Protocols for Food and Feed Safety Assessment of GE crops

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Contents

PREAMBLE

A series of protocols have been developed by the Department of Biotechnology (DBT) as guidance to applicants seeking approval for the environmental release of genetically engineered (GE) plants in India under "Rules for the Manufacture, Use, Import, Export and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells 1989" (Rules, 1989) notified under the Environment (Protection) Act, 1986. Specifically, these protocols address key elements of the safety assessment of foods and/or livestock feeds that may be derived from GE crops. The results of these studies are be submitted to the appropriate regulatory bodies (*i.e*., RCGM and GEAC) as required.

To date, DBT has prepared five protocols. Each of these is based on international best practices, including guidance and peer reviewed publications available from the Codex Alimentarius Commission, the Food and Agriculture Organization, the World Health Organization, the Organization for Economic Cooperation and Development, and the International Life Sciences Institute. These protocols are:

1. Acute Oral Safety Limit Study in Rats or Mice
2. Subchronic Feeding Study in Rodents
3. Protein Thermal Stability
4. Pepsin Digestibility Assay
5. Livestock Feeding Study

Since the genetic material introduced into GE plants may be derived from organisms that have not previously been present in the human diet to any great extent, the corresponding gene products are considered to be novel with respect to human consumption. Therefore, the principal focus of the safety assessment of foods derived from GE plants is on assessing the potential effects of the expression product(s) of the inserted gene(s) as described in "Acute Oral Safety Limit Study in Rats and Mice". For protein products that have a history of significant human dietary exposure, acute safety limit testing is not warranted.

Subchronic whole food feeding studies may be undertaken when: compositional equivalence cannot be established and there is uncertainty over the nutritional and/or health impacts of the difference; if the genetic modification affects multiple metabolic pathways and the potential impact on nutrition is not readily predictable; if the genetic modification results in changes in levels of non-protein metabolites, or the synthesis of new ones; or if other data are insufficient for a complete safety assessment. If feeding studies are warranted, it is recommended that a 90-day feeding study in rodents be performed as the minimum to demonstrate safety (see "Subchronic Feeding Study in Rodents"). All these tests are to evaluate risk/benefit involved with GE crops/foods.

At present, there is no definitive validated biological test involving animals that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, DBT recognises that an integrated, stepwise, case by case approach, should be used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the preponderance of evidence derived from several types of information and data since no single criterion is sufficiently predictive. This includes, but is not limited to, the protocols "Protein Thermal Stability" and "Pepsin Digestibility Assay". DBT is currently developing additional protocols for specific serum screening and amino acid sequence homology comparisons. As scientific knowledge and technology evolves, other methods and tools may be considered in assessing the allergenicity potential of newly expressed proteins as part of the assessment strategy. These include targeted serum screening and the use of animal models.

DBT is committed to reviewing scientific literature and international standards on an annual basis to ensure that the scientific guidance used to support the safety assessment of GE plants and derived foods and feeds keep abreast and up-to-date with internationally accepted best practices.

For additional guidance, applicants should carefully review the “ICMR Guidelines for the Safety Assessment of Foods Derived from Genetically Engineered Plants” which provide a comprehensive summary of the information and data requirements that must be provided to regulatory authorities to demonstrate the human health safety of foods derived from GE plants.

I. ACUTE ORAL SAFETY LIMIT STUDY IN RATS AND MICE

1 INTRODUCTION

Since the genetic material introduced into recombinant DNA plants may be derived from organisms (including microorganisms) that have not previously been present in the human diet to any great extent, the corresponding gene products are considered to be novel with respect to human consumption. Therefore, the principal focus of the toxicological assessment of foods derived from recombinant DNA plants is on assessing the potential toxicity of the protein expression product(s) of the inserted gene(s). For protein products that have a history of significant human dietary exposure, acute toxicity testing is not warranted.

Because proteins exhibiting toxicity generally exert their effect at low dosages and in a short time frame, acute toxicity tests have been considered adequate for evaluating potential toxicity (Jones and Maryanski, 1991; EPA, 2000; NRC, 2000). As indicated by Sjoblad *et al*. (1992), "if toxicity testing of a protein is considered necessary then acute exposure studies in laboratory animals should be sufficient, since – if toxic – proteins are known to act via acute mechanisms." Therefore, when a protein demonstrates no acute oral toxicity in high-dose testing using a standard laboratory mammalian test species, this supports the determination that the protein will be nontoxic to humans and other mammals, and will not present a hazard under any realistic exposure scenario, including long-term exposure.

Among the various routes of exposure to potential toxicants, the oral route is the most relevant for food safety assessment. Oral exposure is usually accomplished by gastric gavage, wherein a tube is inserted through the oral cavity and the esophagus of the test animal and the test substance is injected directly into the stomach. An evaluation of acute toxicity data should include the relationship, if any, between the exposure of animals to the test substance and the incidence and severity of all abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects.

In the assessment of the toxic characteristics of a protein, the determination of oral toxicity is routinely carried out by acute testing. Such studies provide information on the possible health hazards likely to arise from dietary exposure to a novel protein. This method comprises the basic single dose toxicity study that is commonly used for proteins for which low toxicity is expected due to prior knowledge of the source and previous exposure. The duration of post exposure observation is 14 days. Lack of mortality, moribundity or evident toxicity is generally interpreted as a lack of oral toxicity associated with the test substance.

The testing laboratory should consider all available information on the test substance prior to conducting the study. Such information will include the identity and chemical structure of the substance; its physiochemical properties; the results of any other in vitro or in vivo toxicity tests on the substance; toxicological data on structurally related substances; the anticipated use(s) of the substance; and the likely dietary exposure to the substance. This information is

necessary to satisfy all concerned that the test is relevant for the protection of human health, and will help in the selection of an appropriate starting dose.

In experimental studies that involve procedures that could cause clinical symptoms or morbidity in animals, consideration must be given to the selection of the most appropriate endpoint(s). This requires careful consideration of the scientific requirements of the study, the expected and possible adverse effects the research animals may experience (pain, distress, illness, etc.), the most likely time course and progression of those adverse effects, and the earliest most predictive indicators of present or impending adverse effects. The effective use of endpoints requires that properly qualified individuals perform both general and study-specific observations of the research animals at appropriate time points. Optimally, studies are terminated when animals begin to exhibit clinical signs of toxicity if this endpoint is compatible with meeting the research objectives. Such endpoints are preferable to death or morbidity since they minimize pain and distress. Efforts must be made to minimize pain and distress experienced by animals used in research.

Unlike the situation for chemicals, there are no internationally recognized protocols that deal specifically with assessing the potential oral toxicity of proteinaceous substances. In preparing this test protocol, general guidance was drawn from existing practice internationally with respect to acute oral toxicity testing of isolated proteins and other guidance for toxicity testing of chemical substances, including OECD Test Guideline 420 Acute Oral Toxicity – Fixed Dose procedure and the US-EPA OPTTS 870.1100 Health Effects Test Guideline. This test protocol is intended for use in the testing of novel proteins expressed in recombinant DNA plants and foods/products derived from these, and the development of test data that must be submitted to RCGM and/or GEAC as the case may be, for seeking approval under Rules, 1989, of the Environmental Protection Act, 1986.

