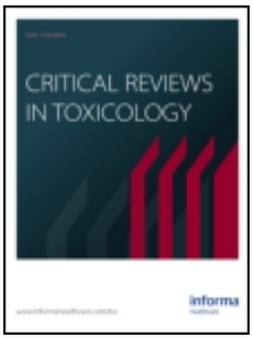

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REVIEW ARTICLE

The use of whole food animal studies in the safety assessment of genetically modified crops: Limitations and recommendations

Andrew Bartholomaeus[1,2], Wayne Parrott[3], Genevieve Bondy[4], and Kate Walker[5] on behalf of the ILSI International Food Biotechnology Committee Task Force on the Use of Mammalian Toxicology Studies in the Safety Assessment of GM Foods*

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Abstract

There is disagreement internationally across major regulatory jurisdictions on the relevance and utility of whole food (WF) toxicity studies on GM crops, with no harmonization of data or regulatory requirements. The scientific value, and therefore animal ethics, of WF studies on GM crops is a matter addressable from the wealth of data available on commercialized GM crops and WF studies on irradiated foods. We reviewed available GM crop WF studies and considered the extent to which they add to the information from agronomic and compositional analyses. No WF toxicity study was identified that convincingly demonstrated toxicological concern or that called into question the adequacy, sufficiency, and reliability of safety assessments based on crop molecular characterization, transgene source, agronomic characteristics, and/or compositional analysis of the GM crop and its near-isogenic line. Predictions of safety based on crop genetics and compositional analyses have provided complete concordance with the results of well-conducted animal testing. However, this concordance is primarily due to the improbability of *de novo* generation of toxic substances in crop plants using genetic engineering practices and due to the weakness of WF toxicity studies in general. Thus, based on the comparative robustness and reliability of compositional and agronomic considerations and on the absence of any scientific basis for a significant potential for *de novo* generation of toxicologically significant compositional alterations as a sole result of transgene insertion, the conclusion of this review is that WF animal toxicity studies are unnecessary and scientifically unjustifiable.

Keywords

Animal-ethics, animal, food, biotechnology, genetically-modified, safety, toxicity, whole-food

History

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Introduction

Commercially cultivated crops have been modified to express traits such as herbicide tolerance or insect/disease resistance. Newer GM crops with nutritional improvements or improved tolerance to environmental stress are being developed by altering endogenous regulatory, metabolic, and signaling pathways. These modifications are accomplished using a variety of techniques, including expression of transferred genes or targeted modifications in the expression of endogenous genes (Delaney et al., 2008b; Hammond & Cockburn, 2008; Parrott et al., 2010). The resulting crops are referred to as genetically modified (GM) crops. GM crops have been grown commercially on over 1 billion hectares cumulatively over the past 15 years, and are now grown in 28 countries (James 2011, 2012).

Foods and feeds derived from GM crops must be shown, prior to commercialization, to be as safe as those derived from conventional crops that have an established history of safe use. This principle was initially referred to as *substantial equivalence,* but is now more typically called *comparative safety assessment* (Codex Alimentarius Commission, 2009; ILSI, 2004). It is based on the concept that it is not possible to demonstrate with certainty that any food is absolutely safe, but acknowledges that many foods have a long history of safe consumption. The comparison of a GM crop to its most closely related conventional counterpart, based on agronomic performance metrics and comprehensive, crop-specific compositional analysis of known nutrients, anti-nutrients, and toxicants for that crop species, is the primary basis of the safety assessment.

In the 1990s when the technology for producing GM crops was relatively new, concern was expressed that the insertion of a transgene into a crop genome or other mutations that occur during the process might produce unintended, unexpected changes that could be potentially hazardous (NNT, 1991). The term *unintended changes* refers to agronomic, phenotypic, and/or compositional changes that may be unintentionally introduced to a crop, in contrast to *intended changes* which refers to agronomic, phenotypic, and/or compositional changes that are intentionally introduced to the crop by genetic manipulation. Unintended changes may be either explicable or inexplicable based on our current knowledge of plant biology, genetics, and/or metabolism (Cellini et al., 2004) and are therefore not necessarily unexpected. Although any unintended, but expected or explicable, effects related to the known biochemistry of the donor and recipient organisms could be readily investigated using analytical chemistry techniques, the argument was made that any unintended effects that were unexpected and/or unrelated to the genome of either donor or recipient organisms might not be detected by these techniques. Consequently, animal studies have also been conducted with the intention of supporting GM crop safety assessment in the belief that such studies could detect the presence of unexpected unknowns of toxicological significance. Against this background, recent information on the natural plasticity of plant genomes and the natural frequency of mutations and transposons in widely cultivated non-GM crops, such as maize, reinforces the implausibility of a simple insertion of a transgene generating *de novo* production of toxic proteins or secondary metabolites unrelated to either the parent crop or the source of the transgene (Weber et al., 2012).

For crops modified to express a specific protein made by a transgene, the purified protein itself has been subjected to toxicology testing using acute and/or short-term repeated-dose rodent studies (Codex Alimentarius Commission, 2009; Delaney et al., 2008b; EFSA, 2008a; Hammond & Cockburn, 2008; Rice et al., 2008). In some cases, the safety of intended changes in specific nutrients or anti-nutrients in a GM crop has been assessed in an animal model (Hammond et al., 2008). Finally, animal studies have been conducted on a whole food or feed derived from a GM crop with the intent of identifying potential adverse effects associated with crop consumption and/or to provide assurance that the GM crop is as safe and nutritious as a conventional comparator. Digestibility, intake, general health and reproductive performance have been assessed in target livestock and poultry where the GM crop was intended for feed. Rodent studies on WF derived from GM crops have been adapted from studies used to identify and characterize the adverse health effects of chemicals. Although they are based on chemical toxicology studies, they are sometimes referred to as ''safety'' studies because they are intended to assess whether GM crops are as safe as their conventional counterparts. Because these studies are generally based on the Organization for Economic Cooperation and Development (OECD) guideline for 90-day toxicity studies (Test Guideline 408), largely employing the same study parameters, and because the European Food Safety Authority (EFSA) guidance on their conduct refers to studies of this type as WF toxicity studies (EFSA, 2011), the term ''toxicity studies'' will be retained in this paper.

Whole food toxicity studies, or a justification for their omission, are explicitly required as a routine component of the data package for regulatory approval of GM food in some jurisdictions (e.g. Europe), but are not required in others (e.g. Australia), while still others do not regulate GM food separately to food developed by other processes (e.g. USA). Table 1 provides a summary of international regulatory guidelines for GM crops and requirements for WF toxicity studies, as of January 2013. Commercialized GM crops were planted in 28 countries in 2012, of which 18 countries grew more than 50 000 hectares (James, 2012). Of these ''mega-producers'', countries with publicly accessible guidelines are included in the table. In many jurisdictions regulatory guidelines do not specify a requirement for WF toxicity studies but are generally sufficiently flexible to allow for such a study to be requested on a case-by-case basis. Although EFSA (2011) has published guidance on how to conduct 90-day WF studies for GM crop safety assessment, it is clear from Table 1 that there is no international consensus on the need for animal studies in support of the safety of GM food or feed or on the circumstances that might trigger a requirement for a WF toxicity study, on the design of such studies, or on how the results might be reliably interpreted.

This lack of consensus reflects differences in regulatory philosophy, founded in sociopolitical as well as scientific considerations, and differences in perceptions of both the

Table 1. Summary of global regulatory requirements for WF studies in support of approval to import/plant GM crops.

Country/region*	Regulatory requirement for WF toxicity studies†	Regulatory guidance (if publically available)‡
North America		
Canada	Not routinely required.	Regulatory guidelines available at http://www.hc-sc.gc.ca/fn-an/gmf-agm/guidelines-lignesdirectrices/index-eng.php
United States	Not routinely required.	Regulatory guidelines are not published online, but the following link provides examples of regulatory decisions taken without requirement for a WF study: http://www.epa.gov/oppbppd1/biopesticides/pips/pip_list.htm
Mexico	Not routinely required.	Regulatory guidelines available at: http://www.hgm.salud.gob.mx/descargas/pdf/dirgral/marco_juridico/reglamentos/regla_22.pdf
Central and South America		
Argentina	Not routinely required/case-by-case.	Regulation of GM crops in Argentina has been summarized by Burachik (2012).
Brazil	Not routinely required.	Regulatory guidelines for biotechnology products available at: http://www.ctnbio.gov.br/index.php/content/view/142.html
Asia/Pacific		
Australia/New Zealand	Not routinely required; however, if a WF study is submitted to the EU it is also provided to Australia/New Zealand.	Regulatory guidelines available at: http://www.foodstandards.gov.au/code/changes/pages/applicationshandbook.aspx
China	Requirement to conduct WF studies in country.	Huang & Yang (2011)
India	Not routinely required.	Guidelines and protocols for the safety assessment of foods derived from available at: http://www.icmr.nic.in/guide/Guidelines%20for%20Genetically%20Engineered%20Plants.pdf; http://igmoris.nic.in/files%5CCoverpage1.pdf
Indonesia	Not routinely required.	The National Biosafety Framework of the Republic of Indonesia is available at http://www.unep.org/biosafety/files/IDNBFrep.pdf
Japan	Not routinely required.	Standards for the Safety Assessment of Genetically Modified Foods (Seed Plants) are available at: http://www.fsc.go.jp/english/standardsforriskassessment/gm_kijun_english.pdf
Philippines	Not routinely required.	http://docs.biotecsur.org/informes/en/inventario/4_normativa_ms.pdf http://www.unep.org/biosafety/files/KRNBFrep.pdf
South Korea	Not routinely required.	The National Biosafety Framework of the Republic of Korea, which includes an example of a safety evaluation of a GM food in Korea, is available at: http://www.unep.org/biosafety/files/KRNBFrep.pdf
Taiwan	Not routinely required.	Specific guidance/regulations not readily available and/or not available in English.
South Africa	Not routinely required.	Specific guidance/regulations not readily available and/or not available in English.
European Union/Europe	WF studies are required for all single trait GM crops; not routinely required for multiple trait (''stacked'') GM crops when single trait crops have already been tested.	For the EU, EFSA (2011) provides specific advice for performing WF studies, available at: http://www.efsa.europa.eu/de/efsajournal/pub/2438.htm The recommendation to perform WF studies is on a case-by-case basis according to OECD Test No. 408 (OECD, 1998) and on EFSA's review of the role of animal trials in GM crop safety assessment (EFSA, 2008a).
Russia	Requirement to conduct WF studies in country.	Specific guidance/regulations not readily available and/or not available in English.

Codex, Codex Alimentarius Commission; EFSA, European Food Safety Authority; FAO, Food and Agriculture Organization of the United Nations; GM, genetically modified; IFBiC, International Food Biotechnology Committee; OECD, Organization for Economic Cooperation and Development; WF, whole food; WHO, World Health Organization.

*Selected countries growing 50 000 hectares or more of GM crops officially approved for planting in 2012 are included (James, 2012). The following countries also grew more than 50 000 hectares of GM crops but are not included because their regulatory documents are either not finalized or are evolving or are not readily accessible: Bolivia, Burkina Faso, Chile, Myanmar, Paraguay, and Uruguay. This does not mean that there is no regulatory process in place for GM crops in these countries. The inclusion of Russia is not based on the number of hectares planted but on knowledge of GM crop registration requirement in this country through the experience of IFBiC Task Force 10 members.

