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Core and Supplementary Studies to Assess the Safety of Genetically Modified (GM) Plants Used for Food and Feed

Phil Brune[a,*], Suma Chakravarthy[b,1,*], Gerson Graser[a,*], Carey A. Mathesius[c,*], Scott McClain[a,2,*], Jay S. Petrick[d,*], Alaina Sauve-Ciencewicki[a,*], Barry Schafer[c,*], Andre Silvanovich[d,*], Kent Brink[c,**], Kristina Burgin[a,**], Dean Bushey[d,**], Matthew L. Cheever[e,**], Thomas Edrington[d,**], Huihua Fu[e,**], Veerle Habex[f,**], Rod Herman[c,**], Emir Islamovic[e,**], Elizabeth A. Lipscomb[e,**], Shawn Motyka[e,**], Laura Privalle[e,**], Rakesh Ranjan[e,**], Jason Roper[c,**], Ping Song[c,**], Gregory Tilton[d,**], John Zhang[c,**], Stephen Waters[g], Adela Ramos[c], Angela Hendrickson Culler[d], Penny Hunst[e], Rachel Gast[a], Debbie Mahadeo[d], Laurie Goodwin[b,3]

[a] *Syngenta, Crop Protection, LLC., Research Triangle Park, NC*
[b] *CropLife International, Washington, DC*
[c] *Corteva Agriscience ™, Johnston, IA*
[d] *Bayer, Crop Science Division, Chesterfield, MO*
[e] *BASF Corporation, Research Triangle Park, NC*
[f] *BASF BBCC, Innovation Center Gent, Belgium*
[g] *Waters International Consulting, Belgium*

Abstract

Genetically modified (GM) plants used for food and feed have an established history of safe use over more than 25 years of their commercialization. Developers and regulatory authorities have accumulated extensive experience in evaluating their safety over time. The studies required for the safety assessment of GM plants used for food and feed should now be re-defined to leverage this experience and increased scientific knowledge. This paper, a companion paper for Waters et al. also published in this issue, presents a systematic approach for the safety assessment of newly expressed proteins (NEPs) in GM plants by evaluating the two components of risk: hazard and exposure. Although the paper focuses on NEPs, the principles presented could also apply to other expression products that do not result in a NEP. A set of core studies is recommended, along with supplementary studies, if needed, to evaluate whether the GM plant poses risk. Core studies include molecular and protein characterization and hazard identification encompassing toxicity and allergenicity. In the absence of hazard, core studies are sufficient to conclude that GM plants are as safe as their conventional counterparts. Depending on the GM trait and intended use, supplementary studies should be performed to characterize hazard and exposure when a hazard is identified. Problem formulation should be used to identify hypothesis-driven supplementary studies. Acute toxicity studies, compositional assessment, and dietary exposure assessment are recommended to be hypothesis-driven supplementary studies. Further discussion on the current food and feed safety assessment landscape for GM plants and the use of problem formulation as a tool for identifying supplementary studies can be found in the companion paper [62].

Keywords: genetically modified, safety assessment, food and feed, hazard, exposure, risk, core studies, supplementary studies

Abbreviations: Codex, Codex Alimentarius Commission; FAO, Food and Agriculture Organization of the United Nations; GM, genetically modified; HPP, hydroxyphenylpyruvate; NEP, newly expressed protein; OECD, Organization for Economic Co-operation and Development; ORFs, open reading frames; RNAi, RNA interference; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SNPs, single nucleotide polymorphisms; WHO, World Health Organization; WOE, weight-of-evidence

*These authors contributed equally to the paper, are listed alphabetically and considered co-first authors.

**These authors contributed equally to the paper, are listed alphabetically and considered co-second authors.

[1] This work was performed when Suma Chakravarthy was employed by CropLife International. Present affiliation: United States Department of Agriculture, Animal and Plant Health Inspection Service, Riverdale, MD.

[2] This work was performed when Scott McClain was employed by Syngenta Crop Protection, LLC. Present affiliation: SAS Institute, Inc, Cary, NC.

[3] Corresponding author: Laurie Goodwin,

1. Introduction

Since the commercial introduction of genetically modified (GM) plants in 1994, regulatory decisions have been made internationally to authorize their use for food and feed and for cultivation [34, 35]. In 2003, the Codex Alimentarius Commission (Codex) published guidelines for conducting safety assessments of GM plants [8] that have constituted the basis for developers and global regulatory authorities to evaluate their safety. To date, regulatory agencies have issued over 3,500 approvals for the use of GM plants for food and feed [34]. Although experience and scientific knowledge about GM plants has expanded, regulatory requirements for scientific data have been increasing disproportionately with the observed potential for risk [33, 71]. Even with the continued relevance of the Codex guidelines, there is an opportunity to leverage both the familiarity and established history of safety of GM plants to revise the safety assessment approach, given the expanded experience of product developers, regulatory authorities, and researchers. As further discussed in Waters et al., current scientific understanding and experience warrants redefining the studies that are sufficient to evaluate whether a GM plant is as safe as its conventional counterpart [71].