Following is one of a series of test protocols for use in the testing of novel proteins expressed in recombinant DNA plants and foods derived from these, and the development of test data that must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under *Rules, 1989*, of the *Environmental Protection Act, 1986*.

The source materials used in developing this protocol include the OECD Test Guideline 420 Acute Oral Toxicity – Fixed Dose procedure and the US-EPA OPTTS 870.1100 Health Effects Test Guideline.

2 DEFINITIONS

Relevant definitions to this test protocol are as follows:

2.1 ACUTE ORAL TOXICITY

Refers to those adverse effects occurring following oral administration of a single dose of a substance, or multiple doses given within 24 hours.

2.2 DELAYED DEATH

Means that an animal does not die or appear moribund within 48 hours but dies later during the 14-day observation period.

2.3 DOSE

Is the amount of test substance administered and is expressed as weight of test substance per unit weight of test animal (*e.g.*, mg/kg).

2.4 EVIDENT TOXICITY

Is a general term describing clear signs of toxicity following the administration of test substance, such that at the next highest fixed dose either severe pain and enduring signs of severe distress, moribund status (criteria are presented in the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation), or probable mortality in most animals can be expected.

Clinical signs that may be indicative of toxicity include, but are not limited to: rapid weight loss; diarrhea (if debilitating); progressive dermatitis; rough hair coat; hunched posture; lethargy or persistent recumbency; labored breathing; nasal discharge; jaundice or anemia; neurological signs; bleeding from any orifice; self-induced trauma; any condition interfering with eating or drinking (*e.g.*, difficulty moving); or excessive or prolonged hyperthermia or hypothermia.

2.5 GENETICALLY ENGINEERED (GE) PLANT

A plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant-deoxyribonucleic acid (r-DNA) and direct injection of nucleic acid into cells or organelles.

2.6 LD50

Median lethal oral dose is a statistically derived single dose of a substance that can be expected to cause death in 50 per cent of animals when administered by the oral route. The LD50 value is expressed in terms of weight of test substance per unit weight of test animal (mg/kg).

2.7 LIMIT DOSE

Refers to a dose at an upper limitation on testing (*e.g.*, limit dose of 2000 mg/kg body weight or when this cannot be achieved in the recommended volume for administration, the dose used should be the maximum possible based on the solubility of the protein).

2.8 MORIBUND STATUS

Being in a state of dying or inability to survive, even if treated.

3 PRINCIPLE OF THE TEST

This test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, *i.e.*, having toxicity only above a regulatory limit dose.

Information about the toxicity of a protein can be gained from knowledge about similar tested proteins, taking into consideration the source organism and history of prior use. When prior data suggest the protein has low toxicity, a limit test using a single dose equivalent to at least 10X the estimated dietary exposure of the test protein may be administered to a single group of five male and/or five female animals using the procedures described under section 4 of this guideline.

In situations where data on plant-expressed protein concentration and/or potential consumption are inadequate to predict a realistic estimated dietary exposure for the target protein, a limit dose of 2000 mg/kg should be employed. On a case-by-case basis, a limit dose less than 2000 mg/kg may be justified either on the basis of valid scientific rationale or on the basis of practical considerations, such as limits of solubility of the test protein. Where there is limited solubility of the test material, the highest dose that can be practically administered in the maximum recommended volume is used. If treatment-related mortality, morbidity or clinical symptoms result, then further study may have to be considered for ascertaining the cause of toxicity.

4 DESCRIPTION OF THE METHOD

4.1 SELECTION OF ANIMAL SPECIES

The preferred rodent species include the rat and the mouse. Commonly used laboratory strains of young healthy adult animals, male and female in equal numbers (*e.g.*, five per sex), should be employed. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are 9 weeks old. At the commencement of the study the weight variation of animals used should be minimal and not exceed ±20% of the mean weight of each sex.

4.2 ACCOMMODATION AND HUSBANDRY

The temperature of the experimental animal room should be 22ºC (±3ºC). Relative humidity should be maintained between 50–60%, and in any event should be at least 30% and not greater than 70%, except during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with drinking water supplied *ad libitum*. Animals may be housed individually, or be caged in

small groups of the same sex; for group caging, no more than five animals should be housed per cage.

4.3 PREPARATION OF ANIMALS

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely (*i.e.*, via ear punch) and acclimatized for at least 5 days in their cages prior to the start of the study.

4.4 TEST PROTEIN DOSE PREPARATION

It is recommended that, wherever possible, the use of an aqueous solution/suspension of the test protein be considered first, followed by consideration of a solution/emulsion in oil (*e.g.* corn oil) and then by possible solution in other vehicles. For vehicles other than water the toxic characteristics of the vehicle must be known.

The maximum volume of liquid that can be administered at one time depends on the size of the test animal and must not be exceeded. In rodents, this volume should not normally exceed 10 ml/kg of body weight: however in the case of aqueous solutions 20–25 ml/kg body weight can be considered.

Where the limit dose of 2000 mg/kg body weight cannot be achieved in the recommended volume for administration, the dose used should be the maximum possible based on the solubility of the protein.

Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

4.5 TEST PROTEIN DOSE ADMINISTRATION

The test protein is administered in a single dose by gavage using a stomach tube or a suitable intubation canula.

In the unusual circumstance that a single dose is not possible, the dose may be given in smaller fractions over a maximum period of 12 hours normally and in any case not exceeding 24 hours.

Animals should be fasted prior to dosing (*e.g.* with the rat, food but not water should be withheld overnight; with the mouse, food but not water should be withheld for 3-4 hours). Following the period of fasting, the animals should be weighed and the test substance administered. After the substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice.

Where a dose is administered in fractions over a period of time, it may be necessary to provide the animals with food and water depending on the length of the period.

4.6 OBSERVATIONS

4.6.1 Visual

Animals are observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and euthanized for animal welfare reasons or are found dead. All observations are systematically recorded, with individual records being maintained for each animal.

Additional observations will be necessary if animals display signs of toxicity. Observations should include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behaviour pattern. Attention should be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma. Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be euthanized. When animals are euthanized for humane reasons or found dead, the time of death should be recorded as precisely as possible.

4.6.2 Body Weight and Feed Consumption

Individual weights of animals should be determined shortly before the test substance is administered (Day 0) and on Days 7 and 14. Measurements of feed consumption should be made at least weekly.

4.6.3 Pathology

All test animals (including those that die during the test or are removed from the study for animal welfare reasons) should be subjected to gross necropsy. All gross morphological changes should be recorded for each animal. In the event that gross morphological changes are observed, the relevant tissues should be subject to histopathological examination.

