order to offer the possibility of establishing a relatively high margin of safety for the consumer.

In the groups given GM rice, effects were observed on the small intestine, stomach, pancreas and mesenteric lymph nodes. These effects were consistent with the observed toxicological profile of the PHA-E lectin dosed by itself. Moreover, most of the changes observed were either statistically significant or more prominent in the group fed PHA-E rice spiked with PHA-E lectin, which provides strong evidence that the treatment-related effects were caused by the presence of the gene product and *not* by secondary effects of the genetic modification *per se*. In terms of sensitivity, the study showed that the biologically relevant effects induced by PHA-E lectin with a known LOAEL of approximately 50 mg/kg bw, can be picked up in a 90-day rat feeding study when dosed at a (spiked) level of 0.1%.

6.8 Alternatives for safety and nutritional testing of GM plant derived foods/feed

In vitro methods have clear advantages with respect to savings in terms of time, costs and animal use but equally may suffer a number of limitations including complications in the direct use of complex matrices such as food, metabolic potential and problems of extrapolation to man. *In vitro* methods are best suited to the study of defined substances or extracts of whole foods, rather than whole foods *per se*. During the last two decades significant progress has been made in reducing pain and distress of animals in regulatory testing and some *in vitro* tests have been developed with ring testing for validation and have been accepted by regulatory authorities, without compromising the extent of safety assurance for defined chemicals and finished products such as cosmetics.

So far only few *in vitro* tests have gained regulatory acceptance, the exceptions being tests for skin and eye irritation, sensitisation, phototoxicity, allergenicity markers and genotoxicity. Of these, allergenicity and genotoxicity tests are potentially relevant for new substances expressed in GM foods. Thus, in general, *in vitro* tests should be considered as complementary to current *in vivo* testing methods and as early warning systems which provide a quick and inexpensive way for assessing potential toxicity.

As detailed in Chapters 2 and 3 and above, a number of *in vitro* and *in silico* tests can be applied on a routine basis during the initial phase of the safety and nutritional assessment of GM plant derived foods/feed or ingredients. Among others, structure-activity relationship studies, structural homology searches for known toxins and allergens, biodegradation studies under simulated gastro-intestinal conditions, and application of the new genomic technologies can yield important information that will further guide the risk assessment and may possibly reduce the requirement for animal studies. It is recognised that a number of these tests lack validation as well as uniform application, which should be pursued with priority.

No progress has so far been made in reducing or replacing the use of animals in repeated dose studies, such as 28-day or 90-day studies, with the important exception for GM plant derived foods/feed stated above, that repeated dose studies in the tiered approach should normally only be undertaken when triggered by likelihood of unintended effects.

Regarding the analytical detection of unintended effects, profiling technologies such as transcriptomics, proteomics and metabolomics are promising tools, which will broaden the spectrum of detectable compounds and supplement current targeted analytical approaches. These technologies are still under development, and need validation before they can be used for routine safety assessment purposes (see Chapter 2).

6.9 Uncertainty analysis

Uncertainty analysis is an essential part of the risk assessment process in order to arrive at final conclusions on the safety and nutritional value of the GM foods/feed (see EFSA GM Plant Guidance Document, chapter IV; EFSA, 2006a).

The risk assessment involves generating, collecting and assessing information on a GMO and its derived foods/feed in order to determine its impact on human/animal health and the environment relative to current equivalents, and thus its *relative* safety. In order to carry out the risk assessment sufficient available scientific data must be available in order to arrive at qualitative/quantitative risk estimates. It should explain clearly what assumptions have been made during the risk assessment, and what is the nature and magnitude of *uncertainties* associated with establishing these risks.

Uncertainties should be highlighted and quantified as much as possible. Distinction should be made between uncertainties that reflect natural variations in biological parameters (including variations in susceptibility in populations), and possible differences in responses between species.