†Regulatory requirements for WF studies are based primarily on the experience of IFBiC Task Force 10 members with GM crop safety assessment in the countries included in Table 1 and secondarily on available regulations and guidelines. For the purpose of this table, regulatory experience is critical because regulations and guidances do not necessarily specify requirements for WF toxicity studies. In countries/regions where WF studies are not routinely required, a range of options may be pursued, including the option to request a WF or other toxicology study if such a study will address a data gap in the safety assessment.

‡Published regulatory documents are not publically available or readily accessed for all countries.

need for safety assessment of GM foods and feeds in general and the scientific value of WF toxicity studies specifically. The USA, for example, regulates food commodities, not the specific technologies used to produce them, whereas Europe has essentially chosen to regulate the technology itself. Similarly, among jurisdictions that do require formal safety assessment of GM food and feed, differences exist between those that have concluded that WF toxicity studies are not scientifically justified, such as Australia, and those that have concluded they provide useful information for safety assessment, such as Europe.

Given the lack of consensus among food regulators with respect to WF toxicity studies, the cost of conducting animal studies, the extensive experience gained from the conduct of large numbers of such studies, the increased concern for experimental animal ethics across most regulatory jurisdictions, and the implausibility of *de novo* generation of toxic substances as a result of defined genetic manipulation, it is timely to consider the ongoing scientific merit of WF toxicity studies of new GM crops and to what extent they are able to inform the safety assessment of GM foods and feeds.

Key issues and sources of data

A considerable number of WF toxicity studies have now been conducted on GM food and feed, which provide sufficient data to consider the nature, extent, and value of the results of those studies and whether they have materially added to the safety assessment of GM crops. Thus it is possible to determine whether WF studies provided outcomes not predicted from a consideration of basic plant genomic science, agronomic, and compositional analysis.

More generally, a large body of research and experience enables a comparison of the relative strengths and limitations of WF toxicity studies and crop compositional analyses to predict risks to human health. A wealth of highly relevant data has also been generated from animal studies of WFs treated by irradiation. The question of the value and utility of WF toxicity studies in the safety assessment of GM foods can therefore be couched in terms of a hypothesis testable against the body of data available both for GM foods and for that from irradiated food. In essence, the perceived requirement for WF toxicity studies can be re-expressed as a related series of hypotheses: (1) commercial production of GM crops can and does produce unintended, unexpected, and unpredictable compositional effects unrelated to both the parent line of the crop and to the inserted transgene; (2) these unintended, unexpected, compositional effects resulting from the production of GM crop varieties are potentially of toxicological significance; (3) compositional analysis of new GM varieties is insufficiently sensitive to reliably detect these differences at levels of toxicological significance; and (4) WF toxicity studies are capable of detecting toxicologically significant differences that would be missed by agronomic and compositional analysis.

Thus, in exploring the value of WF toxicity studies, when they are justified, and how to conduct them if they are justified, the range of data sources and considerations that are pertinent include: (1) previous experience with food irradiation and the value of WF toxicity studies in addressing any safety concerns; (2) current principles and practices of compositional and agronomic analysis of GM crops; (3) results from WF toxicity studies on GM crops that have been conducted to date and what those results have added to the safety assessment; (4) studies on the optimization of WF toxicity study design; (5) exploration of the theoretical limitations of WF toxicity studies; (6) exploration of the likelihood of toxicologically significant unintended effects escaping agronomic and compositional assessment; (7) the relative power of analytical chemistry and WF toxicity studies to detect altered composition of human health significance; and (8) the compatibility of WF toxicity studies with animal ethics guidance.

Historical experience of WF toxicity studies in the assessment of new food technologies – Food irradiation

Prior to the use of recombinant DNA techniques, irradiated foods raised similar concerns centered around unintended effects on food composition. Food irradiation technology also elicited divergent opinions on the need for animal testing of food and feed, with many parallels to the current discourse surrounding safety assessments of GM crops. The following summarizes the discussion drawn from a joint study group report from the Food and Agriculture Organization of the United Nations (FAO), International Atomic Energy Agency (IAEA), and the World Health Organization (WHO) that provides a comprehensive summary of the safety studies conducted on food irradiation; the interested reader is referred to that report for further details (WHO, 1999).

Food irradiation was known to produce a range of unintended compositional effects through the production and reaction of radiolytic products and through damage to micronutrients. The history of research on food irradiation to preserve wholesomeness dates back to the early 1900s (US EPA, 2012). Irradiation of food was first proposed in the 1890s, with the first rodent bioassay conducted on an irradiated food in the 1920s (Giddings, 1992). Since that time, a large number of animal studies, including more than 30 lifetime studies, have been conducted to address the concern that irradiation may unintentionally alter food composition, resulting in the generation of substances capable of causing adverse effects in humans, a concern closely analogous to that postulated for GM foods. Over a number of decades, animal studies were conducted on whole irradiated foods, involving the sacrifice of tens of thousands of laboratory animals, mostly rats and mice, but also dogs, hamsters, quail, primates, and chickens. Overwhelmingly, these studies yielded no data that would call into question the adequacy of safety assessments based on compositional analysis and comparisons with untreated food. Elias (1980), considering the results of these studies, summarized the major limitations of irradiated food toxicity testing, including:

> . . . the impossibility of physically or chemically identifying what was being tested; the inability to incorporate sufficient irradiated food into the animal diet without seriously disturbing the nutrition of the test animals giving rise to secondary toxicological findings totally unrelated to

> irradiation effects, and the obvious impossibility of using sufficiently large numbers of animals in each experimental group to permit ascribing with an acceptable degree of statistical confidence any observed variations to the effect of radiolytic products present in minute amounts. . . . It is more convincing to be able to state that certain likely effects have been searched for and found absent than to admit that one did not know quite what to look for – but found it absent nevertheless.

The Joint FAO/IAEA/WHO Study Group (WHO, 1999) did not directly address the strengths or limitations of whole food studies, but considered that animal studies were suitable models and concluded that subchronic, chronic, and carcinogenicity studies demonstrated no short- or long-term toxicity due to irradiated food consumption. This conclusion draws heavily on analytical data to support the absence of effects in toxicological studies, as indicated in the following:

> . . . [A]bundant and convincing data indicate that high-dose irradiated foods do not contain either measurable levels of induced radioactivity or significant levels of any radiolysis products distinct from those found in un-irradiated foods. The theoretical maximum levels that might be formed would be so low as to be of no toxicological consequence.

The FAO/IAEA/WHO expert group also concluded, however, that ''the determination of wholesomeness for a representative food could be extrapolated to other foods of similar composition on the basis of available chemical data'' (i.e. without animal testing) and that ''the committee . . . also recognized the value of chemical studies as a basis for evaluating the wholesomeness of irradiated foods'' (WHO, 1999).

The expert group further concluded that although ''several different chemical bonds in the constituents are broken or formed, leading to either desired or undesired effects . . . it is through a consideration of the radiation chemistry of food that these chemical differences and their implications for wholesomeness and product quality can be understood.'' They also noted that the nature of the radiolytic products from food irradiation did not differ substantially than those generated by conventional cooking of foods. Thus, although vast numbers of WF animal studies were conducted on a wide range of irradiated foods using various levels of irradiation, ''none of the toxicological studies . . . had produced evidence of adverse effects . . .'' These WF toxicity studies had continued to be conducted despite the understanding that ''knowledge of the nature and concentration of these radiolytic products indicated that there was no evidence of a toxicological hazard'' (WHO, 1999). Indeed, in earlier deliberations, the committee concluded that the WF toxicity studies were supporting evidence for the chemical analyses rather than the other way around. It was arguably justified to conduct some animal experimentation to confirm the conclusions of safety based on analytical chemistry, particularly in the early years when analytical techniques were less sophisticated than current techniques. Today, however, the scientific merit of, and therefore the ethical justification for, continued extensive animal testing of new types of irradiated food is open to question. Indeed, some authors such as Giddings (1992) have observed that ''application of analytical chemical methods to the question of irradiated food toxicity came along later (i.e. after rodent bioassays), and represents probably the most conclusive proof yet of their toxicological safety.'' In light of the above, the expert committee (WHO, 1999) concluded the following:

> The application of ''risk assessment'' in the currently accepted sense is not appropriate to the toxicological assessment of foods preserved by high-dose irradiation. In this context, the concept of ''substantial equivalence'' may be more appropriate.

Taken together the recognized limitations of WF toxicity studies and the demonstrated absence of irradiation-induced substances at levels of concern in foods, do not make a convincing case for the necessity and appropriateness of animal studies to support irradiated food safety assessment, when more sensitive, reliable and readily interpretable analytical techniques are available.

Experiences with food irradiation technology closely parallel the current controversy surrounding the value of WF toxicity studies in GM crop safety assessment relative to agronomic and compositional analyses. The challenges and limitations of WF toxicity studies identified by critics of this approach based on their use in testing irradiated food remain pertinent to safety assessments of GM food and feed. In the time since food irradiation was first introduced, much has changed both in terms of analytical chemistry capabilities and attitudes towards the use of animals in safety testing. The body of work described in the joint review led to the clear conclusions that even in cases where food irradiation demonstrably caused a wide range of compositional changes detectable by chemical analysis, albeit at low concentrations, WF toxicity studies were generally insufficiently sensitive to identify the altered composition. The principle and most robust methodologies for considering the toxicological safety of treated food are those of analytical chemistry.

Current non-toxicological approaches for assessing the safety of GM crops

For regulators, comparative safety assessment is the driving principal of GM crop safety assessment. The concept of substantial equivalence as applied to GM crop safety assessment was first described by the OECD (1993), and the approach has been supported by international scientific authorities such as the FAO/WHO (2000), Codex Alimentarius Commission (2009), and EFSA (2008a). The goal of comparative assessment is to investigate intended and unintended changes in the GM crop relative to conventional, non-GM comparator crops with respect to agronomic, molecular, and compositional characteristics. Ultimately, the intention is to assess whether the GM crop is as safe and nutritious for humans and animals as a conventional, non-GM comparator, with the key assumption being that the conventional comparator is safe to consume based on a history of safe use. Notably, genetic manipulation by conventional breeding may also introduce unintended changes to crops

(Cellini et al., 2004; Kuiper & Kleter, 2003) as does the natural variation in climatic and agricultural conditions for a crop grown across dispersed regions of the world. Non-GM soybeans grown in 2002, for example, had more than an order of magnitude difference in their levels of bioactive isoflavones such as genistein (Kitta et al, 2005). Indeed, the environmental effects on crop composition are the basis for the generally desirable variations in organoleptic properties of food attributed to the ''terroir'' by gastronomes and wine buffs. The prevailing use of comparative safety assessment for GM but not for conventional crops does not therefore appear to have a sound, or indeed any, scientific basis, and evidence of a greater risk of potential toxic unintended changes in GM crops compared to conventionally bred crops is absent.

Safety by design – Initial development and selection of GM crops

Agricultural biotechnology is an extension of conventional plant breeding that offers new tools to alter plant DNA and produce plants with desired traits. Conventional plant breeding relies on the generation of random genetic variation in a large number of plants followed by extensive selection to identify a single (or a small population of) plant(s) that is then multiplied to produce the final commercial product. Poorly performing lines and off-types are removed prior to commercialization. The final product of conventional plant breeding is the result of a series of selections in which plants with undesirable characteristics, such as poor yield, are removed from the breeding pool. The process of developing a GM plant variety is closely analogous to conventional crop breeding. In a typical commercial transgenic plant breeding program, hundreds of cells and plants derived from any one initially transformed cell are produced. Through each selection step, the number of plants carried forward is reduced by removing poorly performing lines and off-types, just as with conventional breeding. For sexually reproducing species, the remaining plants are typically backcrossed repetitively to the same or to newer or more agronomically desirable varieties, again as in a conventional breeding program. This repetitive backcrossing to the parent or other established line results in an inbred that is greater than 90% homologous to that of the recurrent parent, essentially isolating genetic differences to the desired transgene. Those genetic changes caused by the transformation process that might produce deleterious off-types or other undesirable phenotypic changes will generally be eliminated.