4.7 DATA AND REPORTING

Individual animal data should be provided. Additionally, all data should be summarized in tabular form, showing for each test group the number of animals used, the number of animals displaying signs of toxicity, the number of animals found dead during the test or euthanized for humane reasons, time of death of individual animals, a description and the time course of toxic effects and reversibility, and necropsy findings. When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

The test report must include the following information, as appropriate:

4.7.1 Test protein

Physical state, purity, concentration, source, batch/lot reference number, and storage conditions. When the test protein has been isolated from a source other than the GE plant, a characterization of the test protein and demonstration of equivalence with the plant-expressed form of the protein is required (normally as a separate study and report).

4.7.2 Control substance and vehicle

Identification of the vehicle (*e.g.*, water, 10% aqueous carboxymethyl cellulose, etc), and justification for choice of vehicle substance if other than water.

4.7.3 Test animals

Species and strain used, including: source of animals; number; age and sex (including, where appropriate, a rationale for use of males instead of females); accommodation conditions; and diet.

4.7.4 Test conditions

- Details of test substance formulation, including details of the physical form of the material administered.
- Details of the administration of the test substance including dosing volumes and time of dosing;
- Details of food and water quality (including diet type/source, water source); and
- The rationale for the selection of the starting dose.

4.7.5 Results

- Tabulation of response data and dose level for each animal (*i.e.* animals showing signs of toxicity including mortality, nature, severity and duration of effects);
- Tabulation of body weight and body weight changes;
- Individual weights of animals at the day of dosing, in weekly intervals thereafter, and at time of death or sacrifice;
- Date and time of death if prior to scheduled sacrifice;
- Time course of onset of signs of toxicity and whether these were reversible for each animal; and
- Necropsy findings and histopathological findings for each animal, if available.

4.7.6 Discussion and Interpretation of Results.

The significance and likely impacts of any abnormal findings should be discussed. Where there are statistically significant differences in parameters (*e.g.*, body weight) between test and control groups, these should be discussed in terms of their biological significance and impact on safety. The need, or not, of any additional or follow up studies should be discussed.

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II. SUBCHRONIC FEEDING STUDY IN RODENTS

1 INTRODUCTION

Animal studies are a major element of the safety assessment of many compounds, including pesticides, pharmaceuticals, industrial chemicals, and food additives. In most cases, the test substance is well characterized and of known purity. Human exposure is generally low. It is relatively straightforward to feed such compounds to animals at a range of doses (some several orders of magnitude above expected human exposure levels) in order to identify any potential adverse effects of importance to humans. However, foods are complex mixtures of compounds characterized by wide variations in composition and nutritional value. Due to their bulk and effect on satiety, they can usually be fed to animals only at low multiples of the amounts that might be present in the human diet.

Animal studies cannot, therefore, be readily applied to testing the risks associated with whole foods and detecting any potential adverse effects and relating these conclusively to an individual characteristic of the food can be extremely difficult. Nevertheless, there may be circumstances where chronic or subchronic whole food feeding studies may contribute to an assessment of potential toxicity. Such circumstances could include: when compositional equivalence cannot be established and there is uncertainty over the nutritional and/or health impacts of the difference; if the genetic modification affects multiple metabolic pathways and the potential impact on nutrition is not readily predictable; if the genetic modification results in changes in levels of non-protein metabolites, or the synthesis of new ones; or if other data are insufficient for a complete safety assessment.

If animal feeding studies are warranted, it is recommended that a 90-day feeding study in rodents be performed as the minimum to demonstrate safety. In order to avoid introducing adverse effects not directly related to the material itself, the nutritional value and balance of the diets used must be considered. Therefore, the chosen dose level should be one that does not cause nutritional imbalance and, minimally, should be comparable to anticipated human intake.

The 90-day whole food feeding study is not intended to assess the potential toxicity of the protein expression product(s) of the inserted gene(s) as this is accomplished via the 14-day acute oral toxicity study in rodents.

In experimental studies that involve procedures that could cause clinical symptoms or morbidity in animals, consideration must be given to the selection of the most appropriate endpoint(s). This requires careful consideration of the scientific requirements of the study, the expected and possible adverse effects the research animals may experience (pain, distress, illness, etc.), the most likely time course and progression of those adverse effects, and the earliest most predictive indicators of present or impending adverse effects. The effective use of endpoints requires that properly qualified individuals perform both general and study-specific observations of the research animals at appropriate time points. Optimally, studies are terminated when animals begin to exhibit clinical signs of toxicity if this endpoint is

compatible with meeting the research objectives. Such endpoints are preferable to death or moribundity since they minimize pain and distress. Efforts must be made to minimize pain and distress experienced by animals used in research.

Following is one of a series of test protocols for use in the testing of novel proteins expressed in recombinant DNA plants and foods derived from these, and the development of test data that must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under *Rules, 1989,* of the *Environmental Protection Act, 1986*.

The source materials used in developing this protocol include the OECD Test Guideline 408 Repeated Dose 90-day Oral Toxicity Study in Rodents

2 PURPOSE

For the assessment and evaluation of potential toxicity associated with a whole food derived from a GE plant. The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth well into adulthood. The study will provide information on the major toxic effects, including possible target organs, and the possibility of cumulative effects. These data may also be useful in assessing whether there have been any unintended effects as a result of the genetic modification process.

The need for careful clinical observations of the animals, so as to obtain as much information as possible, is stressed. This study should allow for the assessment of potential to cause neurotoxic, immunological or reproductive organ effects, which may warrant further in-depth investigation.

3 DEFINITIONS

Relevant definitions to this test protocol are as follows:

3.1 DOSE

In the context of this guideline, the dose refers to the level of incorporation of the GE plant material in the test diet, usually expressed as a percentage by weight, or as constant dietary concentrations (ppm). The chosen dose level should be one that does not cause nutritional imbalance while, minimally, being comparable to anticipated human intake.

3.2 TEST MATERIAL

The test material includes those plant parts that are considered edible by humans or farm animals at any time during the life cycle of plant.

3.3 EVIDENT TOXICITY

Is a general term describing clear signs of toxicity following the administration of test substance, such that at the next highest fixed dose either severe pain and enduring signs of severe distress, moribund status (criteria are presented in the OECD Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation), or probable mortality in most animals can be expected.

Clinical signs that may be indicative of toxicity include, but are not limited to: rapid weight loss; diarrhea (if debilitating); progressive dermatitis; rough hair coat; hunched posture; lethargy or persistent recumbency; coughing; labored breathing; nasal discharge; jaundice or anemia; neurological signs; bleeding from any orifice; self-induced trauma; any condition interfering with eating or drinking (e.g., difficulty moving); or excessive or prolonged hyperthermia or hypothermia.

3.4 GENETICALLY ENGINEERED (GE) PLANT

A plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant-deoxyribonucleic acid (r-DNA) and direct injection of nucleic acid into cells or organelles.

4 PRINCIPLE OF THE TEST

The test substance is administered by incorporation of one, or more, dose levels of the recombinant-DNA plant material into the test diet of experimental animals. At least 20 animals (ten female and ten male) should be used at each dose level, with additional group(s) of 20 animals receiving control diet(s). If interim euthanasia of animals is planned, the number should be increased by the number of animals scheduled to be euthanized before the completion of the study.