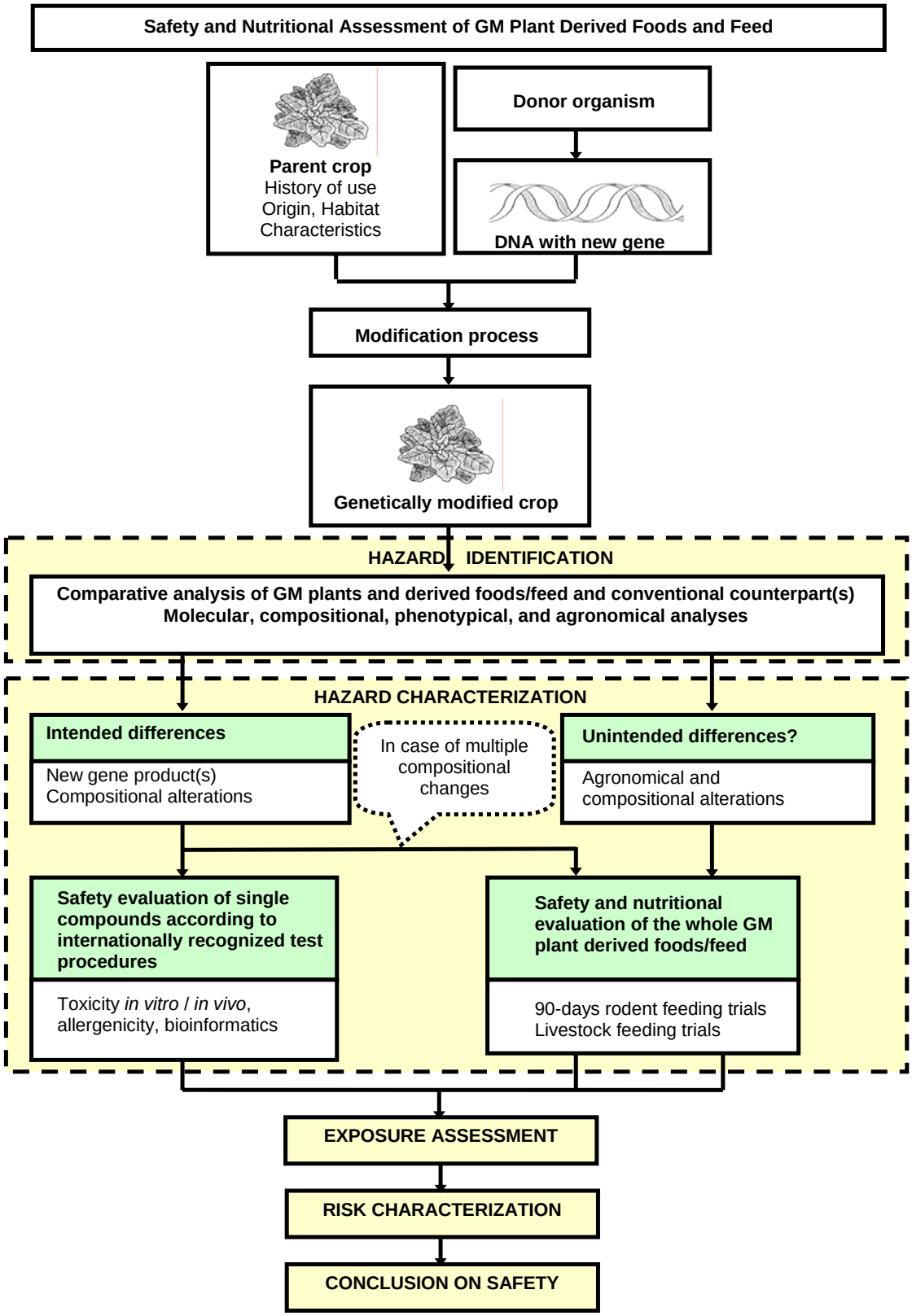
Estimation of uncertainties in experimental data should be handled by proper statistical analysis, while quantification of uncertainties in assumptions (e.g. extrapolation of data from *in vitro* studies to humans, from animals to humans, extrapolation from environmental laboratory studies to complex ecosystems) may be more difficult. Furthermore absence of data essential for the risk assessment should be indicated and it should be made clear how this has been taken into account.