For the plants that survive the initial steps of this intense breeding and selection process, key agronomic and phenotypic characteristics are compared with those characteristics of the non-transgenic counterpart. The compositional, morphological, and agronomic characteristics of a crop plant are the culmination of the coordinated expression of multiple genes that produce enzymes, structural components, regulatory proteins and nucleic acids, and metabolites. All of these components create the phenotypic characteristics of the crop, and changes in these can be expected to be reflected in changes in composition or agronomic behavior. Thus, from the perspective of the GM crop developer, safety assessment begins at the conceptual or design phase, continues through development and agronomic selection cycles, and is confirmed by compositional analysis once a new product has been selected for commercialization.

Evidence has been presented in the literature in support of the generation of unintended effects in GM plant development. Haslberger (2003) presents a number of transgenic plant examples claimed to illustrate such unintended effects; however, none of the cited examples are truly representative, as they had not gone through the selection processes associated with the transformation, lead event selection and commercialization of GM crops. First, only a limited number of plants were described in most cases, in contrast to the large number of events and subsequent breeding steps customary in a commercial program. Second, in many cases, the phenotype was consistent with the targeted pathway or due to better plant health. Most importantly, the unintended effects cited as a concern were within the range of natural differences that currently exist among conventional, non-GM crops, and therefore, neither indicative of a food safety concern nor supportive of a differential regulatory requirement for crops produced by conventional compared to biotechnology techniques (Filipecki & Malepszy, 2006). Assessment of the rigor of this crop development process is a normal aspect of risk assessment by regulatory authorities.

Comparative safety assessment of GM crops

Comparative safety assessment requires characterization of all elements that contribute to the development of a GM crop. The parent crop must be characterized for phenotype, agronomic performance, history of safe use, and composition. The source of the transgene, the transgene itself, and the process used to introduce the transgene to the parent crop are also characterized. This includes characterization of the introduced DNA and the insertion site(s) in the genome. The gene product(s) must also be characterized for identity, structure, mode of action, specificity, toxicity, and allergenicity (Cockburn, 2001; Codex Alimentarius Commission, 2009; EFSA, 2008a; König et al., 2004). Gene insertion is often carried out to bring about the introduction of a protein that imparts the plant with a desired trait. Safety assessment of the introduced protein is conducted early in the crop development process in accordance with established principles (Codex Alimentarius Commission, 2009; Delaney et al., 2008b; Hammond & Cockburn, 2008; Rice et al., 2008) to guard against allergenicity and toxicity.

The final phase of comparative safety assessment focuses on the GM crop itself. The agronomic and phenotypic qualities of the crop are assessed, and crop composition is analyzed. Compositional assessments evaluate the concentrations of key nutritional, toxicant, and anti-nutritional components in candidate plants and compare them with the most closely related conventional inbred plant or variety for which a history of safe use has been established. These comparisons are done in replicated trials on crops grown under the same environmental conditions using similar agronomic practices. The key components measured for GM crops are based upon species-specific OECD consensus documents that define observed ranges for proximates (protein, fat, carbohydrate, and moisture), amino acids, fatty acids, micronutrients

(minerals, vitamins), anti-nutrients, and known toxins naturally present in the crop (OECD, 2001, 2002, 2004, 2006). As an example, the approximately 100 analytes listed in the OECD consensus document for maize, plus starch, account for >95% of a maize kernel biomass (OECD, 2002; Watson, 2003). Therefore, any unintended changes in the plant not detected by compositional analysis would comprise a fractional component of the remaining 5% of the biomass, which contains thousands of low abundance compounds. Any single unknown substance or novel substance that theoretically may be present would therefore constitute only a small fraction of the WF and would need to be a relatively potent toxin to present a human health concern. Similar values for other crops can be estimated from OECD Consensus Documents for the Work on the Safety of Novel Foods and Feeds (OECD, 2012), which provide composition data for 16 plants to date, or from the ILSI Crop Composition Database for maize, cotton, and soybeans (Alba et al., 2010). For example, the ILSI Crop Composition Database shows that approximately 78% of soybean biomass can be accounted for by OECD analytes, without counting free sugars that are not in the database. Once reported values of free sugars (Dornbos & McDonald, 1986; Kim et al., 2006; Yazdi-Samadi et al., 1977) are added to the mix, compositional analysis accounts for an additional 10–15% of dry weight; thus, at a minimum, 88–93% of soybean biomass is analyzed in compositional studies.

Depending on the nature of the introduced trait (e.g. improved nutrition), further compositional analyses may include individual amino acid and fatty acid profiles or other metabolites of specific interest in that crop (ILSI, 2004, 2008; Kok et al., 2008). It should be recognized that if compositional variation is detected, this should not be inferred as representing a de facto hazard, but could lead to further evaluation to determine if the observed difference poses a risk to food and feed safety.

Past and current use of WF animal studies in the assessment of the safety of GM crops

A substantial number of animal studies have been conducted on commercial GM crops as a component of their safety assessment, and as a component of research on the design and utility of WF studies for food risk assessment. Animal studies are conducted with the intention of assessing the safety of both intended and unintended changes in GM crops. For evaluation of intended changes, these have included acute and repeated-dose studies on any intentionally introduced protein and studies on the health effects of specific components such as nutrients or anti-nutrients that are known to be altered in the GM crop relative to the conventional comparator. WF toxicity studies to assess intended and unintended changes have been recommended by some, for crops that have been extensively modified relative to a conventional comparator, such as crops modified to cope with environmental stress (EFSA, 2008a), although neither convincing evidence nor plausible argument for the generation of potentially hazardous unintended, unpredictable, compositional changes in such circumstances have been identified. More commonly, WF toxicity studies have been used to address uncertainties about unintended, unexpected changes in a crop. These studies are triggered by concern that an intended change may be accompanied by unintended change(s) not identifiable by compositional, agronomic, or molecular comparison of the GM crop with appropriate conventional comparators.

For the purpose of the present review, references incorporated in the tables were identified using the following databases: PubMed, SCOPUS, Google Scholar, AGRICOLA, PASCAL, CABA, FROSTI, LIFESCI, PQSCITECH, BIOSIS, FSTA, CA, ESBIOBASE, BIOENG, COMPENDEX, EMBASE and SCISEARCH for articles published between January 2004 and June 2013. These databases were selected based on their coverage of scientific literature for relevant subjects including, but not limited to, food and feed composition, nutrition, food, agriculture, chemistry, toxicology, allergy, dietetic, clinical and biotechnology. Detailed information about the databases searched (e.g. list of subjects covered, coverage dates, update schedule, and sources for data) can be obtained from the website of the Chemical Abstracts Service (CAS) at http://www.cas.org/products/stn/dbss.

Searches were conducted periodically throughout the preparation of the review to ensure that new publications were identified as they entered the open literature. Furthermore, manual searches were conducted to pursue references of references if warranted (Chapman et al., 2009). Only feeding studies and not studies using gavage or other routes of administration were considered. References consisting only of an abstract were not considered. Similarly where a study was available both as an opinion or evaluation by a regulatory agency and as a paper in the peer reviewed literature, the latter has been cited but both have been considered. The search algorithm contained the following keywords; ''whole food'', ''toxicity study'', ''subchronic'' and ''90-day'', in combination with the keywords: ''genetically modified'', ''GM'', ''rodents'', ''rats'', ''safety''. Some of the above were also searched with terms ''cow'', ''cattle'', ''pig'', ''swine'', ''chicken'', and ''poultry'', in various combinations. Additional combinations of terms or individual search terms not on this list may have also been used in the course of preparing this review, as required to gain a comprehensive snapshot of the literature. This search was completed by a review of each paper to complete Tables 2–4, to identify study parameters of interest in the context of this review, including number of animals, endpoints, duration, and others.

WF studies used to assess GM feed nutrition and safety in livestock and poultry

Although compositional analyses are the foundation for the nutritional assessment of GM crops, WF studies have been conducted in target domestic animals to confirm that feed produced from the GM crop is as nutritious as feed produced using the conventional comparator, and supports the same pattern of growth and development of the target livestock. In this respect such studies are closely analogous to clinical studies that compare two active drugs to demonstrate that a new drug is not less effective than an existing treatment (FDA, 2010). Feed studies in the target species are essentially nutritional non-inferiority studies, rather than toxicity studies, because both the test and ''control'' groups are nutritionally functional and no true ''placebo'' or control group is therefore

Table 2. Summary of peer-reviewed nutrition, performance and/or safety studies of genetically modified (GM) feeds conducted on livestock and poultry*.