Test and control diets are administered to respective groups of test animals for a period of 90 days, during which time feed intake and body weights are measured at intervals and animals are observed closely for signs of evident toxicity. Animals which die or are euthanized during the test are subject to necropsy and, at the conclusion of the test, all surviving animals are euthanized and subject to necropsy. Samples of blood and urine are drawn for clinical chemistry analysis at the end of the test period.

5 DESCRIPTION OF THE METHOD

5.1 SELECTION OF ANIMAL SPECIES

The preferred species is the rat, although other rodent species (e.g., the mouse), may be used. Commonly used laboratory strains of young healthy adult animals should be employed. The females should be nulliparous and non-pregnant. Dosing should begin as soon as possible after weaning and, in any case, before the animals are nine weeks old. At the commencement

of the study the weight variation of animals used should be minimal and not exceed ± 20 % of the mean weight of each sex. Where the study is conducted as a preliminary to a longer term chronic toxicity study, animals from the same strain and source should be used in both studies.

5.2 ACCOMMODATION AND HUSBANDRY

The temperature of the experimental animal room should be 22ºC (±3ºC). Relative humidity should be maintained between 50–60%, and in any event should be at least 30% and not greater than 70%, except during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with drinking water supplied *ad libitum*. Animals may be housed individually, or be caged in small groups of the same sex; for group caging, no more than five animals should be housed per cage.

5.3 PREPARATION OF ANIMALS

Healthy young adult animals are randomly assigned to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimized. The animals are identified uniquely (i.e., via ear punch) and acclimatized for at least 5 days in their cages prior to the start of the study.

5.4 TEST SUBSTANCE DOSE PREPARATION

The maximum level(s) of incorporation of the recombinant-DNA plant material in the test diet(s) should be determined such that the test diet still meets established nutritional requirements for the animal species. Control diet(s) should include the same level of incorporation of plant material derived from conventional non-genetically engineered plants and nutritional analysis of test and control plant material should be performed to confirm that levels of key nutrients (e.g., proximates and amino acids) are similar.

5.5 DIET ADMINISTRATION

All test and control diets should be available 7 days per week for at least 13 weeks, *ad libitum*, until the day prior to scheduled necropsy.

5.6 OBSERVATIONS

5.6.1 Visual

General clinical observations should be made at least once a day, preferably at the same time(s) each day, taking into consideration the peak period of anticipated effects after dosing. The clinical condition of the animals should be recorded. At least twice daily, usually at the beginning and end of each day, all animals are inspected for signs of morbidity and mortality.

At least once prior to the first exposure (to allow for within-subject comparisons), and once a week thereafter, detailed clinical observations should be made in all animals. These

observations should be made outside the home cage, preferably in a standard arena and at similar times on each occasion. They should be carefully recorded, preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the observation conditions are minimal. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g., lacrimation, piloerection, pupil size, or unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypes (e.g., excessive grooming, repetitive circling) or unusual behaviour (e.g., self-mutilation, walking backwards) should also be recorded

Animals found in a moribund condition and animals showing severe pain or enduring signs of severe distress should be humanely killed. When animals are killed for humane reasons or found dead, the time of death should be recorded as precisely as possible.

5.6.2 Body Weight and Feed Consumption

All animals should be weighed at least once a week. Measurements of feed consumption should be made at least weekly. Water consumption may also be considered for dietary or gavage studies during which drinking activity may be altered.

5.6.3 Clinical Pathology

Blood and urine samples should be taken at the end of the test period, just prior to or as part of the procedure for euthanizing the animals. Overnight fasting of the animals prior to blood sampling is recommended.[1] The following haematological examinations should be made on blood samples: haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential. Urine analysis should include: appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from each animal just prior to or as part of the procedure for euthanizing the animals (apart from those found moribund and/or intercurrently euthanized). In a similar manner to haematological investigations, interim sampling for clinical biochemical tests may be performed. Determinations in plasma or serum should include sodium, potassium, glucose, total cholesterol, urea, blood urea nitrogen, creatinine, total protein and albumin, and more than two enzymes indicative of hepatocellular effects (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transferase, and sorbitol dehydrogenase).

[1] For a number of measurements in serum and plasma, most notably for glucose, overnight fasting would be preferable. The major reason for this preference is that the increased variability that would inevitably result from non-fasting could mask more subtle effects and make interpretation difficult. On the other hand, however, overnight fasting may interfere with the general metabolism of the animals and, particularly in feeding studies, may disturb the daily exposure to the test substance. If overnight fasting is adopted, clinical biochemical determinations should be performed after the conduct of functional observations of the study.

5.6.4 Gross Necropsy

All animals in the study should be subjected to a full, detailed gross necropsy, which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. The liver, kidneys, adrenals, testes, epididymides, uterus, ovaries, thymus, spleen, brain and heart of all animals (apart from those found moribund and/or intercurrently euthanized) should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying.

The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination: all gross lesions, brain (representative regions including cerebrum, cerebellum and medulla/pons), spinal cord (at three levels: cervical, mid-thoracic and lumbar), pituitary, thyroid, parathyroid, thymus, oesophagus, salivary glands, stomach, small and large intestines (including Peyer's patches), liver, pancreas, kidneys, adrenals, spleen, heart, trachea and lungs (preserved by inflation with fixative and then immersion), aorta, gonads, uterus, accessory sex organs, female mammary gland, prostate, urinary bladder, gall bladder (mouse), lymph nodes (preferably one lymph node covering the route of administration and another one distant from the route of administration to cover systemic effects), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, a section of bone marrow (and/or a fresh bone marrow aspirate), skin and eyes (if changes were observed during gross necropsy). The clinical and other findings may suggest the need to examine additional tissues.

5.6.5 Histopathology

Full histopathology should be carried out on the preserved organs and tissues of all animals in the test diet (where test diets with differing levels of incorporation of recombinant-DNA plant material have been used, this is the diet with the highest level of incorporation) and control groups. These examinations should be extended to animals of all other dosage groups if treatment-related changes are observed in the high dose group. All gross lesions should be examined.

5.7 DATA AND REPORTING

Individual animal data should be provided. Additionally, all data should be summarized in tabular form showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or euthanized for humane reasons and the time of any death or euthanasia, the number showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the number of animals showing lesions, the type of lesions and the percentage of animals displaying each type of lesion. When possible, numerical results should be evaluated by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

The test report must include the following information, as appropriate:

5.7.1 Diets

The identification, source, including breeding history, of recombinant-DNA plant material and control plant material used during the preparation of the test and control diet(s). Compositional analysis of the recombinant-DNA plant material and control plant material, including nutritional and anti-nutritional components.

5.7.2 Test animals

Species and strain used, including: source of animals; number; age and sex (including, where appropriate, a rationale for use of males instead of females); accommodation conditions; and diet.