Normally a conservative approach is taken by scientists in any risk assessment by application of relatively large uncertainty or extrapolation factors. Inevitably there are uncertainties, divergences of view, unknowns and gaps in knowledge. Nevertheless by recognising that uncertainty and hypothesis testing is intrinsic to data evaluation, a peer review by experts employing scientific judgement, can be considered as a form of "scientific quality control".

In cases where scientific information is insufficient, inconclusive, or uncertain, or where there are indications that the possible adverse effects on the environment, or animal, human or plant health may be potentially dangerous and inconsistent with the chosen level of protection, the precautionary approach may be invoked (EC, 2000b). Application of the precautionary approach is the responsibility of the risk manager.

6.10 Strategic scheme for pre-market safety and nutritional testing of GM Plant derived Foods/Feed

The generation of the studies for the pre-market assessment of the safety and nutritional properties of foods/feed from GM plants should follow a structured approach with stepwise development and consideration of the obtained data at each step in order to formulate the precise questions to be asked and answered at the next step. As discussed in Chapter 5 each study to be performed should have its clear objective(s), subsequent study design, protocol, dose level selection, sensitivity, statistical validity, compliance, data analysis and science based interpretation. The strategic scheme given below illustrates the type of the questions asked in the course of the process. It is essential to identify the hazard(s) of the new gene product(s) before embarking on safety and nutritional evaluation of the whole GM plant derived foods/feed.


Safety and Nutritional Assessment of GM Plant Derived Foods and Feed
Parent crop
History of use
Origin, Habitat
Characteristics
Donor organism
DNA with new gene
Modification process
Genetically modified crop
HAZARD IDENTIFICATION
Comparative analysis of GM plants and derived foods/feed and conventional counterpart(s)
Molecular, compositional, phenotypical, and agronomical analyses
HAZARD CHARACTERIZATION
Intended differences
New gene product(s)
Compositional alterations
In case of multiple compositional changes
Unintended differences?
Agronomical and compositional alterations
Safety evaluation of single compounds according to internationally recognized test procedures
Toxicity in vitro / in vivo, allergenicity, bioinformatics
Safety and nutritional evaluation of the whole GM plant derived foods/feed
90-days rodent feeding trials
Livestock feeding trials
EXPOSURE ASSESSMENT
RISK CHARACTERIZATION
CONCLUSION ON SAFETY

6.11 Post-market monitoring

Post-marketing monitoring (PMM) could follow the pre-market risk assessment of GM plant derived foods where appropriate, but is a separate process, with limited practical use. As stated in the EFSA Guidance Document on the risk assessment of GM plants and derived foods/feed (EFSA, 2006a), a PMM should seek to address questions like (i) is the use of the product as expected/recommended, (ii) are known effects and side-effects as predicted, and (iii) does the product induce unexpected side effects. However the difficulties associated with the methodologies required, using heterogeneous populations to detect findings in the presence a very low signal to noise ratios, should not be underestimated.

Prospective nutritional monitoring utilising large, market-research food consumption databases combined with sufficiently comprehensive food composition data, could be capable of describing patterns of novel ingredient or food exposure at household level. It could also be used to monitor temporal changes in consumption. However, both ethical and information technology constraints suggest that linkage to health data is not feasible (Chapter 3).

Knowledge gained through PMM might therefore at best describe only broad patterns of human nutritional exposure. It does may not always have the sensitivity to estimate individual intakes, or intakes of particular age groups. It should not be considered a feature of the risk assessment but a later step which may additionally inform risk management. Thus in general it cannot be relied upon as a technique for monitoring adverse events or other health outcomes related to the consumption of GM plant derived foods. Specifc hypothesis driven studies may be required to relate adverse events to the consumption of these foods.

7. CONCLUSIONS AND RECOMMENDATIONS

The comparative approach to safety and nutritional testing of foods/feed derived from GM plants

1. The risk assessment of GM plants and derived foods/feed follows a comparative approach, *i.e.* the derived foods/feed are compared with their non-GM near isogenic counterparts in order to identify differences which subsequently are assessed with respect to their potential impact on the environment, safety for humans and animals, and nutritional quality (Concept of Substantial Equivalence or Comparative Safety Assessment). This approach has been developed and accepted by international organisations like the European Union, the FAO/WHO, Codex Alimentarius and OECD.