Test species	Test crop	Study duration	Control	Reference group†	% in feed‡	References
Cattle (dairy cows)	HT soy (Gly)	28 d	Parental	1	10.2	Hammond et al. (1996b)
	Bt maize	21–28 d	Iso	0	75–80	Donkin et al. (2003)
	HT maize (Gly)	28 d	Iso	2	63	Grant et al. (2003)
	Bt maize	28 d	Iso	2	66.7	Grant et al. (2003)
	HT maize (Gly)	28 d	Iso	2	57.3	Ipharraguerre et al. (2003)
	Bt maize	35 d	Conventional	0	35	Yonemochi et al. (2003)
	HT maize (Gluf)	84 d	Iso	2	33.1	Phipps et al. (2005)
	Bt + HT maize (Gly)	28 d	Iso	0	45.1	Calsamiglia et al. (2007)
	HT alfalfa (Gly)	28 d	Conventional	2	39.7	Combs and Hartnell (2008)
	Bt cottonseed	28 d	Iso	0	40	Mohanta et al. (2010)
	Bt maize	25 mo	Iso	0	71	Steinke et al. (2010)
	Bt + HT maize (Gluf)	28 d	Iso	0	44	Brouk et al. (2011)
	Bt cottonseed	28 d	Iso	0	40	Singhal et al. (2011)
Cattle (steers)	HT maize (Gly)	92 d	Iso	2	75	Erickson et al. (2003)
	HT maize (Gly)	94 d	Iso	2	73	Erickson et al. (2003)
	HT maize (Gly)	144 d	Iso	2	79.5	Erickson et al. (2003)
Cattle (bulls)	Bt + HT maize	246 d	Parental	0	*Ad lib*.sileage	Aulrich et al. (2001)
Cattle (calves)	Bt maize	84 d	Iso	0	43.3	Shimada et al. (2006)
Swine	Bt + HT maize	14 d	Parental	0	50	Aulrich et al. (2001)
	HT maize	24 d	Parental	0	30	Böhme et al. (2001)
	HT sugar beet	24 d	Parental	0	30	Böhme et al. (2001)
	Bt + HT maize	98–114 d	Parental	0	70	Reuter et al. (2002a)
	Bt + HT maize	NI¶	Parental	0	70	Reuter et al. (2002b)
	HT soy (Gly)	4 mo¶	Iso	0	14–24.3‡	Cromwell et al. (2002)
	HT maize (Gly)	103 d	Iso	2	68.1–81.8	Hyun et al. (2004)
	HT maize (Gly)	NI¶	Iso	2	65–77	Hyun et al. (2004)
	HT rice (Gluf)	98 d	Iso	1	72–85.8	Cromwell et al. (2005)
	Bt maize	104 d	Iso	2	68.7–82.5	Hyun et al. (2005)
	Bt maize	NI¶	Iso	2	65–76	Hyun et al. (2005)
	Bt maize	NI¶	Combined§	0	78–83	Custodio et al. (2006)
	Bt maize	NI¶	Combined§	0	70–76.5	Custodio et al. (2006)
	HT wheat (Gly)	NI¶	Iso	4	70–85	Peterson et al. (2008)
	Bt + HT maize (Gluf)	4 mo¶	Iso	1	69.1–81.9	Stein et al. (2009)
	Bt maize	NI¶	Iso	0	70	Yonemochi et al. (2010)
	Bt maize	30 d	Iso	0	38.9	Walsh et al. (2012)
Poultry (broiler chickens)	HT soy (Gly)	42 d	Parental	0	26.6–32.9	Hammond et al. (1996b)
	Bt maize	38 d	Iso	0	61.4–67.4	Brake & Vlachos (1998)
	HT maize (Gly)	38–40 d	Parental	5	50–60	Sidhu et al. (2000)
	Bt + HT maize	30–35 d	Parental	0	50	Aulrich et al. (2001)
	Bt maize	49 d	Conventional	0	70	Yonemochi et al. (2002)
	Bt maize	42 d	Iso	1	48.2–63.6	Brake et al. (2003)
	Bt maize	42 d	Iso	5	57.1–62.7	Taylor et al. (2003)
	Bt + HT maize (Gly)	42 d	Iso	5	55.2–60.5	Taylor et al. (2003)
	Bt maize	35 d	Parental	0	73.6	Tony et al. (2003)
	HT canola (Gly)	42 d	Iso	6	25	Taylor et al. (2004)
	Bt maize	39 d	Iso	0	60	Aeschbacher et al. (2005)
	IP maize	49 d	Iso	2	55.0–66.0	Brake et al. (2005)
	Bt maize	42 d	Iso	0	48.7–62.7	Rossi et al. (2005)
	Bt + HT maize (Gly)	43–44 d	Iso	5	54.7–59.4	Taylor et al. (2005)
	HT soy (ALSi, Gly)	42 d	Iso	3	22.5–31	McNaughton et al. (2007)
	HT soy (Gly)	42 d	Iso	6	61.4–64.8	Taylor et al. (2007a)
	Bt maize	42 d	Iso	4	55.1–59.6	Taylor et al. (2007b)
	Bt + HT maize (Gly)	42 d	Iso	4	54.8–58.5	Taylor et al. (2007b)
	Bt + HT maize (Gly)	42 d	Iso	6	57.3–59.4	Taylor et al. (2007c)
	HT maize (ALSi, Gly)	42 d	Iso	3	58.5–71.5	McNaughton et al. (2008)
	HT maize	42 d	Iso	3	50–60	Herman et al. (2011a)
	HT soy	42 d	Iso	3	32–40	Herman et al. (2011b)
	HT maize + HT soy	42 d	Iso	3	91.5–94.2	McNaughton et al. (2011a)
Poultry (laying hens)	Bt + HT maize	10 d	Parental	0	50	Aulrich et al. (2001)
	Bt maize	6 months	Iso	0	60	Aeschbacher et al. (2005)
	Bt + HT maize (Gluf)	3 months	Iso	1	64.8	Jacobs et al. (2008)
	High oleic soy	3 months	Iso	2	23.5	Meija et al. (2010)
	HT maize + HT soy	3 months	Iso	3	84.6–86.3	McNaughton et al. (2011b)

ALSi, acetolactate synthase inhibitor (herbicide tolerant); Bt (*Bacillus thuringiensis*), crop expresses one or more Cry proteins; Gluf, glufosinate tolerance; Gly, glyphosate tolerance; GM, genetically modified; HT, herbicide tolerant (if Gluf, Gly, or ALSi is not specified, crop is tolerant to one or more alternative herbicides); Iso, near-isogenic line (has similar background genetics to test line but lacks the gene insert for the GM trait); IP, insect-protected (expresses non-Cry protein); NI, not indicated.

*Peer-reviewed, published livestock and poultry feeding studies on insect pest-protected and herbicide-tolerant crops are summarized. The list is not intended to be comprehensive, and small numbers of studies using sheep or quail are not included. A review by Flachowsky et al. (2007) includes some studies not summarized in this table.

†Non-GM reference groups used for establishing historical control normal range.

‡Percent incorporation (w/w) of all forms of the test crop(s) in feed (i.e. grain and/or silage); ranges represent changes in test crop levels in feed over the course of the study in grower/finisher pigs and starter/grower/finisher broiler chickens.

¶Duration approximate or not indicated for some swine studies in which animals were terminated when they reached a specific body weight.

§Control corn was a mixture of several non-transgenic inbred lines.

Table 3. Summary of experimental designs in peer-reviewed, published short-term (21-to-30-day) rodent toxicology studies conducted on whole foods derived from genetically modified crops*.

Crop	Sponsor	Dose group	Group size	Reference group†	Control	% in diet‡	References
Bt potato	Monsanto	NI	NI	0	Parental	NI	Lavrik et al. (1995)
HT soy, processed	Monsanto	1	10/sex	0	Iso	24.8	Hammond et al. (1996b)
HT soy, unprocessed	Monsanto	2	10/sex	0	Iso	5/10	Hammond et al. (1996b)
Bt cotton	China	2	6/sex	0	Parental	5/10	Chen et al. (1996)
Sweet pepper, virus resistant	China	3	10/sex	0	Rodent diet	NI	Chen et al. (2003)
Tomato, virus resistant	China	3	10/sex	0	Rodent diet	NI	Chen et al. (2003)
HT oilseed rape	Monsanto	2	10/sex	0	Parental	5/15	EFSA, 2004
HT oilseed rape	Monsanto	2	10/sex	8	Parental	10	EFSA, 2004
Bt potato	NI	1	12 males	1	Iso	30	El Sanhoty et al. (2004)
Potato, virus resistant	NI	1	8; sex NI	2	Iso	40	Juskiewicz et al. (2005)
Potato, non-browning	NI	1	NI	0	Iso	NI	Llorente et al. (2011)

Bt (*Bacillus thuringiensis*), crop expresses one or more Cry proteins; HT, herbicide tolerant; Iso, near-isogenic line (has similar background genetics to test line but lacks the gene insert for the GM trait); NI, not indicated.

*The duration of most studies was 28–30 days; the study duration was 21 days for Juskiewicz et al. (2005). Test GM foods were administered in feed. A selection of clinical observations, hematology, blood chemistry, organ weights, and gross and microscopic pathology were performed.

†Non-GM reference groups used for establishing historical control normal range.

‡Percent incorporation (w/w) of test crops in the rodent diet.

Table 4. Summary of experimental designs in peer-reviewed, published subchronic (90-day) rodent toxicology studies conducted on whole foods derived from genetically modified crops*.

Crop	Sponsor	Dose group	Group size	Reference group†	Control	% in diet‡	References
Bt tomato†	RIKILT	1	12/sex	0	Iso	10	Noteborn et al. (1995)
HT soy	Japan	1	5/sex	0	Iso	30	Teshima et al. (2000)
Bt maize	Japan	1	8/sex	0	Iso	5/50	Teshima et al. (2002)
Ht maize	Monsanto	2	20/sex	6	Iso	11/33	Hammond et al. (2004)
HT soy	China	3	10/sex	0	Iso	30/60/90	Zhu et al. (2004)
Bt/HT maize	Monsanto	2	20/sex	6	Iso	11/33	EFSA (2005a)
Bt/HT maize	Monsanto	2	20/sex	0	Iso	11/33	EFSA (2005b)
Bt/HT maize	Monsanto	2	20/sex	0	Iso	11/33	EFSA (2005c)
Bt maize	Monsanto	2	20/sex	0	Iso	11/33	EFSA (2005d)
HS potato (amylopectin)	BASF	3	5/sex	0	Iso	5	EFSA (2006a)
HT sugar beet	KWS SAAT AG/Monsanto	2	NI	4	Iso	2/5	EFSA (2006b)
Bt maize	Monsanto	2	20/sex	6	Iso	11/33	Hammond et al. (2006a)
Bt maize	Monsanto	2	20/sex	6	Iso	11/33	Hammond et al. (2006b)
Bt cotton	Dow	1	12/sex	3	Iso	10	Dryzga et al. (2007)
HT maize	Syngenta	2	12/sex	0	Iso	10/41.5	EFSA (2007)
Bt/HT maize	Pioneer	2	12/sex	3	Iso	11/33	MacKenzie et al. (2007)
Bt/HT maize	Pioneer	1	12/sex	2	Iso	35	Malley et al. (2007)
Bt rice	EU and Canada	1	16/sex	0	Iso	60	Schrøder et al. (2007)
Lectin rice (snowdrop)	EU, China, India	1	16/sex	0	Iso	60	Poulsen et al. (2007a)
Lectin rice (PHA-E)	EU and China	1	8/sex	0	Iso	60	Poulsen et al. (2007b)
HT soy	Pioneer	1	12/sex	3	Iso	20	Appenzeller et al. (2008)
High oleic soy	Pioneer	1	12/sex	3	Iso	20	Delaney et al. (2008a)
HT soy	Monsanto	2	20/sex	6	Iso	5/15	EFSA (2008b)
Bt maize	Monsanto	2	20/sex	0	Iso	11/33	EFSA (2008c)
Bt maize	Pioneer	2	10/sex	0	Iso	50/70	He et al. (2008)
Bt/HT maize	Monsanto	2	20/sex	6	Iso	11/33	Healy et al. (2008)
HT maize	Pioneer	1	12/sex	3	Iso	35–38	Appenzeller et al. (2009b)
Bt/HT maize	Pioneer	1	12/sex	3	Iso	34	Appenzeller et al. (2009a)
Bt maize	Syngenta	2	12/sex	0	Iso	10/41.5	EFSA (2009b)
Lysine maize	Pioneer	2	10/sex	0	Iso	30/76	He et al. (2009)
High amylose rice	China	1	10/sex	0	Iso	70	Zhou et al. (2011)
HT soy	BASF	2	10/sex	4	Iso	11/33	Chukwudebe et al. (2012)
IP maize	Syngenta	2	12/sex	0	Conventional	10/41.5	EFSA, 2012a
High oleic, HT soy	Monsanto	1	12/sex	3	Conventional, HT soy	30	EFSA, 2012b
Bt/HT maize	China	3	10/sex	1	Iso	12.5/25/50	Liu et al. (2012)
High oleic/HT soy	Pioneer	3	10/sex	0	Iso	7.5/15/30	Qi et al. (2012)
rhIGF-1 rice	China	2	16/sex	0	Iso	20	Tang et al. (2011)
HT maize	China	3	10/sex	0	Iso	12.5/25/50	Zhu et al. (2013)

Bt (*Bacillus thuringiensis*), crop expresses one or more Cry proteins; HS, high starch; HT, herbicide tolerant; IP, insect protected (non-Bt); Iso, near-isogenic line (has similar background genetics to test line but lacks the gene insert for the GM trait); NI, not indicated; PHA-E, phytohemagglutinin-E; rhIGF-1, recombinant human growth factor-1.

*Test GM foods were administered in feed. A selection of clinical observations, hematology, blood chemistry, organ weights, and gross and microscopic pathology were performed.

†Non-GM reference groups used for establishing historical control normal range.