5.7.3 Test conditions

- Details of test and control diet formulation and feeding schedule;
- Details of food and water quality (including diet type/source, water source); and
- The rationale for the selection of the test substance incorporation rate.

5.7.4 Results

- Body weight and body weight changes;
- Food consumption, and water consumption in cases where the observation is optionally included;
- Toxic response data by sex and dose level, including signs of toxicity;
- Nature, severity and duration of clinical observations;
- Haematological tests with relevant base-line values;
- Clinical biochemistry tests with relevant base-line values;
- Organ weights and organ/body weight ratios;
- Necropsy findings;
- Detailed description of all histopathological findings; and
- Statistical treatment of results, where appropriate.

5.7.5 Discussion and Interpretation of Results.

The significance and likely impacts of any abnormal findings should be discussed. Where there are statistically significant differences in parameters between test and control groups, these should be discussed in terms of their biological significance and impact on safety. The need, or not, of any additional or follow up studies should be discussed.

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III. PROTEIN THERMAL STABILITY

1 INTRODUCTION

Investigations on the thermal (heat) or processing stability of newly expressed proteins in foods derived from recombinant-DNA plants are part of a "weight-of-evidence" approach to assessing potential allergenicity. In addition to exhibiting stability in the peptic and acidic conditions of the digestive system, known protein allergens also tend to be stable to heat and processing, while labile proteins in foods that are eaten cooked or undergo other processing before consumption are of less concern.

Typically, heat stability assays are appropriate for proteins that exhibit a known enzymatic activity or biological activity for which there exist appropriate assay systems. Examples of novel dietary proteins meeting this criterion would include glyphosate resistant forms of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, herbicide detoxification enzymes such as phosphinothricin acetyltransferase (PAT), or various insecticidal proteins (e.g., Cry1Ab, Cry1Ac, etc). Since heat denaturation does not necessarily result in protein degradation, heat stability studies are not generally applicable to structural proteins or proteins without known enzymatic or biological activities that can be tested.

Following is one of a series of test protocols for use in the testing of novel proteins expressed in recombinant DNA plants and foods derived from these, and the development of test data that must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under *Rules, 1989*, of the *Environmental Protection Act, 1986*.

The source materials used in developing this protocol are listed in the literature section. There is currently no standard protocol, although recommendations for some testing parameters are included in the Report of a Joint FAO/WHO Expert Consultation on the Allergenicity of Foods Derived from Biotechnology (2001).

2 PURPOSE

All newly expressed proteins in recombinant-DNA plants that could be present in the final food should be assessed for their potential to cause allergic reactions. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case by case approach, is used in the assessment of possible allergenicity of newly expressed proteins.

The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation and heat stability.

Resistance to heat denaturation has been observed in several food allergens; thus a correlation exists between heat stability and allergenic potential. Therefore, the retention of biological activity after incubation under high temperature conditions may indicate that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic.

The assay of biological activity of a protein depends on the protein and thus cannot be specified in this guideline, however a standard temperature regime should be followed wherever possible to aid the comparison of heat stability properties between different proteins.

3 DEFINITIONS

3.1 ALLERGENIC POTENTIAL

Refers to the potential to induce sensitization and cause food allergy.

3.2 RECOMBINANT-DNA PLANT

Means a plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

4 PRINCIPLE OF THE TEST

Purified protein samples are dissolved in a buffer and incubated at a range of temperatures from 25°C to 95°C for up to 30 minutes. This is followed by rapid cooling of the sample and assays of the biological activity of the protein are performed. Proteins which show less than 10% of the non-treated activity after 30 minutes incubation are considered heat labile at that temperature, although there needs to be consideration of the relevance of the particular temperature to human exposure, for example, whether the food is processed or cooked before consumption.

5 DESCRIPTION OF THE METHOD

5.1 TEST SYSTEM

The protein is prepared as a 1 mg/ml solution in a buffer relevant to that used for the biological assay. Separate samples are incubated at 25, 37, 55, 75 and 95°C for 30 minutes before cooling on ice.

5.2 ASSAY PARAMETERS

The samples are assayed using a method applicable to the protein and compared to control samples of the protein maintained on ice. Where possible, quantitative assays should be

performed to enable determination of a threshold level of 10% of the activity of the untreated sample.

Samples may be analyzed by SDS-PAGE along with molecular weight markers, untreated test protein equivalent to the initial test protein sample and a 10% test protein sample to determine if there is any degradation of the sample, but this should be included in addition to the biological assay, not as a replacement.

5.3 INTERPRETATION OF RESULTS

The stability of the protein at a defined temperature is determined from the biological activity remaining after 30 minute incubation at that temperature.

Proteins with more than 50% biological activity remaining are considered stable at that temperature. Proteins with between 50 and 10% biological activity are considered partially stable and proteins showing less that 10% biological activity are considered labile at the relevant temperature. Determination of the biological relevance of this result should be made in consideration of the processing and cooking procedures commonly followed prior to consumption of the plant product containing the protein.

5.4 DATA AND REPORTING

Assay results for each temperature should be presented, along with control samples held on ice and stored. The variability of the biological assay should be determined prior to the test to determine the number of replicates needed to provide statistical significance.

The test report must include the following information, as appropriate:

5.4.1 Test protein

Physical state, purity, concentration, source, batch/lot reference number, storage conditions, identity of the test protein, and, where relevant, physiochemical properties. When the test protein has been isolated from a source other than the recombinant DNA plant, a characterization of the test protein and demonstration of equivalence with the plant-expressed form of the protein is required (normally as a separate study and report).

5.4.2 Detailed testing protocols

The full experimental procedures should be detailed including the source of all chemicals, reagents, controls and standards.

5.4.3 Results

- All assay results should be included, with raw data for analysis;
- A table of comparison between the different heat treatments should be provided;

- A statistical analysis of the variability of the biological assay to show the level of confidence in the results should also be provided.

6 LITERATURE

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IV. PEPSIN DIGESTIBILITY ASSAY

1 INTRODUCTION

Unlike ingested chemicals, novel dietary proteins have a predictable metabolic fate in the human or animal gut that is similar to the fate of conventional dietary proteins. To test this metabolic prediction for novel proteins, *in vitro* studies with simulated digestive solutions have been widely used. Typically, most food allergens tend to be stable to the peptic and acidic conditions of the digestive system in order to reach and pass through the intestinal mucosa to elicit an allergic response (Metcalfe *et al*., 1996; Taylor *et al*., 1987; Taylor, 1992). Although some researchers (Veiths *et al*., 1999; Kenna and Evans, 2000; Fu, 2002; reviewed by Fu *et al*., 2002) have questioned the validity of digestion stability as a criterion for protein allergenicity assessment, it is one component of a comprehensive weight-of-evidence approach to assessing allergenic potential (Codex, 2003).