2. The comparative risk assessment approach for GM plant derived foods/feed, is a stepwise procedure and considers two main categories of potential safety issues, *i.e.* those related to the intrinsic properties and function of the introduced trait(s), and those resulting from insertion and expression of the introduced gene(s) into the plant genome that might cause unintended effects. Key elements of this assessment procedure are the molecular, compositional, phenotypic, agronomic and other analyses (e.g. metabolic pathway considerations) that identify the similarities and differences between the GM plant and its non-GM near isogenic counterpart needing further evaluation.

   i) The GMO Panel considers that this comparative approach, using molecular, compositional, phenotypic, agronomic and other analyses, remains appropriate as the basis for deciding whether animal feeding studies are needed for the safety and nutritional assessment of GM foods/feed.

Experience from testing of non-GM and GM whole foods

3. Extensive experience with the risk assessment of whole foods has been built up in recent decades from the safety and nutritional testing in animals of irradiated foods, novel foods and fruit and vegetables. Many subchronic feeding studies in rodents have also been conducted over the past 15 years on foods/feed derived from GM plants developed so far. The majority of these experiments did not indicate clinical effects or histopathological abnormalities in organs or tissues of exposed animals. The results obtained from the testing of GM foods/feed in rodents indicate that large safety margins can be established between the levels of animal exposure without adverse effects and the estimated human daily intakes.

4. Numerous livestock feeding studies have also been performed in food-producing animals with feed derived from GM plants, modified for agronomic input traits. Results indicate that animals fed with feed derived from GM plants do not differ with respect to uptake of nutrients, health and performance, hatchability, milk yield, milk quality, etc., compared to animals fed with conventional comparable feed.

In silico and *in vitro* tools available for safety and nutritional testing of GM plant derived foods/feed

5. The scientific tools available for studies on the safety and nutritional aspects of GM foods/feed include *in silico, in vitro* and *in vivo* methods. However, few *in vitro* tests have so far met the necessary criteria of validation and reproducibility required to gain regulatory acceptance, and little progress has been made in reducing or replacing the use of animals in repeated dose studies, such as 28-day or 90-day studies. At present, *in vitro* tests should be considered as complementary to current *in vivo* testing

methods and as early warning systems which may provide a quick and inexpensive way for gaining additional insights into potential toxicity endpoints.

6. A number of *in silico* and *in vitro* tests can be applied during the initial phase of the safety assessment, in particular to characterise the properties of newly expressed proteins and/or metabolites in GM plant derived foods/feed. Among them are structure-activity relationship studies, structural homology searches for known toxins and allergens, and biodegradation studies under simulated gastro-intestinal conditions. Results of these studies will further guide the risk assessment and possibly refine, reduce or replace the need for animal studies.

ii) It is recommended that any programme for the risk assessment of GM foods/feed should first consider what safety and nutritional aspects need to be investigated and whether initial studies using in silico and in vitro approaches may answer some of the safety questions and enable subsequent in vivo studies, and hence the use of animals, to be better focused and possibly reduced.

iii) More efforts should be invested in the development of in vitro tests suitable for safety and nutritional evaluation of whole (GM) foods/feed and derived ingredients.

Testing of defined single substances from GM plant derived foods/feed in *in vivo* studies

7. A comprehensive range of *in vivo* laboratory animal tests are available to evaluate the toxicity of ***defined single substances***, in cases where such substances present in GM foods/feed need to be tested (EFSA, 2006a). Methods for such studies are described in OECD Test Guidelines or in European Commission Directives concerning the testing of chemicals. Guidelines are available for a range of repeat-dose toxicity tests, reproductive and developmental toxicity tests, while models for allergenicity testing are in development.

8. These methods may be applied, on a case-by-case basis, in order to characterise the safety of newly expressed proteins and metabolites in GM plant derived foods/feed. Single dose acute toxicity testing contributes very little to the risk assessment of dietary exposure to substances present in foods. Subchronic toxicity studies in general reveal most major toxic effects of defined substances and are often sufficient in themselves to allow safety assessment to proceed to a conclusion.