‡Percent incorporation (w/w) of test crops in the rodent diet.

possible. In addition, it is fundamentally impossible to prove that two treatments or foods are identical nutritionally or that they have identical safety profiles. The aim of such studies is to demonstrate that any differences, if present, are less than some commercially acceptable margin, or are less than that identifiable by a livestock producer under normal production circumstances. In the case of animal feeds, a reasonable margin would be based on the likely differences that would be observed from the use of two nutritionally similar conventional feeds. In this context, an absence of identifiable differences in growth and development between test and control groups confirms the non-inferiority of the GM feed with respect to the conventional feed. Table 2 provides a summary of livestock and poultry feeding studies conducted to evaluate the use of GM crops in feed.

Because they are conducted in the species that the GM crop is destined to feed, issues of extrapolation for human health risk assessment are not applicable. Furthermore, since the parameters of interest are largely confined to those related to normal growth and development, statistical challenges related to multiple comparisons and a lack of toxicological power are not generally applicable. Although the value that such studies add to compositional analysis of GM feed is open to question, they are at least readily and reliably interpretable in terms of nutritional non-inferiority. These types of studies are not considered further in this review, although notably, no adverse findings have been identified in studies of this type.

WF toxicity studies using laboratory rodents

Rodent toxicity studies on WF derived from GM crops vary in study design and duration, but all are based to some extent on studies used in chemical hazard characterization. Many of the WF toxicity studies that have been conducted on GM crops have been 28- or 90-day subchronic rat studies in which the GM food was administered by incorporation into the diet. Tables 3 and 4 provide summaries of a number of published 28- and 90-day studies. In many of these cases, the experimental design was adapted from OECD Test Guidelines 407 or 408, of which the latter has been recommended for the safety testing of WF obtained from GM crops in cases in which plant composition is either substantially modified or there are indications of potential unintended changes in the plant (EFSA, 2008a; OECD, 1998, 2008). The studies in Tables 3 and 4 are illustrative of the approaches used to adapt animal toxicology studies for use with WF test material and will be used in the present review to further discuss issues associated with the design and interpretation of WF toxicity studies.

Studies longer than 90 days, including multigeneration and reproduction studies, have also been conducted on GM crops (Brake et al., 2004; Brake & Evenson, 2004; Kiliç & Akay, 2008; Rhee et al., 2005; Snell et al., 2012; Velimirov et al., 2008; Wainwright et al., 2003). A justification for longer studies has been that they are necessary to address limitations in subchronic studies that may obscure the detection of adverse effects and/or the interpretation of biological changes as adverse (de Vendômois et al., 2009). However, a conclusion of a recent review of long term WF toxicity studies is that GM food is not revealed to be harmful when the duration of feeding is increased to well over 90 days (Snell et al., 2012). For the purpose of the present review, the limitations associated with the use of WF as a test material would be expected to apply equally to lifetime studies and reproduction studies and these studies will not be considered in depth here.

Basic principles of toxicology studies applicable to WF studies

Animal ethics

Attitudes and legislative requirements around the ethics of animal studies have changed considerably over the years since the bulk of the irradiated food studies were conducted. A consideration of the contemporary requirements of ethical animal studies is therefore an appropriate entry point to a review of the conduct and value of WF studies on GM crops.

A consideration of the welfare of experimental animals, and the ethics of conducting a specific study, by institutional animal research ethics committees is a requirement in most nations. Given the general comparability of GM to non-GM food observed to date, safety testing of GM foods and food constituents is unlikely to lead to unacceptable pain and/or suffering in the experimental animals used, other than as a result of normal experimental and investigative practices, and provided that the researchers conducting the experiments adhere to normal humane practices. Unfortunately, this is not always the case as illustrated by the recent study by Séralini et al. (2012). In describing the egregious errors of design, conduct, and interpretation of the study by these authors, the European Society of Toxicologic Pathology expressed shock at the treatment of the animals and questioned the legality of the study conduct under European law (Schorsch et al., 2013).

Despite the negligible potential for adverse effects in well-designed and conducted WF animal studies, there are nonetheless substantial ethical considerations that need to be taken into account in any deliberation on the circumstances under which studies will be required, or permitted, and experimental animals sacrificed, and on how they are to be conducted. A key consideration in this context is the international consensus on the need to *reduce* the number of animals used in research, to *refine* the way they are used in order to reduce pain and suffering, and to *replace* animal experiments with alternative methods. These objectives are commonly known as the three ‘‘Rs’’ of experimental animal use. An equally critical consideration in the current context is that of scientific merit, or the potential of a specific study design for a given range or class of substances to yield robust, interpretable results not obtainable through other means. This principle is reflected in the EU directive on the protection of animals used for scientific purposes (European Commission, 2010a), articles 11 and 39 of which state:

> The principles of replacement, reduction and refinement should be implemented through a strict hierarchy of the requirement to use alternative methods. Where no alternative method is recognised by Community legislation, numbers of animals may be reduced by resorting to other methods which are reasonably and practically available, and by implementing testing strategies, such as use of

in vitro and other methods that would reduce and refine the use of animals.

It is also essential, both on moral and scientific grounds, to ensure that each use of an animal is carefully evaluated as to the scientific or educational validity, usefulness and relevance of the expected result of that use. The likely harm to the animal should be balanced against the expected benefits of the project.

These principles are reiterated by a range of national agencies and scientific associations around the world, including the Federation of European Laboratory Animal Science Associations, the Australian National Health and Medical Research Council, and the US National Institutes of Health. Thus, from an animal ethics perspective, the following critical questions should be explored when considering the necessity and value of a WF toxicity study on a GM crop:

(1) Is sufficient information from other sources unavailable and unobtainable, such as compositional analysis, to demonstrate the safety of the specific GM event under consideration?
(2) Is there a reasonable scientific basis from which to postulate that the GM event has the potential to produce a specific alteration to the food portion of the crop that cannot be identified and quantified by analytical techniques or that may reasonably be presumed to be associated with increased toxicological potential over that of a non-transgenic crop?
(3) Does previous experience with the transgene and the transformed crop provide any scientifically credible basis for postulating a potentially significant toxicological concern not addressable through compositional analysis?
(4) Does the nature of the specific potential change to the food portion of the crop have a potential to lead to an adverse effect on the consumer beyond what might arise from consumption of a non-GM variant of that food?
(5) Are the potential toxicological effects due to changes in the GM crop amenable to exploration using WF toxicity studies, i.e. is it likely a WF study could detect these effects?
(6) Is the design and conduct of the proposed study such that they are likely to lead to findings that are

- robust (i.e. repeatable),
- relevant to a safety assessment of the GM event,
- reliably interpretable, and
- able to be extrapolated to an assessment of human health and safety?

These key criteria are the basis for establishing triggers for the ethical conduct of WF toxicity studies on new GM crops. When each of these questions cannot be answered in the affirmative, the animal ethics of conducting a WF toxicity study is at least questionable. In such circumstances, a reasonable conclusion is that alternative, generally more sensitive, approaches to safety assessment should be explored instead of the conduct of a WF toxicity study.

Basic principles of toxicology study design

It is recognized that animal toxicology studies intended for human risk assessment have inherent limitations and challenges due to the use of a surrogate species and relatively small numbers of animals in each study group. Differences in physiology (toxicokinetics, toxicodynamics, nutritional requirements, and other biochemical characteristics), anatomical structure, and behavior can lead to positive or negative findings that may not accurately reflect human responses; however, toxicology study design seeks to overcome these limitations by utilizing compensatory design elements (Creton et al., 2012; Gad, 2007).

All biological studies are inherently ''noisy'' to varying degrees. That is, the interaction between the environment and the natural variation between individuals inherent in any species population will lead to random, treatment independent, variation in a number of parameters between treatment groups. The nature and source of that variation may be environment and species or strain specific, and differences in physiology and anatomy between the test species and humans can exaggerate or mask the relevance of treatment effects to humans. The core objective when designing animal studies is the maximization of the treatment ''signal'' so that it is readily distinguishable from background biological ''noise'' in studies utilizing relatively small group sizes. Where dose escalation is practicable, as in the testing of pure substances, a dose range finding study provides a ''calibration'' of the bioassay system to ensure doses are selected for the main study that provide a clear signal above background noise. For compounds of low toxicity, very high, or ''limit'', doses, hundreds or thousands of times greater than presumptive human exposure levels, may be used. Where such doses do not produce evidence of toxicity in the experimental animals this is taken to provide further support for the conclusion that adverse effects in humans are not likely, provided species differences in toxicokinetics do not preclude extrapolation of the results across species.

Thus, when we speak of power in toxicological studies, we are not just considering the ability of the study to discriminate between true effects and differences due to simple variation, we are also concerned with the ability to differentiate between the following:

- adaptive and pathological effects;
- species-specific effects and those of relevance to other species, primarily humans;
- reversible or short-term effects from irreversible or longer-term effects;
- physicochemical concentration-related effects from biological, toxicological effects; and
- true negative results due to a lack of toxicity from false negatives due to species insensitivity.

In this context, the interpretive power of a study encompasses all of the factors that enable the identification of real treatment-related effects from random background variation and to determine the relevance of observed effects, or their absence, first to the animal model being studied and then to humans.

Statistical versus biological significance

The interpretation of all animal toxicology studies must take into consideration biological, toxicological, and statistical significance. For example, if a particular parameter is altered

in the same direction in multiple studies in multiple species with a temporal and dose relationship, it may be interpreted as biologically significant even if none of the changes achieve statistical significance. Equally, a statistically significant alteration in the value of a given parameter may not be interpreted as biologically and toxicologically significant if the finding is discordant with correlating parameters, temporally isolated, and/or within historical control ranges for the species. If a positive finding is reported, a general tenet of scientific methodology and an expectation of high-quality testing would be reproducibility of that finding. Although toxicity studies are not commonly repeated, a typical package of toxicology studies for a new, agricultural, pharmaceutical or food chemical would comprise a number of studies of various durations and more than one species, providing some capacity to test reproducibility of findings. Even for studies in which the test material is a single chemical of known purity, interpretation of responses for multiple endpoints is not necessarily straightforward (Lewis et al., 2002). This is particularly true given that statistical significance at a given magnitude of effect is dependent on, or an artifact of, study design.

The statistical power of a toxicology study is determined by the interplay of three key parameters: the numbers of animals in each group to be compared, the underlying background variation in the value of a given parameter in the species population being studied, and the size of the effect produced by the test material administered. For reasons related to both study cost and animal welfare, the number of animals in a toxicology or safety study is kept relatively low. For OECD Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity Study in Rodents) (OECD, 1998), only 10 animals per sex per dose are employed, although the study designer can opt for additional animals per group if necessary to detect effects of lesser magnitude. As a direct consequence, the capacity of such studies to detect subtle treatment-related changes in biological parameters is low. In prospectively determining the statistical power of a study, the researcher will give thought to the likely magnitude of change in any given parameter in comparison to the known background variation for that parameter in the test species; however, this is not possible where the test substance is unknown and the potential target organ(s) or effects(s) is also unknown. In animal studies, however, the dose of a test substance administered can be increased until the maximum tolerated dose (MTD) is reached. Thus, to compensate for the effect of constrained animal numbers on statistical power, strategies are employed to increase the magnitude of any treatment-related effect. Dose range finding studies are conducted before longer-term (generally lifetime carcinogenicity studies) or other pivotal studies (developmental or reproduction studies) are initiated to ensure the dose ranges used are of sufficient magnitude such that effects are unequivocally manifested without excessive mortality or morbidity, while at the same time determining a suitable dose range that includes a no-observed-adverse-effect level (NOAEL). In other words, the doses are selected to ensure generation of effects of sufficient magnitude to be detected as statistically and biologically significant in the number of animals included in the study design. For a whole GM food, neither dose ranging nor attainment of an MTD is possible.