The test method for the assessment was first described by Astwood *et al.* (1996). The assay is not meant to predict whether a given protein will always be digested in the stomach of the human consumer, but does provide a simple *in vitro* correlative assay to evaluate protein digestibility. Investigation of proteins that have been tested, suggest a strong positive predictive value that food allergens causing systemic reactions are relatively stable in the assay, while non-allergenic food proteins are typically digested relatively quickly (Bannon *et al*., 2002).

Following is one of a series of test protocols for use in the testing of novel proteins expressed in recombinant DNA plants and foods derived from these, and the development of test data that must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under *Rules, 1989,* of the *Environmental Protection Act, 1986.*

The source materials used in developing this protocol are listed in the literature section. There is currently no standard protocol, although recommendations for some testing parameters are included in the Report of a Joint FAO/WHO Expert Consultation on the Allergenicity of Foods Derived from Biotechnology (2001).

2 PURPOSE

All newly expressed proteins in recombinant-DNA plants that could be present in the final food should be assessed for their potential to cause allergic reactions. At present, there is no definitive test that can be relied upon to predict allergic response in humans to a newly expressed protein, therefore, it is recommended that an integrated, stepwise, case by case approach is used in the assessment of possible allergenicity of newly expressed proteins. This approach takes into account the evidence derived from several types of information and data since no single criterion is sufficiently predictive.

The initial steps in assessing possible allergenicity of any newly expressed proteins are the determination of: the source of the introduced protein; any significant similarity between the amino acid sequence of the protein and that of known allergens; and its structural properties, including but not limited to, its susceptibility to enzymatic degradation and heat stability.

Resistance of a protein to degradation in the presence of pepsin under appropriate conditions indicates that further analysis should be conducted to determine the likelihood of the newly expressed protein being allergenic.

3 DEFINITIONS

3.1 ALLERGENIC POTENTIAL

Refers to the potential to induce sensitization and cause food allergy.

3.2 RECOMBINANT-DNA PLANT

Means a plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant deoxyribonucleic acid (DNA) and direct injection of nucleic acid into cells or organelles.

4 PRINCIPLE OF THE TEST

Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in nine laboratories (Thomas *et al*., 2004). Porcine pepsin is an aspartic endopeptidase with broad substrate specificity. Pepsin is optimally active between pH 1.2 and 2.0, but inactive at pH 3.5 and irreversibly denatured at pH 7.0 (Collins and Fine, 1981; Crevieu-Gabriel *et al*., 1999).

The assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. The original assay described by Astwood *et al*. (1996) recommends performing the digestion at pH 1.2, however, the FAO/WHO (2001) recommends using two pH conditions (pH 1.2 and pH 2.0). The assay is performed at 37°C and samples are removed at specific times and the activity of pepsin is quenched by neutralization with carbonate buffer and sodium dodecyl sulfate (SDS-) polyacrylamide gel electrophoresis (PAGE) loading buffer, then heating to more than 70°C for 3 to 5 minutes. The timed digestion samples are separated by SDS-PAGE and stained with Coomassie or colloidal blue to evaluate the extent of digestion.

A review of the digestibility assay by Bannon *et al.* (2002) and by Thomas *et al.* (2004) indicates that most of the non-allergenic food proteins that have been tested are digested by approximately 30 seconds, while major food allergens are stable, or produce pepsin-stable fragments that are detectable for from eight to 60 minutes.

5 DESCRIPTION OF THE METHOD

5.1 TEST SYSTEM

The test system is an *in vitro* digestion model using porcine pepsin in simulated gastric fluid (SGF) at pH 1.2. SGF preparation and digestion procedures are based on the methods described by Thomas *et al*. (2004). The pepsin activity assay is based on the method described by Sigma for determining the activity of pepsin.

5.2 PREPARATION OF SGF

The SGF reaction buffer is prepared by adding 122.8 mg of NaCl to 59.2 ml of distilled water and adjusting the pH to either pH 1.2 or pH 2.0 using 6 N HCl. The HCl content is approximately 0.084 N, and the NaCl concentration is 35 mM. The amount of pepsin used to prepare SGF is calculated from the specific activity of the product. One unit of activity is defined as a change in $A_{280\ nm}$ of 0.001 at 37°C, measured as trichloracetic acid (TCA)-soluble products using hemoglobin as the substrate. The assay is designed for fixed volumes and a fixed amount of test protein so the amount of pepsin diluted in SGF is adjusted to provide the appropriate ratio of 10 units of pepsin activity per microgram of test protein in the digestion mixture.

5.3 ASSAY PARAMETERS

At predetermined times (*e.g.*,0, 0.5, 1, 2, 5, 10, 20, 30, 60 minutes) a fixed volume of the digestion reaction mixture is withdrawn and added to sample tubes containing neutralization and denaturing reagents, which stop the digestion. Samples are then heated to ~ 95°C before analysis by SDS-PAGE, or storage at -20°C for later analysis. All samples from a single digestion experiment are applied to wells of the same SDS-PAGE gel along with molecular weight markers. Control samples include: test protein in SGF reaction mixture without added pepsin, T=0 min; test protein in SGF reaction mixture without added pepsin, T=60 min; SGF with added pepsin but without test protein, T=0; SGF with added pepsin but without test protein, T=60; and a 10% test protein sample and quenched pepsin without SGF reaction mixture (to verify detectability of at least 10% of the original protein concentration). Samples are separated by electrophoresis, fixed, stained with Coomassie or colloidal blue G-250, destained and analyzed.

Western blot analysis of the digested samples with antibodies specific to the test protein can be used to illustrate specific digestion of the target protein and the presence or absence of lower molecular weight digestion products. Western blot analysis should be included in addition to colloidal blue stained gels and not as a replacement.

5.4 INTERPRETATION OF RESULTS

The stability of the protein is defined as the time required to reach 90% digestion, which is estimated based on the shortest time-digested sample with a band intensity equal to, or less than the 10% undigested standard. Any new bands above approximately 3,000 MW that are

generated as intermediate products of digestion would be noted as stable (or partially stable) intermediate proteolytic fragments and would be analyzed in addition to the test protein. Western blot analysis would identify if any of the intermediate products are derived from the test protein.

Proteins with more than 10% stainable full-length protein band remaining at > 30 to 60 minutes are considered stable. Proteins reduced to < 10% stainable band at 5 to 30 minutes are considered of intermediate stability. Proteins reduced to < 10% stainable band by 2 minutes are considered labile (rapidly digested).

5.5 DATA AND REPORTING

Individual gel images should be presented, along with digital analysis of band intensities for quantitative evaluation of digestion. Control gels should be used to establish the limits of detection of protein amount using the staining and detection methods.

The test report must include the following information, as appropriate:

5.5.1 Test protein

Physical state, purity, concentration, source, batch/lot reference number, storage conditions, identity of the test protein, and, where relevant, physiochemical properties. When the test protein has been isolated from a source other than the recombinant DNA plant, a characterization of the test protein and demonstration of equivalence with the plant-expressed form of the protein is required (normally as a separate study and report).

5.5.2 Detailed testing protocols

The full experimental procedures should be detailed including the source of all chemicals, reagents, controls and standards.