9. In some instances, effects on particular tissues or target organs may need to be investigated further in specially designed studies, like reproductive and developmental toxicity testing, immunotoxicity testing and/or allergenicity testing. Long-term studies, extending over most of the lifetime of the test species, can be used, if needed, to assess the potential of defined single substances for chronic toxicity and/or carcinogenicity.

iv) It is recommended that, where needed, laboratory animal feeding studies on defined single substances should follow OECD Test Guidelines and should be carried out according to the principles of Good Laboratory Practice (GLP).

v) Further development and validation of test models, including animal models, for the detection and evaluation of allergenicity of proteins expressed in GM plant derived foods (and of the whole modified food), is recommended, since so far no validated animal tests to detect potential allergenicity of foods for humans are available.

Testing of whole GM plant derived foods/feed in animal feeding studies

10. In cases where testing of the safety and nutritional value of ***whole*** GM plant derived foods/feed is indicated, either because the composition of the GM plant is modified substantially, or there are indications for the potential occurrence of unintended effects, the testing program should include at least a subchronic, 90-day toxicity test in rodents (EFSA, 2006a).

vi) A subchronic, 90-day rodent feeding study on whole GM plant derived foods/feed is considered to have sufficient specificity, sensitivity and predictivity to act as a sentinel study in order to detect in a comparative manner potential adverse effects that may be due to the expression of new substances, alterations in levels of natural compounds or unintentional effects.

11. The current generation of GM plants cultivated for commercial purposes, has been modified through the introduction of one or a few genes coding for herbicide tolerance, insect resistance or a combination of these traits. In these plants the genetic insert leads to the production of a gene product, which does not interfere with the overall metabolism of the plant cell, and does not alter the composition of the GM plant except for the introduced trait

*vii) In cases where molecular, compositional, phenotypic, agronomic and other analyses have demonstrated **equivalence** between the GM plant derived foods/feed and their conventional counterpart, except for the inserted trait(s), and results of these analyses do not indicate the occurrence of unintended effects, the performance of animal feeding trials with rodents or with target animal species adds little if anything to the overall safety assessment, and is not recommended.*

12. More extensive genetic modifications of plants are targeted at specific alterations of the plant's metabolism leading to improved responses to environmental stress conditions, like salt or metal tolerance, or drought resistance. Moreover GM plants are under development with quality or output traits with the purpose to improve human or animal nutrition and/or health. In these cases relatively complex genetic modifications are applied, through for instance the insertion of gene cassettes, leading to substantial changes in the metabolism and composition of the GM plants and derived foods/feed.

*viii) In cases where molecular, compositional, phenotypic, agronomic and other analyses have demonstrated **relevant differences** between the GM plant derived foods/feed and their conventional counterpart, apart from the inserted trait(s), or if there are any indications or remaining uncertainties for the potential occurrence of unintended effects, animal feeding studies with rodents should be considered.*

13. Livestock feeding studies with target animal species should be conducted, on a case-by-case basis and be hypothesis driven. The focus should be on the safety of expressed products, on the identification and characterisation of unintended effects, and on the nutritional impact of any intentional, substantial, compositional modifications of the GM plant. In the case of GM plant derived feed with claimed nutritional/health benefits, their purpose is to establish the growth, performance and potential nutritional benefits that might be expected.

ix) Where livestock feeding studies with target animal species are indicated for a GM plant derived feed, possible effects of the new feed resource on animal performance, animal health and welfare, efficacy, and acceptability of the new feed ingredient should be investigated. Studies should span either the finishing period to slaughter for chickens, pigs, and beef cattle, or a major part of a lactation cycle for dairy cattle. Further development of test protocols at an international level for livestock feeding studies is recommended.