In a toxicology study such as that described in OECD Test Guideline 408, numerous clinical, hematological, histological, and health-related parameters are typically measured. If statistical significance is set at the standard default value of $p \leq 0.05$, then on average 1 parameter in 20 (i.e. 5%) is expected to be statistically significantly different between treatment groups purely by chance, and a few of these might even be expected to show evidence of a dose relationship, again purely by chance (McDonald, 2009). So common and predictable are these random statistically significant differences that every study can be expected to report these. Indeed, as Goodman (1992) has pointed out, the probability of obtaining a statistically significant result with the same or better p value in a duplicate experiment when the p value in the first experiment was 0.05 may only be around 50%.

A further interpretive complication is that statistical differences in individual response variables are often observed in only one sex, which may raise suspicion of a random variation even though that is not necessarily the cause. Rats have far greater inter-sex metabolism differences than do many other species, including humans, so marked differences in response between sexes at a given dose resulting from differences in metabolism are not uncommon (Mugford & Kedderis, 1998). In classical toxicology packages for a new chemical, toxicokinetic studies, dose response patterns and/or knowledge of the structure of the test substance facilitates interpretation of apparent sex differences in metabolism. This approach, however, is not possible in WF toxicity studies in which the test substance(s) are unknown and meaningful dose escalation is not achievable.

The importance of recognizing that normal variation results in statistically significant differences between treatment groups about 5% of the time is illustrated by the critical comments of Food Standards Australia and New Zealand (FSANZ, 2011) and EFSA (2009a) on the re-analysis of data by de Vendômois et al. (2009) on 90-day rat feeding studies on three different varieties of biotechnology-derived maize products (Hammond et al., 2004, 2006a,b). In reviewing the re-analyses, it was concluded that the incidence of statistically significant differences in animals fed GM corn (MON863) was entirely consistent with normal background variability, and that there was a lack of concordance of the statistics with other investigative processes used in the studies such as pathology, histopathology, and histochemistry. Thus, the re-analyses placed undue emphasis on the statistical treatment of data, and failed to take other relevant factors into account. It was concluded that changes attributed to GM maize were neither sex- nor dose-related and were primarily due to chance alone (EFSA, 2009a; FSANZ, 2011).

When there is a true treatment-related effect that results in the increased incidence or severity of a pathological finding, or causes a real difference in a response variable with high inter-individual variability, it may not achieve statistical significance due to limitations of the statistical power of the study design, but may be ‘‘real’’ nonetheless. That is, a lack of statistical significance does not necessarily mean that an effect is due to chance alone. In such cases, the study interpretation must consider correlative endpoints to add, or subtract, toxicological ‘‘weight’’ to the observation. A well-designed toxicology study will include multiple endpoints

indicative of biological effect on a single organ or tissue. For example, liver weight, liver histopathology, and clinical indices of liver function are among those endpoints assessed to characterize the hepatic effects of a test substance. A statistically significant change in any one parameter may not, in isolation, have biological significance in the context of all parameters assessed.

Ioannidis (2005) has discussed the statistical basis for the poor reproducibility of many types of studies in the literature and notes a number of factors contributing to the likelihood of study results being reliable. Key contributors to unreliable results include; small study size, small effect sizes in relation to background variability, and large numbers of parameters being tested that are unrelated to a specific prior hypothesis. All are characteristics of the WF toxicity studies reviewed here. Whether they are due to random variation or whether they are indicators of adverse effects, the biological and toxicological relevance of statistically significant differences should always be evaluated and interpreted in the context of the following (Doull et al., 2007):

- the magnitude of the difference, and the potential influence of any outliers on mean values either in the GM or control group;
- the presence or absence of dose response, if more than one dose was used in the study;
- the consistency over time and between sexes (considering there can be genuine sex differences in response, especially if there are sex differences in metabolism);
- the co-occurrence of changes in related indicators of toxicity, such as morphological changes in organ histology, concurrent with changes in body and organ weight, blood chemistry, and/or hematology;
- biological plausibility;
- the reversibility of adverse effects;
- the possible mechanisms of toxicity; and
- historical control data for each parameter's normal range of variability in the same strain of animal, ideally at the test facility concerned, and at a relevant period close to the time of the study concerned.

All of these factors contribute to the interpretation of a study and ultimately provide a scientific basis for the utility, or lack thereof, of a WF toxicity study.

Interpretive power

Interpretive power in the current context is the extent to which the results of a study are able to be robustly interpreted to identify true treatment-related effects and characterize the nature of the effects on the animal species being used in the study. Key study design considerations to maximize discriminative power include the range of parameters to be investigated in the study as well as basic design issues such as pre-treatment, or baseline, determination of amenable parameters (e.g. blood chemistry, urinalysis); dose escalation; age and sex of animals on test; and availability of contemporary normative (historical control) data for the species, strain, and source of animal; and estimated margin of exposure of the achieved systemic dose in the test species over that likely in humans exposed to the test material.

Extensive historical control data are available for some strains of experimental animals sourced from major animal supply houses (e.g. Charles River, 2012). These data may be critical in interpreting random clusters of potentially significant findings or discounting random variation in endpoints with a high background rate and variability (Keenan et al., 2009). For example, chronic progressive nephropathy of the kidney and microscopic histocytosis in the liver naturally occur at a high rate in control rats (Appenzeller et al., 2008; Hard et al., 2009). In many studies cited in Table 3, some statistical differences were observed in clinical chemistry, hematology, or other variables between the GM and the comparator groups, as expected when large numbers of statistical comparisons are made. Recognition of common pathologies is essential in avoiding misinterpretation of changes in treated animals that may be inherent in the animal model and not due to treatment. This will be a particular issue where marked treatment-related effects are unlikely and/or implausible, such as with WF toxicity studies.

The purpose of conducting safety studies in animals, regardless of the test substance, is to predict effects in humans. For many toxicological endpoints, even closely related species such as the mouse and rat do not predict effects in each other (Gold et al., 1989; Tomatis et al., 1973). Similar effects observed in unrelated species such as dog and rat are likely to be more relevant to humans than effects observed in only one species, although this is not invariably true. Equally, studies in only one species are a weak basis for extrapolation to humans because an absence of effects in one species does not mean that effects will not be seen in another species. In classical, non-pharmaceutical, toxicology, species differences in metabolism and kinetics are to some extent compensated for by the use of escalating doses to achieve an MTD and testing in multiple species, and achievement of a margin of exposure of that anticipated, or permitted, in humans. Lack of sensitivity of a specific animal model due, for example, to differences in the production of a toxic metabolite or due to lower sensitivity of the target organ are addressed by including dose levels that approach or meet the definition of an MTD. Potential, but unknown, differences in toxico-dynamics are compensated through the use of multiple species such as teratogenicity testing in both rabbits and rats. Differences in the physiology of the test species to that of humans must be carefully considered both in the design and the interpretation of a toxicological study.

Dose response

In chemical toxicology studies, dose escalation is used to demonstrate the dose dependency of responses to the test chemical, to increase the potential to identify its hazards, to overcome interspecies differences in toxicodynamics and/or toxicokinetics of the chemical, and to establish a NOAEL. In WF studies, the test substance(s) are the unknown substances that may or may not be present and may or may not be of toxicological significance. The WF itself is simply a carrier for any unknown. Dose selection for a WF is therefore limited by the bulk, palatability, and nutrient composition of the test crop. Generally, the highest tolerable level of the test crop has been incorporated into the diet, with the maximum dietary

concentration depending on the type and nutritional composition of the GM crop. In general, test crops may only be introduced to rodent diets at levels which do not result in dietary nutrient imbalance. Furthermore, some human foods such as onions and chilies are not suitable for administration to rats as a large proportion of diet, because they are known to lead to adverse effects that are attributable to endogenous constituents (Elias, 1980; Hammond et al., 1996a). Dietary incorporation of test crops at levels greater than 60% (w/w) have been achieved, although levels in the range of 30% (w/w) are more common (Table 4). In some WF toxicity studies, two or more levels of test food have been incorporated into the diet with the goal of generating a ''dose response'' (Chen et al., 2003; Chukwudebe et al., 2012; EFSA, 2005a,b,c, 2007, 2008a; Hammond et al., 2004, 2006a,b; He et al., 2008, 2009; Healy et al., 2008; Liu et al., 2012; Qi et al., 2012; Zhu et al., 2004, 2013). When no effect is observed at any dose, the additional dose groups provide a further basis for evaluating and dismissing the inevitable random statistically significant findings. Other investigators have used a single ''limit dose'' approach, comparing test animal responses to GM versus comparator crop at maximum levels in the diet (Appenzeller et al., 2008, 2009a,b; Delaney et al., 2008a; Dryzga et al., 2007; Malley et al., 2007; Momma et al., 2000; Teshima et al., 2000, 2002). The assumption behind the limit dose strategy is that if there are no adverse effects due to the test diet with the maximum possible level, the use of graduated doses to establish a NOAEL is unnecessary. For all of the studies in Table 4, regardless of the dose selection strategy, no adverse effects were observed.

The use of escalating doses to increase the magnitude of treatment effects and minimize the number of animals required per group is also an economical and ethical approach to reducing the cost of a toxicology study. Increasing the duration of a study will also tend to increase the level of effect at a given dose, or decrease the dose required to reveal a given effect (Batke et al., 2011), but will also tend to introduce age related pathologies that may complicate or confound interpretation and is generally a less cost-effective strategy than using higher doses over a shorter period. Furthermore, the use of escalating doses facilitates the use of additional statistical tools such as trend analysis. For WF, the ability to escalate doses to levels approaching an MTD to assess the presence and safety of unintended changes is limited by the bulk and complexity of the test material. This must be considered when interpreting the statistical versus biological or toxicological significance of any small differences in responses in the GM WF test groups relative to one or more non-GM WF control groups.

Experience gained from WF toxicity studies on GM crops

Table 4 provides a summary of 90-day WF toxicity studies conducted on commercialized GM crops to date. In each of the 38 studies, the design of the new crop, the selection of lead variants, and agronomic and compositional analysis of the variant selected for commercialization predicted that no intended or unintended effects of human health significance would be detectable in WF toxicity studies. In each of the studies listed, no biologically or toxicologically relevant effects were observed. Thus, none of the WF toxicity studies conducted on commercialized GM crops to date have produced a result that might call into question the adequacy, sufficiency, and accuracy of a risk assessment based on agronomic and compositional analysis, in which the GM crop under consideration has been through normal commercialization processes and considerations. If the WF toxicity studies are considered valid scientifically, there is an unparalleled 100% concordance between the prediction of agronomic and compositional characterization and the outcome of the rodent bioassay. This conclusion is consistent with those of Ricroch (2012) who compared the results of 17 long term (greater than 90 day) animal studies with that from targeted ''omics'' techniques and found that the ''-omics'' comparisons showed less impact on gene expression in genetically modified crops than in conventionally bred crops and that neither the animal studies nor the ''-omics'' studies raised safety concerns.