5.5.3 Results

- All gel images should be included;
- Data from digital analysis of band intensities;
- A comparison of digestion time points with amount of protein should be provided.

6 LITERATURE

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V. LIVESTOCK FEEDING STUDY

1 INTRODUCTION

The aim of livestock feeding trials in the safety assessment of a food derived from a recombinant DNA plant is primarily to evaluate the nutritional parameters (*e.g.*, wholesomeness and nutrient bioavailability) of the food and/or feed under relevant circumstances. Generally, livestock feeding trials are not designed, nor are they sufficiently sensitive, to evaluate the potential toxicity of individual proteins or the potential toxicity associated with the whole food. These latter questions are more appropriately addressed through 14-day acute toxicity studies, in the case of individual proteins, or, if warranted, whole food 90-day sub-chronic feeding studies in rodent species.

Compositional analysis is the cornerstone of the nutritional assessment of a food derived from a recombinant DNA plant. Once the compositional equivalence between the GE food and its conventional counterpart has been established, the results of numerous published livestock feeding trials with GE varieties of maize, soybean, canola, cotton, or sugarbeet, also confirmed no significant differences in digestibility of nutrients, animal health or animal performance (Flachowsky *et al.*, 2005). Therefore, once compositional and phenotypic equivalence has been established, nutritional equivalence may be assumed, and livestock feeding trials are conducted only to confirm the same.

The need for conducting livestock feeding trials should be carefully evaluated on a case-by-case basis. Generally, there are two situations in which livestock feeding trials may be of value: (1) if significant compositional differences are observed between the GE food and its comparator, then feeding trials may be used to investigate the biological significance of such differences; and (2) in the case of a GE food with enhanced nutritional characteristics, livestock feeding trials may be used to demonstrate that the expected nutritional benefit is achieved.

Following is one of a series of test protocols for use in the testing of novel proteins expressed in recombinant DNA plants and foods derived from these, and the development of test data that must be submitted to regulatory bodies as the case may be for seeking approval for commercial release of a GE plant under *Rules, 1989*, of the *Environmental Protection Act, 1986*.

2 DEFINITIONS

Relevant definitions to this test protocol are as follows:

2.1 FEED CONVERSION EFFICIENCY

Feed conversion efficiency (FCE) is a composite measure that combines feed intake with growth rate to estimate the effectiveness by which feed is converted to saleable meat product, and is a major determinant of production system efficiency. Mathematically, FCE can be calculated by: kg feed/kg weight gain; or kg feed/litre milk; or kg feed/dozen eggs.

2.2 GENETICALLY ENGINEERED PLANT

A plant in which the genetic material has been changed through *in vitro* nucleic acid techniques, including recombinant-deoxyribonucleic acid (r-DNA) and direct injection of nucleic acid into cells or organelles. In this document, genetically engineered (GE) plant is used synonymously with recombinant-DNA plant.

3 PRINCIPLE OF THE TEST

The GE plant product (*e.g.*, grain, forage, meal etc) is incorporated into livestock feed rations and provided as feed to an appropriate livestock species for a period of time approximating a normal production cycle. Measurements of body weight and feed consumption are taken periodically and at the end of the study, animals may be slaughtered and carcass yield data collected. Control animals receive diet formulated with plant product from the conventional comparator. The experimental design of the study should be sufficient to detect, at $P<0.05$, a 5–10% difference in animal performance.

4 DESCRIPTION OF THE METHOD

As different types of livestock feeding trials may be appropriate, on a case-by-case basis, the intent of this section is only to provide general guidance to be considered in the design of a livestock feeding trial.

4.1 SELECTION OF LIVESTOCK SPECIES

The livestock species chosen should be relevant to the final end-use of the product, and in some cases, multiple studies with different livestock species may be desirable. Ideally, the animal species should be one which: (1) can be used in relatively large numbers to increase the statistical power of the study; (2) can be obtained as a nearly genetically uniform population in order to negate any effects of genetic background; and (3) is sensitive to the effect of small changes in nutritional quality on its growth and performance.

One example of an animal species that meets these requirements is the broiler chicken, which has emerged as a useful animal model for assessing the nutritional value of foods and feeds derived from GE maize or soybean crops. Under typical conditions, a broiler chicken has a

daily consumption of *ca*. 60 g maize kernel/kg body weight, compared to 45 g/kg body weight for a growing pig and *ca*. 0.2 g/kg body weight for an adult human. Hence, the broiler chicken model offers the advantage of significantly higher exposure over nearly the complete life span of the animal. Fast growing species such as the broiler chick increase their body weight approximately 45-fold during the approximately 40 days they take to reach market weight. Because of this rapid weight gain, broilers are particularly sensitive to any change in nutrient supply or the presence of toxic elements in their feed (OECD, 2003). Broilers have advantages over many other species used in commercial production, as they tend to provide a genetically homogeneous population and can be used in relatively large numbers to increase the statistical power of the experiment.

4.2 ACCOMMODATION AND HUSBANDRY

Animals should be kept under the normal conditions of animal husbandry recommended for the species and in common use under typical production practice. Experiments conducted under outdoor conditions (*e.g.*, open-front buildings, pastures, dry lots) should include a daily report of the climatic conditions. Water is a key nutrient and research locations should have their water source tested periodically for microbial contamination and toxicants that could affect animal performance and health.

4.3 PREPARATION OF ANIMALS

Generally, all animals used in a study should be healthy, free of parasites, and have a similar genetic history. Each animal should be individually identified using a method appropriate to the animal species (*e.g.*, ear tag, ear notch, brand, neck tag, etc). All animals should be individually weighed at the start of the study. Grouping of animals into test and control groups should be randomized taking into account sex and initial weight. Animals should be assigned to treatment blocks (or pens) by sex, and the initial body weights (treatment group mean and variance) of the test and control groups should not be statistically significantly different.

4.4 DIET PREPARATION

For preparation of test and control diets, the GE and control plant material should be grown under identical environmental conditions and harvested and processed at the same time, using the same equipment and under the same conditions. Nutrient analysis should be carried out on the harvested plant material, including any processed products, used for diet formulation, and on the final formulated test and control diets. The nutrients to be analyzed are those that are important for meeting the requirements of the recipient livestock or poultry species. Knowing the nutrient content is critical to formulating the final prepared feed as nutrient deficiency or imbalance may result in decreased animal performance. Table 1 lists some crop species and the nutrients to be considered.