14. Ninety-day studies with rodents are normally of sufficient duration for the identification of general toxicological effects of compounds that would also be seen after chronic exposure. In general, long term, chronic toxicity testing of whole GM foods/feed is not expected to generate information additional to what is already known from *in silico/in vitro* testing and from subchronic testing. However, the subchronic, 90-day toxicity study is not designed to detect effects on reproduction or development, other than effects on adult reproductive organ weights and histopathology. Thus, in some cases, testing of the whole food/feed beyond a 90-day toxicity study may be needed.

x) In cases where structural alerts, indications from the subchronic study or other information on whole GM plant derived foods/feed are available that suggest the potential for reproductive, developmental or chronic toxicity, the performance of such testing should be considered.

15. There is a need for a more uniform approach to the design and analysis of animal feeding trials, and in particular for appropriate statistical analysis of data. The process of data interpretation requires extensive professional experience of the field, together with a thorough understanding of the concept of causality. One of the pivotal requirements is to distinguish those effects which are potentially treatment-related from spurious occurrences or result from normal individual biological variation.

xi) The suitability and possible application of bioequivalence and difference testing approaches for the comparative safety assessment of GM plants and derived foods/feed should be further explored. Data should be analysed by appropriate statistical methods, e.g. the use of multivariate rather than univariate data analysis should be considered in cases where several variates are involved.

16. It can be anticipated that in the future the predictive value of a 90-day rodent feeding studies used for the safety assessment of whole foods/feed will be enhanced by the integration of new technologies like transcriptomics, proteomics and metabolomics into the experimental risk assessment approach. Moreover, the use of 'profiling' technologies may also facilitate a non-targeted approach in compositional analysis in order to aid the detection of unintended effects in GM plant derived foods/feed due to the genetic modification.

xii) Further validation of these technologies and experience with their interpretation will be needed and standardisation of experimental procedures etc is recommended, before they can be utilised in routine safety assessment of foods/feed derived from GM plants.

17. Ninety-day studies are not suited for identification of potential allergenicity. An integrated, stepwise approach for the assessment of potential allergenicity of newly expressed proteins has been put forward by the Codex Alimentarius (2003).

18. OECD methods for subchronic, reproductive, developmental and chronic toxicity testing can be adapted for the testing of whole GM plant derived foods/feed.

xiii) It is recommended that OECD should develop supplementary guidelines for safety and nutritional testing of whole foods/feeds (e.g. type of control and test diets, spiking regimes, number of animals per test group, toxicological and nutritional endpoints to be measured).

Importance of a structured approach for development of data for the pre-market safety and nutritional testing of GM plant derived foods/feed

19. Each GM plant is unique and therefore each study necessary for the pre-market assessment of the safety and nutritional properties of the derived foods/feed need to be designed a case-by-case basis using knowledge already available or generated. The strategic scheme in Chapter 6 proposes the sequence of questions to be raised and answered by the appropriate scientific studies discussed in this report.

xiv) The structured approach in testing is important in order to improve the outcome and save resources in the assessment process. In accordance with this, each study to be performed in the overall sequence of studies should be based upon a thorough examination of already generated data, leading to well designed studies with clear objective(s), precise study designs, protocols, dose level selection, sensitivity, statistical validity, data analysis and science based interpretation of the results.

Role of post market monitoring

20. Post market monitoring (PMM) is not a substitute for thorough pre-marketing risk assessment, neither should it be considered as a routine need. It is a later step which may additionally inform risk management. Knowledge gained through PMM might at best describe only broad patterns of human nutritional exposure. It may not always have the sensitivity to estimate individual intakes, or intakes of particular age groups. Thus in general it cannot be relied upon as a technique for monitoring adverse events or other health outcomes related to the consumption of GM plant derived foods. Specifc hypothesis driven studies may be required to relate adverse events to the consumption of these foods.

xv) Models for prospective nutritional monitoring in humans should be further developed, utilising market-research food consumption databases combined with comprehensive food composition data, in order to describe patterns of food/food ingredient exposure at household level. The possibilities for linkages of exposure information to health data should be further explored.