A number of other studies and re-analyses of published WF toxicity studies on GM crops have claimed to demonstrate adverse effects. Three notable examples are the re-analyses of rat feeding studies with MON863, MON810, and NK603 corn (de Vendômois et al., 2009; Séralini et al., 2007, 2009), a chronic rodent feeding study on NK603 corn (Séralini et al., 2012), and a series of reproduction and development studies on maize NK603xMON810 in mice commissioned by the Austrian government and published on the Internet (Velimirov et al., 2008). The conclusions of the Séralini studies have been comprehensively rebutted in the literature and elsewhere by both academicians and regulatory authorities throughout the world (Arjo et al., 2013; FSANZ, 2011; Grunewald & Bury, 2012). Careful examination of the design, conduct, and results of the studies by Velimirov et al. (2008), which were in themselves of good quality, demonstrated that these studies, to the limited extent possible for studies of this type, were in fact supportive of the absence of novel hazards of the crops studied, contrary to the conclusions of the authors (FSANZ, 2011).

The considerable available data indicate that for those GM crops currently available, WF toxicity studies have been an unnecessary component in the assessment of their safety, yielding no results not predicted from other analyses, with essentially 100% concordance with the predictions based on agronomic and compositional analyses. A proponent of WF toxicity studies could reasonably argue, however, that the observation that such studies were unnecessary in these instances does not of itself demonstrate that WF toxicity studies are unhelpful in general or that they do not add ''assurance'' that agronomic and compositional analyses have in fact yielded the correct conclusions. A number of studies relevant to address these concerns have been conducted to explore the capability of WF toxicity studies to detect unintended events of toxicological interest. In this respect, the study by Poulsen et al. (2007b) is especially pertinent. This 90-day study in rats established what might be considered a worst case scenario, in which a crop (rice, in this case) is deliberately genetically engineered to produce a known toxin and the totality of the novel toxin protein produced (red kidney bean lectin or PHA-E lectin) is a known

rodent toxin. The GM rice was added to the rodent diet at 60% by weight. To assist in the interpretation of the findings with the GM rice, a third dose group received the GM rice at the same level of addition to the feed, but was spiked with additional PHA-E lectin. Although the study included many good design elements, such as careful balancing of nutrient intakes between study groups, the findings in the unspiked GM rice group were unremarkable, with no effects observed on histopathology, clinical chemistry, growth, or food consumption relative to body weight. The only findings consistent with the presence of lectin were small changes in relative organ weights of the pancreas, small intestine, and stomach, with all differences somewhat less than 1 standard deviation in magnitude. Without the inclusion of the additional PHA-E spiked feed group, providing the normally absent and unobtainable dose response, and knowledge of the nature of the toxin expressed, those mild findings that were consistent with PHA-E toxicity would not have been reliably discernible despite the very high proportion of the diet composed of the GM rice and the totality of the protein expressed by the transgene being PHA-E lectin.

Triggers for the conduct of WF toxicity studies

A WF toxicity study in animals of a highly toxic plant such as castor bean (ricin) (Balint, 1974) or *Strychnos nux vomica* (strychnine) would be generally able to unequivocally demonstrate the effects of the toxic constituent contained within them. Strychnine, with an LD50 of around 20 mg/kg bw in the rat (INCHEM, 2012), for example is present in *Strychnos nux vomica* seeds at approximately 1.5% (Mitra et al. 2011) or 15 mg per gram weight. To provide a dose of strychnine close to the LD50 for a rat, which normally consumes 10–20 g of food a day, nux vomica would have to be incorporated at an achievable, but not insignificant, level of 5 to 10% of the feed by weight dependent on the weight of the rat and the concentration of strychnine in the nux vomica. Such an amount of a novel toxic compound would lead to a noticeable alteration of the compositional analysis.

In the unlikely hypothetical situation in which genetic material from a toxic plant or its near relative was used in the production of a GM crop, there are specific, sensitive analytical techniques available to exclude the possibility of toxin production at far lower levels of presence than would be possible using a WF animal study. For example, relatively unsophisticated methods for the detection of strychnine are reported to be able to detect 50 ng of strychnine per sample (Kamal et al., 2012). Furthermore, there is no evidence that the insertion of a transgene from a non-toxic source into a GM crop has any greater propensity to result in the *de novo* generation of novel toxic compounds than does the range of conventional plant breeding techniques that currently require no toxicity testing (Steiner et al. 2013). Thus, it presents a considerable challenge to identify circumstances under which the conduct of a WF study is scientifically and ethically justified, taking into account the negligible probability that the use of recombinant DNA technology to produce a GM crop will randomly produce novel, unintended toxic substances *de novo*, the implausibility of any such substances being highly toxic, the low concentration of unintended substances likely to be produced, the inherent limitations of WF toxicity studies, and the lack of knowledge of what is actually being tested. The converse, identification of circumstances under which WF toxicity studies are clearly not justified or ethical, is somewhat easier. WF toxicity studies are essentially bioassays and are inherently less sensitive than the range of analytical chemistry techniques. Use of a less sensitive experimental animal method to provide reassurance of the accuracy of more sensitive analytical methods is difficult to justify scientifically or ethically. Furthermore, in circumstances in which a plausible basis for suspecting potential toxicity exists, analytical chemistry techniques of considerably higher sensitivity could be targeted toward the compound(s) or class of compound(s) of concern. A WF toxicity study is a poor analytical tool and therefore should be considered at best a last resort rather than a normal aspect of GM food safety assessment. Other more reliable, robust, and interpretable strategies should be considered first and in preference to a WF study.

WF toxicity study design considerations

Regardless of the scientific merits of WF toxicity studies, some jurisdictions are likely to continue to maintain a requirement for their conduct. The EFSA scientific opinion on the risk assessment of GM crops, for example, places substantial emphasis on the use of WF toxicity studies as a key component of risk assessment. This opinion establishes a very broad trigger for the conduct of such studies and indicates the following (EFSA, 2008a):

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

The decision to test a WF, or the fraction thereof consumed by humans or livestock, using a rodent model, presents a challenge for the application of commonly used toxicology protocols for safety assessment (Codex Alimentarius Commission, 2009; EFSA, 2008a; IFBiC, 1990; OECD, 1997). The study designer has no knowledge of the nature of the test substance or even if a test substance is actually present, in addition to a lack of knowledge of potential target organs and systems. Range finding studies, if conducted at all, are likely to be negative and so no basis for dose selection is available. In such circumstances, a negative result is uninterpretable because the investigator cannot determine whether the animals were actually exposed orally or systemically to a test substance not in the control feed; nor can they consider the likely toxicokinetics or toxicodynamics of any test substance that might be present. Consequently, such studies cannot differentiate between a negative due to species insensitivity, inadequate exposure, or lack of toxicity.

These challenges are further compounded by the fact that, with the exception of fruits and some vegetables, humans

generally do not consume raw WFs as such. More commonly, humans consume specific crop components, such as tofu or oil prepared from soybeans. Consequently, studies on WFs are of questionable relevance to normal patterns of human consumption and theoretical margins of exposure based on the whole food are unlikely to be relevant to any specific ''unknown,'' especially where that substance may be isolated to one specific fraction of the whole food.

Although these limitations cannot be readily eliminated, some strategies are available to somewhat mitigate their impact. The bulk of the composition of GM crops is well characterized. Rather than incorporate a relatively unprocessed form, such as ground corn grain or cottonseed meal (Dryzga et al., 2007; Hammond et al., 2004, 2006a,b), a more processed form, such as de-hulled, defatted, toasted soybean meal, may be more appropriate (Appenzeller et al., 2008; Hammond et al., 1996b). For soybeans, the use of toasted soybeans is necessary because anti-nutritive trypsin inhibitors present in raw soybeans cause adverse pancreatic effects in rats (Hammond et al., 1996b). Reduction of bulky WFs may be further reduced by lyophilization and grinding (Chen et al., 2003). Removal of a significant proportion of well characterized components (e.g. starch, oil), removal of components not normally consumed by humans, and processing in a manner consistent with normal food preparation practices before testing will reduce the bulk of the WF, permitting higher exposures, and ensure that the test material is consistent with that actually consumed by humans.

Once a suitable crop fraction for study has been identified, careful selection and production of comparator (control) crop(s) is required. One of the control diets should be prepared using the closest genetically related (near-isogenic) comparator crops with a history of safe use. Since geography, climate, and seasonal environmental conditions influence levels of endogenous plant constituents (Harrigan et al., 2010), it is essential that the primary comparator and the GM crop be grown in the same growing season and location with the same management conditions. With few exceptions, the 90-day studies summarized in Table 4 specify that the GM and near-isogenic comparator crops were grown in the same location and season. The GM and comparator diets must be characterized analytically prior to any actual testing to identify compositional differences that will influence interpretation of the study results. Consideration should be given to the inclusion of additional control groups utilizing related varieties of the GM crop and/or the same crops grown in different locations, to ensure that the effects of normal intercrop variation is reflected in the spread of findings across the treatment groups. In many of the published studies in Table 4, one or more additional groups of ''reference'' diets prepared with conventional non-GM crops were also included (Appenzeller et al., 2008, 2009a,b; Chukwudebe et al., 2012; Delaney et al., 2008a; Dryzga et al., 2007; EFSA, 2005a,b, 2008b, 2012b; Hammond et al., 2004, 2006a,b; Healy et al., 2008; Liu et al., 2012; MacKenzie et al., 2007; Malley et al., 2007).

A further challenge in controlling differences between treatment and comparator diets is that compositional variation may occur when growth conditions are advantageous to the GM crop but not its comparator. This includes treatment with herbicide in the field for an herbicide-tolerant crop, or growth under environmentally stressed conditions for a crop developed to withstand environmental stress. In such situations, the resulting intended compositional variation between the GM crop and its comparator can be expected to complicate interpretation of the WF toxicity study. Intentional alterations in the nutrient composition of a GM crop can also make the choice of comparator difficult, requiring the selection of an alternative crop in addition to a near-isogenic comparator. To address these variables, compositional analyses must be used to identify major deficiencies or differences in macronutrients and micronutrients. Both GM and comparator diets must be balanced to be iso-nitrogenous, to be iso-caloric, and to meet the minimum nutrient requirements of the test animals. Care must also be taken to ensure diets are free of mycotoxins, and other natural or synthetic chemicals, and that levels of phytoestrogens and other biologically active components naturally present in the test species have been determined. Failure to provide adequate nutrients in test diets will result in nutritional imbalance, and consequent alterations in test parameters, being an artifact of the treatment (ILSI, 2003).

Examples of studies with inherent limitations

The inherently low toxicological power of WF toxicity studies and/or flawed study design and failure to consider the limitations of WF toxicity studies have resulted in a number of studies of GM crops in which the data have been misinterpreted, or misrepresented, as showing adverse effects (Table 5). For example, one of the first and most widely cited reports claiming adverse effects from GM crops is that of Ewen & Pusztai (1999), which claimed that potato expressing an inserted gene encoding snowdrop lectin affected parts of the intestine when fed to rats. Some of the effects were attributed to the lectin *per se*, whereas the remainder was attributed to unknown and unintended effects resulting from transformation. Potato glycoalkaloids, known to be toxic to monogastric animals, were not measured in the potatoes used for feed. Moreover, in a critical review of the study, the Royal Society (1999) noted that other components of the diet were not measured, the sample size was too small, there were possible dietary deficiencies in the group fed the GM potato, the results were not consistent across treatments, and the statistical treatment used was inappropriate. The Royal Society review concluded, taking study design limitations into consideration, that the study conclusions were not supported by the data.