Table 1 Recommendations for nutrient analysis

Crops/grain/co-products	Livestock type	Analytes†
Grain: maize, wheat, barley	Non-ruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, ash, starch, lysine, methionine, cystine, threonine, tryptophan, isoleucine arginine, phenylalanine, histidine, leucine, tyrosine, valine
Oilseed meals: soybean, linseed, cottonseed, canola meal, full-fat oilseeds	Non-ruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, ash, fatty acids (full-fat oilseed), lysine, methionine, cystine, threonine, tryptophan, isoleucine, arginine, phenylalanine, histidine, leucine, tyrosine, valine
Grain: maize, wheat, barley	Ruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, starch, ADIN, soluble protein, NPN, degradable protein, NDICP, ADICP
Seeds or oilseed meals: soybean, linseed, cottonseed, canola meal, sunflower	Ruminants	DM, CP, EE, ADF, NDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, ADIN, soluble protein, NPN, degradable protein, NDIN
Silage: maize, grass, legumes	Ruminants	DM, CP, EE, ADF, NDF, ADIN, ADL, DNDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, soluble protein, NPN, degradable protein, NDIN, starch, sugar, pH, organic acids such as lactic, acetic,butyric, isobutyric
Fresh/dry forages: grass, legumes	Ruminants	DM, CP, EE, ADF, NDF, ADIN, ADL, DNDF, Ca, P, Mg, K, S, Na, Cl, Fe, Cu, Mn, Zn, Mo, ash, soluble protein, NPN, degradable protein, NDIN, starch, sugar

† ADF, acid detergent fiber; ADIN, acid detergent insoluble nitrogen; ADL, acid detergent lignin; CP, crude protein; DM, dry matter; DNDF, digestible neutral detergent fiber; EE, crude fat; NDF, neutral detergent fiber; NDIN, neutral detergent insoluble nitrogen, NPN, non-protein nitrogen. Table adapted from ILSI (2003).

In the case of a GE crop with enhanced nutritional characteristics, additional compositional analysis may be warranted. As appropriate for the crop species, the plant material and final formulated diets should also be tested for the presence of pesticide residues, mycotoxins, and antinutrients (*e.g.*, trypsin inhibitors and lectins in soybean; glucosinolates in canola meal; gossypol and cyclopropenoid fatty acids in cottonseed) that could affect animal health and performance.

Test and control diets should be formulated according to published guidelines or minimum accepted nutrient requirements for the animal species. Each diet should contain the same level of incorporation of plant material derived from either GE or control non-genetically engineered plants, and this level should not result in nutritional imbalance. Test and control diets should be normalized with respect to digestible amino acid content, proximates, and total dietary energy. The diets should be processed to a physical form (meal, pellets, crumbles, etc) that is common to local standard practices and the processing should be documented; test and control diets should be fed in the same form. If additives (*e.g.*, growth promoters, antibiotics, enzymes) are included in the diet, this should be documented and the inclusion rate should be the same for both test and control diets.

4.5 EXPERIMENTAL DESIGN AND ALLOTMENT OF ANIMALS

Important factors to be considered when designing any particular livestock feeding study are as explained below. However, it may be noted that any additional factors/parameters may be added on case by case basis:

- **The treatments that should be included.** In general, it is advisable to keep the number of treatments to a minimum – *e.g.*, comparison of plant material from the GE and non-transformed parental line. The inclusion of one or more commercial reference varieties **may** be important in providing a context for the animal performance data. For example, while there may be some statistically significant differences between the test (GE) and control (non-GE) material, these differences may be due to mere chance and may not be biologically relevant. Reference varieties help establish the normal range of variation for the crops species.

- **The experimental unit.** This is the smallest unit to which a treatment is applied and it may be the individual animal or all the animals in a pen if they share a common food source. This is important because it is the variation among experimental units treated alike that gives the unbiased estimate of error used to evaluate treatment effects.

- **Parameters to be measured and evaluated.** These include body weight (initially, and periodically throughout the study) and feed intake (daily or weekly). These data are used to calculate feed conversion efficiency during different phases of the study. Depending on the nature of the study, other parameters could include measurements of egg or milk production and quality.

- **Interpretation of results.** The design and analysis of the study should be kept as simple as possible, avoiding unnecessarily complex, sophisticated statistical techniques. If the design is simple, the statistics are likely to give straightforward results. Non-statistical knowledge must be applied in study design and proper interpretation of the biological significance of the results. Just because two treatments are statistically significantly different does not mean that the difference is large enough to have any biological importance or any practical significance.

4.6 OBSERVATIONS

4.6.1 Visual

The animal facility should be checked twice daily for temperature, lighting conditions, proper functioning of feeders and watering systems, overt clinical signs, injured animals, and mortality. A qualified veterinarian should perform or supervise a diagnostic necropsy on any animals that die during the experiment and cause of death should be recorded.

4.6.2 Body Weight and Feed Consumption

Individual animals, or all the animals in an experimental unit (*e.g.*, a pen if it is a poultry feeding study), should be weighed at several pre-determined times throughout the course of the study, including at the study termination. Measurements of feed consumption should be made on the same schedule as collecting body weight data. Water consumption may also be considered for dietary studies during which drinking activity may be altered. In the event that any animal exhibits morbidity or loses weight or gains little weight during two consecutive measurement periods, it should be removed from the experiment and the reasons

for removal documented. The final growth and performance data should not include any animals removed from the study.

4.6.3 Carcass Measurements

At the termination of the study, carcass data from a number of the animals in each treatment group should be obtained, if possible. In the case of poultry trials for meat production, carcass yield data would normally include weights of dressed carcass, fat pad, drums, thighs, wings, *Pectoralis major*, and *Pectoralis minor*.

4.6.4 Other Measurements

Depending on the nature of the study, other parameters may be measured. For example, in a study on the performance of lactating ruminants, it would be appropriate to record milk yield, fat corrected milk yield, milk composition (*e.g.*, fat, protein, and lactose), body weight, body condition score, somatic cell counts in milk, and general observations on animal health.

4.7 DATA AND REPORTING

Data should be provided at the level of the experimental unit, which could be an individual animal or a pen of animals. Data should be analyzed by an appropriate and generally acceptable statistical method. The statistical methods should be selected during the design of the study.

The test report must include the following information, as appropriate:

4.7.1 Diets

- Identification, source, including breeding history, of GE plant material and control plant material used during the preparation of the test diet(s);
- Compositional analysis of the GE plant material and control plant material including nutritional and anti-nutritional components;
- Composition of the final formulated diets listing the identity and amounts of all feed additives.

4.7.2 Test animals

Species and strain used, including:

- Source of animals;
- Number;
- Age and sex;
- Accommodation conditions;
- Diet.

4.7.3 Test conditions

- Details of test and control diet formulation and feeding schedule;
- Details of food and water quality (including diet type/source, water source); and
- The rationale for the study design including choice of treatments, factors, and statistical analysis.

4.7.4 Results

- Body weight and body weight changes;
- Food consumption, and water consumption;
- Feed conversion efficiency; and
- Carcass measurements as appropriate to the study.

4.7.5 Discussion and Interpretation of Results.

The significance and likely impacts of any abnormal findings should be discussed. Where there are statistically significant differences in parameters between test and control groups, these should be discussed in terms of their biological significance and impact on animal performance and health. The need, or not, of any additional or follow up studies should be discussed.

5 LITERATURE

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3. OECD (2003). Considerations for the safety assessment of animal feedstuffs derived from genetically modified plants. Organization for Economic Cooperation and Development, Paris, ENV/JM/MONO(2003)10.