8. GLOSSARY

- Absorption, Distribution, Metabolism, and Excretion (ADME): these are the four basic biological processes that determine how an environmental or food substance is handled by the body's natural physiological processes and defenses. The ADME factors are often referred to collectively as the pharmacokinetic (PK) processes or the toxicokinetic (TK) processes;

- Acceptable Daily Intake (ADI): Estimate of the amount of a substance in food or drinking water, expressed on a body mass basis (usually mg/kg body weight) which can be ingested daily over a lifetime by humans without appreciable health risk;

- *Bacillus thuringiensis* (Bt): soil bacterium used for biological pest control; the bacterium produces a crystalline protein toxic to certain types of insects;

- Balance studies: animal feeding studies that aim at measuring the digestibility and the bioavailability of the product to be assessed;

- Basic Local Alignment Tool (BLAST): a computer program for comparing DNA and protein sequences;

- Benchmark dose (BMD): a standardised reference point derived from animal data by mathematical modelling within the observed range of experimental data. It uses all of the information obtained over the range of doses from the experiment;

- DNA microarray: A microarray composed of nucleic acid molecules of known composition linked to a solid substrate, which can be probed with total messenger RNA from a cell or tissue to reveal changes in gene expression relative to a control sample. This form of microarray technology allows the expression of many thousands of genes to be assessed in a single experiment;

- Enzyme-linked immunosorbent assay (ELISA): an assay in which an enzyme is linked to an antibody and a coloured substrate is used to measure the activity of bound enzyme and, hence, the amount of bound antibody;

- Estimated daily intake (EDI): is estimated taking into account information on food consumption and the nature and amount of the food ingested;

- FASTA: the first widely used algorithm for database similarity searching. The program looks for optimal local alignments by scanning the sequence for small matches called "words";

- Good laboratory Practice (GLP): Fundamental rules incorporated in national regulations concerning the process of effective organization and the conditions under which laboratory studies are properly planned, performed, monitored, recorded and reported;

- *In silico*: data generated and analysed using modelling and information technology approaches;

- *In vitro*: study in the laboratory usually involving isolated organs, tissues, cells or cellular fractions;

- *In vivo*: study performed on a living organism;

- Lowest-observed-effect-level (LOEL): The LOEL corresponds to the lowest administered dose capable of producing a measurable increase in the frequency of biological changes, which may be either pathological (adverse) or non-pathological (adaptive);

- Lowest-observed-adverse-effect level (LOAEL): The LOAEL is the lowest dose of a chemical, in studies on laboratory animals, that produces an observable adverse health effect in the exposed group;

- Margin of exposure (MOE); Margin of safety (MOS): the ratio of the NOAEL (or other measures of toxicological threshold) to the actual level of product exposure experienced by the most highly exposed individuals in the population. MOE is considered a more value-neutral term than MOS, since safety can never by absolutely assured for all exposed individuals;

- Metabolomics: analytical techniques (such as LC-MS, GC-MS, NMR) that generate profiles of the metabolites;

- Near isogenic lines: a group of lines that are genetically identical except at one or a few *loci* (which are the positions occupied by the inserted (transgenic) construct in a chromosome);

- No observed adverse effect level (NOAEL): the highest dose level of a substance administered in a toxicological dose-response study that produces no significant biological effects of a harmful or pathological nature;

- No observed effect level (NOEL): the highest dose level, in a toxicological dose-response study, where no detectable biological effect is found (usually in test animals). Used as an experimental estimate of the threshold dose at which toxic effects begin to appear in the dose-response relationship;

- "Omics" technologies: contrary to targeted analysis, these techniques are indiscriminate in that they do not require prior knowledge of every substance analysed;

- Output traits: traits that increase nutritional value, reduce naturally occurring toxicants, enhance flavor, or yield pharmaceutical products;

- Phenotype: The observable characteristics of an organism;

- Unintended effect: an effect that was not the purpose of the genetic modification;

- Polymerase Chain Reaction (PCR): a method for amplifying a DNA base sequence using a heat-stable polymerase and two primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other end. Because the newly synthesized DNA strands can subsequently serve as additional templates for the same primer sequences, successive rounds of primer annealing, strand elongation, and dissociation produce rapid and highly specific amplification of the desired sequence. PCR also can be used to detect the existence of the defined sequence in a DNA sample;

- Post-market monitoring (PMM): PMM may be an appropriate risk management measure in specific circumstances. It has a role in the validation of estimated exposure assessment and in confirming the pre-market risk assessment;