Safety assessment is part of an overall risk analysis [8]. Risk is a function of hazard and exposure [14]:

(Risk = Hazard x Exposure)

Lack of either hazard or exposure would imply that there is no risk. If it is determined that the newly expressed protein (NEP) presents both a potential hazard and potential exposure risk, a problem formulation approach considering both familiarity and the history of safe use should be used to identify specific questions relevant to the safety of the GM plant [5]. Evaluation of these identified risks should then be conducted according to the Codex science-based process employing a stepwise approach to hazard identification, hazard characterization, exposure assessment. and risk characterization [74]. It is recommended to evaluate hazard and exposure systematically during the safety assessment process using a problem formulation approach [71], where the broad 'problem' (i.e., food and feed safety of the GM plant) must be addressed based on the specific trait introduced, host plant, and intended use. Hypotheses that address specific safety questions must be framed, and study designs developed, to address these questions. Although a hazard-led approach has typically been followed for safety assessment of GM plants [14], exposure-based approaches for risk assessment have also been discussed recently [41, 52]. It is important to perform hazard identification studies as a basis for safety assessment of GM plants, although the approach for assessing potential hazards for these products is reconsidered in this manuscript based on knowledge and experience gained to date. Exposure-led studies, which are performed for small molecules [17], can also be helpful if relevant to the NEP and its expression in the GM plant when hazards are present.

In this paper, a core set of studies is recommended that is focused on characterization and safety assessment of the introduced trait. These recommendations are modified from earlier guidelines and recommendations for the safety assessment of GM plants (e.g., Codex, 2009 [9]; Delaney et al., 2008 [14]). A schematic overview of the recommended core and supplementary studies is available in Figure 1. Using the data resulting from the recommended core studies, and employing a "problem formulation" approach, the need for supplementary hypothesis-driven or case-by-case studies can be determined.

Depending on the nature of the introduced GM trait and intended use, supplementary hypothesis-driven or case-by-case studies may be further needed to complete the safety assessment. As outlined in Waters et al. [71], when the weight-of-evidence from core studies is not sufficient to determine the absence of hazard, supplementary studies may provide additional hazard characterization and/or exposure characterization to better understand the hazard presented by the NEP. As an example, one of the studies proposed to be supplementary is dietary exposure assessment, which is unnecessary if the weight-of-evidence [18] supports a conclusion of low or negligible hazard associated with consumption of a GM plant [41]. However, if the weight-of-evidence failed to provide support for a low or negligible hazard conclusion, a supplemental dietary exposure assessment and other supplemental data may be necessary to conclude on risk.

As previously discussed, thousands of safety assessments conducted globally have been consistent in their outcomes. Consequently, some jurisdictions have chosen to implement a streamlined and pragmatic approach to regulate GM plants for food or feed use by empowering the appropriate governmental body to authorize products based on the safety determinations of authorities in one or more other countries. This allows for efficient use of regulatory resources while maintaining a high level of safety for human/animal health and the environment. This approach to regulation is also embedded in the Codex guidelines which clearly state that "*where appropriate, the results of a risk assessment undertaken by other regulatory authorities may be used to assist in the risk analysis and avoid duplication of work*" [8].

The recommendations presented in this paper build on earlier guidelines and recommendations for the safety assessments of GM plants, and also incorporate the history of safety and familiarity that can be employed after 25 years of commercial use (e.g., Codex, 2009 [9]; Delaney et al., 2008 [14]) towards standardizing those assessments.

Core Studies: Characterization and Safety Assessment

It is noted that there may be alternative newly expressed substances that are not addressed in this manuscript; however, the principles presented could also apply to other expression products that do not result in a NEP.

The suggested core studies for typical (i.e., sexually propagated) GM plants producing a NEP are:

2.1 *Molecular Characterization*

Email: lauriegoodwin@croplife.org, Phone: 202-365-5059

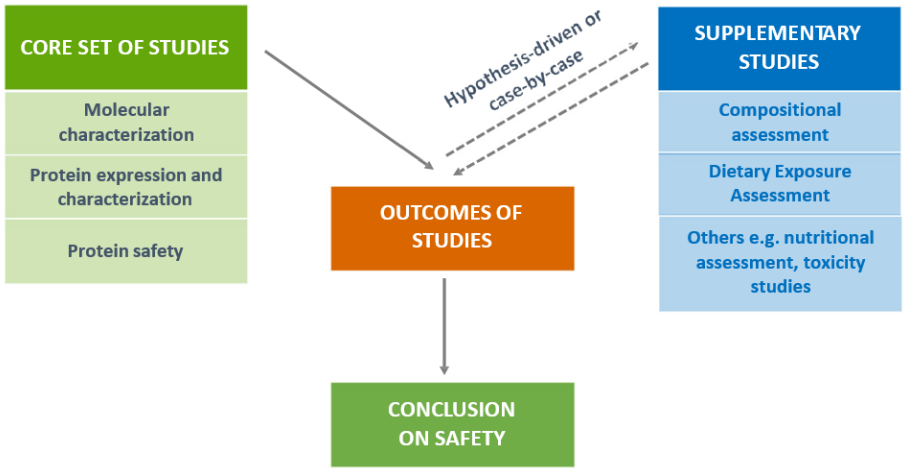

Figure 1: Schematic representation of core and supplementary studies for typical GM plants (reprinted from *Recommendations for science-based safety assessment of genetically modified (GM) plants for food and feed uses* [62]. Core studies are a set of studies necessary for a science-based risk assessment of a GM plant. These are suggested core studies for typical GM plants. There may also be alternative newly expressed substances (e.g. RNAi). Supplementary studies are studies to be conducted upon identification of information and/or hypothesis that indicates increased risk to human or animal health. The conduct of these studies depends on the nature of the introduced trait, intended use and data obtained from core studies.