Several studies, mostly from one laboratory, have reported ultra-structural anomalies based on electron microscopic examination of the liver and pancreas of mice fed 14% GM herbicide-tolerant soybean (Malatesta et al., 2002a,b, 2003, 2005, 2008; Magaña-Gomez et al., 2008). The control mice received 14% ''wild type'' soybean in their diet, with no indication of the variety or origin of either the GM or non-GM soy used. A critical review of these studies revealed many more methodological deficiencies, the most critical of which were as follows: (1) study designs that failed to control for possible litter effects; (2) inadequate methodological procedures to ensure an unbiased, quantitative assessment; (3) inappropriate statistical methods; and (4) failure to

Table 5. Examples of limitations in experimental design, analyses, and interpretation in some WF toxicity studies with GM crops.

Best practices for WF toxicity studies	Deficiencies observed	References
Experimental design		
Identity of test and control substances	The identity of the GM test substance was not confirmed through an appropriate analytical method. Confirmation of correct control and test crop presence in diet was not conducted.	Brake & Evenson (2004), Ermakova (2007), Ewen & Pusztai (1999), Kiliç & Akay (2008), Malatesta et al. (2002a, 2002b, 2003, 2005, 2008)
Use of appropriate control crops	The control crop was not of similar genetic background to the GM test crop. In some studies the control was simply identified as a ''wild'' variety.	Brake & Evenson (2004), Ermakova (2007), Ewen & Pusztai (1999), Malatesta et al. (2002a, 2002b, 2003, 2005, 2008), Rhee et al. (2005)
	The test and control substances were not produced under similar environmental conditions. and/or no information was provided on the production of test and control substances.	Ermakova (2007), Ewen & Pusztai, (1999), Malatesta et al. (2002a, 2002b, 2003, 2005, 2008), Rhee et al. (2005)
Acceptable levels of contaminants (eg pesticides, mycotoxins, other microbial toxins) in control and test crops	Study results were not interpreted in light of differences in antinutrient or mycotoxin levels in test and control diets.	Velimirov et al. (2008), Carman et al (2013)
Nutritionally balanced diet formulations for control and test diets	Compositional analyses were not performed on the test and control substances to confirm that test and control diets had similar nutrient content and were nutritionally balanced.	Ewen & Pusztai (1999)
Description of study design, methods and other details sufficient to facilitate comprehension and interpretation	Inadequate information was provided on the source of animals used, age, sex, animal husbandry practices followed, collection, and evaluation of biological samples to confirm that the procedures followed met accepted practices.	Ermakova (2007), Ewen & Pusztai (1999), Séralini et al. (2012)
Statistical analyses and study interpretation		
Use of appropriate statistical methods for the design of the study	Statistical methods were sometimes not provided in sufficient detail to confirm if they were conducted appropriately for the data that were collected; statistical methods were documented, but were not appropriate. Estimates of statistical power were based on inappropriate analyses and magnitudes of differences.	de Vendômois et al. (2009), Ewen & Pusztai (1999), Malatesta et al. (2003, 2005), Séralini et al. (2007, 2012)
Appropriate interpretation of statistical analyses	Statistical differences were not considered in the context of the normal range for the test species, including data from historical and/or concurrent reference controls; the toxicological relevance of the difference was not considered (i.e., the reported finding is not known to be associated with adverse changes). Observed differences were not evaluated in the context of the entire data collected to determine if changes in a given parameter could be correlated with changes in related parameters.	de Vendômois et al. (2009), Ewen & Pusztai (1999), Kiliç & Akay (2008), Malatesta et al. (2002a, 2002b, 2003, 2005), Séralini et al. (2007, 2012), Carman et al (2013)
Adequate numbers of animals or test samples collected to be able to make meaningful comparisons between test and control groups	Too few animals/group were used to make meaningful comparisons; tissue sampling did not follow acceptable guidelines and was too limited to provide an accurate assessment of what was occurring in the organ being examined.	Ermakova (2007), Malatesta et al. (2002a, 2002b, 2003, 2005, 2008)
Study publication and availability		
Publication of studies in peer-reviewed journals	Circumvention of the peer-review process removes a level of review that may contribute to ensuring that WF studies are appropriately designed and interpreted.	Ermakova (2007), Velimirov et al. (2008)

GM, genetically modified; WF, whole food.

address potential confounding factors, especially those resulting from differences in the phytoestrogen content of the control and GM soybean-based feeds (Williams & DeSesso, 2010). These authors further observed that electron microscopy of selected tissues may be useful in studies designed to elucidate a chemical's mechanism of action, but it is not a recommended approach in OECD testing guidelines because the relatively small amount of tissue evaluated cannot be considered representative (Williams & DeSesso, 2010). Taken together, the conclusions regarding adverse effects of GM soybean-based diets on the subcellular morphology of selected organs were invalid.

The studies in Table 5 are informative because they highlight the importance of understanding the design challenges and inherent limitations of WF toxicity studies. Chief among these is the failure to acknowledge that WF toxicity studies are an inappropriate methodology to detect unintended changes, given the lower detection limits and relatively greater reliability and reproducibility of available analytical methods. Having decided to use a WF toxicity study, the main faults in those studies included a failure to use a near-isogenic comparator appropriate for the GM crop, failure to use GM and comparator crops grown at the same time and under the same environmental conditions, failure to adequately analyze GM and comparator crops, failure to base the studies on internationally accepted protocols for toxicology studies, and over interpretation of statistical significance in studies that lack statistical and toxicological power to distinguish differences. Even in well-designed studies, represented by many in Table 4, the evaluation of subtle effects at low doses is a challenge for which adherence to scientific principles and practical experience in this arena is an absolute necessity for proper scientific interpretation. Design limitations can render it virtually impossible to draw any inferences from some published WF toxicity studies (EFSA, 2008a) (Table 5). The inherently low sensitivity of WF toxicity studies to detect toxicological effects is such that the results of even well-designed and well-conducted studies yielding negative results are of highly questionable value in the risk assessment of GM crops.

Conclusions and recommendations

A resolution of the international regulatory controversy over the necessity, value and ethics of WF toxicity studies on GM crops is likely to involve societal and political considerations that science at times seems unlikely to greatly influence. The wealth of data now available provides a basis for resolving at least the scientific aspects of the controversy. This paper began by breaking down the basis of the controversy into a series of postulates that were discussed in the context of the body of data now available. An examination of these leads to the conclusion that there is currently no evidence that normal commercial GM crop production can or does produce unintended, unexpected, compositional effects of human toxicological significance, and that compositional and agronomic analysis separately or in combination have, without exception, accurately predicted negative results for WF toxicity studies. This outcome was predictable based on nearly a century of experience of WF toxicity studies on irradiated food involving tens of thousands of experimental animals of multiple species. Food irradiation is known to produce unintended compositional changes, albeit at low concentrations, and most particularly to reduce the levels of some micronutrients that are vulnerable to attack by free radicals produced by radiolysis of water and other cellular constituents. Although it has been possible to demonstrate alteration in food composition in animal studies in which a sensitive animal model is available that specifically relates to the depleted vitamin (e.g. cats and vitamin A), chemical analytical methods have been shown to be more sensitive and reliable in each case. The following conclusion of the joint FAO/IAEA/WHO review of these studies (WHO, 1999) remains relevant to current discussions on the testing of GM crops using WF toxicity studies:

> The application of ''risk assessment'' in the currently accepted sense is not appropriate to the toxicological assessment of foods preserved by high-dose irradiation. In this context, the concept of ''substantial equivalence'' may be more appropriate.

The protracted animal testing of multiple combinations of food and irradiation dose, despite clear evidence of a lack of need, and utility of, such studies provides a close parallel to the current situation with data requirements for GM crops in some jurisdictions. Thus, what can be learned from a consideration of the irradiated food experience is that even where a wide range of compositional changes have been demonstrated to have occurred through chemical analysis, albeit at low concentrations, WF toxicity studies were generally insufficiently sensitive to detect the altered composition.

Although advances and new developments continue in the field of plant biotechnology, the technology and its application are no longer new. Concerns expressed 20 years ago around the potential for the unexpected generation of toxic substances, unrelated to either the source of the transgene or the transformed parent line, while arguably tenuous, were understandable in the context of the state of the science at the time. Since the introduction of GM food crops, a wealth of new data has been generated on plant genome plasticity. It is now known that plant genomes change over generations through the natural mechanisms of transposon relocation and background gene mutation. In the context of this understanding, concerns over inadvertent, *de novo* negative effects of targeted transgene insertion are biologically implausible (Weber et al., 2012). The potential for upregulation or alteration of genes responsible for the production of endogenous toxins naturally present in crops such as potatoes is somewhat less tenuous but of equal concern with conventional breeding; these however, are not *de novo* hazards arising spontaneously. Fortunately such possibilities can be readily examined using sensitive analytical techniques and are used in plant breeding to monitor levels of naturally occurring toxins that are identified in OECD consensus documents for specific crops (Steiner et al., 2013). In this context, the requirement for relatively extensive safety evaluation and WF animal toxicity studies is disproportionate to the known risks and is discordant with the extensive data available.

Risk assessment is a multidisciplinary undertaking and toxicology is only one of the many disciplines required for a robust risk assessment. In practice, assessments of potential human health risks of GM plants do not hinge on the findings of WF toxicity studies. For regulators, risk assessment begins at the completion of the commercialization of a new GM crop, whereas for GM crop developers, safety assessment begins at the conceptual or design phase, continues through development and agronomic selection cycles, and is confirmed by compositional analysis once a new product has been selected for commercialization. Although some would argue that the absence of detectable adverse effects over the multitude of WF toxicity studies conducted with a robust study design on GM crops serves to confirm GM crop safety, it is equally arguable that WF toxicity studies have limited sensitivity to detect low levels of toxicologically significant components in whole food and do not provide additional reassurance of GM food safety beyond that which is already provided by more sensitive, reliable, and robust techniques such as comparative molecular, agronomic, and compositional analyses. Indeed, there is no evidence arising from the WF toxicity studies conducted to date that would bring into question the adequacy, reliability, and sufficiency of compositional and/or agronomic assessment as the principle basis for risk assessment of new GM crops. Consequently, the recommendation from this review is that the routine conduct of, or requirement for, WF toxicity studies is not supported scientifically and may be considered unethical given their, at best, limited contribution to the safety assessment of the test material. This conclusion has recently been supported by a review of more than 139 projects conducted by the European Commission over the past 25 years, involving more than 500 independent research groups and representing European research grants of more than €200 million – in which the conclusion of all of that research was that ''biotechnology, and in particular [GM Organisms], are not more risky per se than e.g. conventional plant breeding technologies'' (European Commission, 2010b). Furthermore, increasing the duration of a WF toxicity study from 28- or 90-days to a long term or chronic study cannot be expected to correct for the inherent limitations of WF studies.

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Declaration of interest

The employment affiliation of the authors is shown on the cover page. A. Bartholomaeus was, prior to retirement, the General Manager of the Risk Assessment Branch of Food Standards Australia New Zealand (FSANZ). W. Parrott does public sector research with genetically modified crops. G. Bondy is employed by Health Canada; the contents of this paper do not represent official policy of Health Canada, FSANZ, or other Canadian or Australian government agencies. The preparation of this manuscript was supported by the members of a task force of the ILSI International Food Biotechnology Committee: BASF Plant Science; Bayer CropScience; Dow AgroSciences LLC; Pioneer, a DuPont Company; Monsanto Company, and Syngenta Biotechnology, Inc. This paper has been reviewed by individuals internationally recognized for their diverse perspectives and technical expertise. However, it must be emphasized that the content of this document is the authors' responsibility and not the reviewers, and it does not represent an endorsement by the reviewers' institutions. While the authors carefully considered the views and comments of the various reviewers and the sponsors, the final analyses, interpretations and conclusions drawn are exclusively those of the authors.

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