- Profiling**:** Creation of patterns of the substances within a sample with the aid of analytical techniques, such as functional genomics, proteomics, or metabolomics. The identity of the compounds detectable within the pattern needs not to be previously recognized;
- Proteomics**:** protein profiling using among others 2D-gel electrophoresis and mass spectrometry;
- Radioallergosorbent test (RAST): a solid-phase radioimmunoassay for detecting IgE antibody specific for a particular allergen;
- Sentinel study: a study that would yield alerting signals of potential adverse effects due to consumption of the whole food/feed under investigation;
- Skin-prick test (SPT): an allergy test that involves placing a small amount of suspected allergen to a scratch on the skin;
- Spiking: the novel gene product expressed in the GM plant is added to the control group (which contains the GM or the non-GM plant derived foods/feed) at a certain dose level (for instance, at the level as expressed in the GM plant in the case the control group contains the non-GM plant derived foods/feed to discriminate between intended and intended effects);
- Subchronic studies: an animal study in which the effects produced by the test material, when administered in repeated doses (or continuously in food or drinking water) over a period of about 90 days (less than 10 per cent of the lifespan), are studied;
- Theoretical maximum daily intake (TMDI): is calculated by multiplying the average per capita daily food consumption for each foodstuff or food group by the legal maximum use level of the additive established by Codex standards or by national regulations and by summing up the figures;
- Threshold: dose or exposure concentration of an agent below which a stated effect is not observed or expected to occur;
- Toxicogenomics: “omics” technologies applied to toxicology;
- Transcriptomics: gene expression profiling using RNA detection techniques;
- Wholesomeness: within the evaluation of whole foods, wholesomeness encompass toxic, nutritional, microbiological and environmental effects (Dybing, 2002).

9. ABBREVIATIONS

ADME: Absorption, Distribution, Metabolism, and Excretion
ADI: Acceptable Daily Intake
ARM: Antibiotic resistance marker
ALAT: Alanine-aminotransferase
BLAST: Basic Local Alignment Tool
BMD: Benchmark dose
Bt: *Bacillus thuringiensis*
BW: Body weight
CROs: Contract Research Organisations
DART: Developmental and Reproductive Toxicology database
DHA: Docoshexaenoic acid
EC: European Commission
EFSA: European Food Safety Authority
EPSPS: 5-enolpyruvylshikimate-3-phosphate synthase
ELISA: Enzyme-linked immunosorbent assay
EDI: Estimated daily intake
EPA: Environmental Protection Agency
FAO: United Nations Food and Agriculture Organisation
FCE: Food conversion efficiency
FDA: US Food Drug Administration
GC: Gas chromatography
GLA: γ-linolenic acid
GLP: Good laboratory Practice
GM: Genetically modified
HPLC: High performance liquid chromatography
HPRT: Hypoxanthine-guanine phosphoribosyl transferase
HT: Herbicide tolerance
ILSI: International Life Sciences Institute
IRIS: Integrated Risk Information System database
JECFA: Joint FAO/WHO Expert Committee on Food Additives
LDH: Lactate dehydrogenase
LOEL: Lowest-observed-effect-level
LOAEL: Lowest-observed-adverse-effect level
MOA: Mechanism of action
MOE: Margin of exposure
MOS: Margin of safety
MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium-bromide
NI: Novel ingredient
NOAEL: No-observed-adverse-effect level
NOEL: No-observed-effect level
NTP: United States National Toxicology Program
OECD: Organisation for Economic Co-operation and Development
PAT: Phosphinothricin acetyltransferase
PCR: Polymerase Chain Reaction
PHA-E lectin: Lectin agglutinin E-form
PMM: Post-market monitoring
TK: Thymidine kinase
QA: Quality assurance

RAST: Radioallergosorbent test
RCT: Randomised controlled trials
SCF: Scientific Committee on Food
SD: Standard deviation
SPT: Skin-prick test
TMDI: Theoretical maximum daily intake
XPRT: Xanthineguanine phosphoribosyl transferase
WHO: World Health Organisation

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