2.1.1 *Number of insertion loci and inserts per locus*

2.1.2 *Presence or absence of unintended sequences (e.g., plasmid backbone)*

2.1.3 *Sequence of the inserted DNA*

2.1.4 *Stability of inserted DNA across multiple generations*

2.2 *Protein Expression and Characterization*

2.2.1 *Core characterization of the NEP isolated from the GM plant*

2.2.2 *Determining that the surrogate protein test substance and the plant-produced protein are sufficiently similar: Core comparative studies*

2.2.3 *Quantification of NEP expression levels in planta*

2.3 *Protein Safety: Hazard Identification Encompassing Toxicity and Allergenicity*

2.3.1 *Toxicological Assessment*

2.2.2 *Allergenicity Assessment*

Supplementary Studies

Supplementary studies should be conducted when core studies identify a hazard or are not sufficient to conclude a negligible risk, or when certain GM traits require additional analyses for complete characterization. Problem formulation can be used to design hypothesis-driven studies to answer specific safety questions. Depending on the nature of the NEP, case-by-case studies may be required for complete characterization. Examples of supplementary studies include:

3.1 *Protein abundance*

3.2 *Processing*

3.3 *Resistance to digestion*

3.4 *Toxicity studies*

3.5 *Compositional assessment*

3.6 *Dietary exposure assessment*

3.7 *Case-by-case protein characterization studies*

3.8 *Nutritional assessment*

3.9 *Immunoglobulin E binding*

GM traits could be the outcome of the expression of NEPs, double-stranded RNA to target silencing of a target pest gene, or altered expression of endogenous proteins. Studies described in this paper focus on traits derived from NEPs, and although the other types of modifications are not discussed in detail here, they may be mentioned or referred to, with the principles discussed in this paper still being applicable.

2. Core Studies: Characterization and Safety Assessment

The integrity and genetic stability of the introduced DNA and expression of the trait should be evaluated for all GM plants. Molecular and protein characterization are core characterization studies. Some data obtained from these studies also inform certain aspects of the protein safety assessment.

2.1. Molecular Characterization

Molecular characterization contributes important data and information that underlies the safety assessment of GM plants according to both Codex and FAO/WHO principles and guidelines. While the molecular characterization of GM plants is not a safety assessment in and of itself (nucleic acids are generally regarded as safe), it helps confirm the novel gene product(s) [74]. Molecular characterization elucidates the molecular changes that have been introduced into the plant during the transformation process. There are four primary endpoints for molecular characterization of a GM plant: (1) Number of insertion loci and inserts per locus; (2) Presence or absence of unintended sequences (e.g., plasmid backbone); (3) Sequence of the inserted DNA; and (4) Stability of the inserted DNA across multiple generations.

2.1.1. Number of insertion loci and inserts per locus

Some current established techniques employed to transfer genes into plant cells, including *Agrobacterium*-mediated transformation and particle bombardment, could result in random integration of insert(s) into the recipient genome [2]. Furthermore, using these transformation methods there is no control over the number of integrations (inserts) or whether the DNA transferred is complete, truncated, or rearranged. Determining the number of inserts integrated in the GM plant genome is a necessary molecular characterization endpoint to support risk assessments described in subsequent sections.

Transgene copy number can be positively or negatively associated with transgene expression and associated with inheritance/segregation patterns from generation to generation. Therefore, determining the number of insertion locations (loci) and number of inserts per location (locus) in the GM plant genome is a useful molecular characterization endpoint. For example, confirmation of a single locus containing a single transgene can help ensure that there are no unexpected anomalies in transgene expression levels that could impact expressed trait protein levels. In some GM plants, such a confirmation could provide assurance of heritable product efficacy and quality.

2.1.2. Presence or absence of unintended sequences (e.g., plasmid backbone)

A plant transformation plasmid is usually composed of the DNA that is intended to be transferred to the recipient plant genome for the intended trait, and a plasmid backbone. The plasmid backbone contains origin(s) of replication and selectable marker(s), as well as sequences that allow for the propagation and maintenance of the plasmid in bacteria, including *Agrobacterium*. The microbe-derived origin of replication and selectable marker genes in the plasmid backbone, however, are unnecessary for trait gene expression in the plant cell. Although the presence of the plasmid backbone fragment in an event has not resulted in any safety concerns [56], confirmation that no plasmid backbone DNA or any other plasmids used in transformation process have been inserted into the genome of the transgenic plant remains an important characterization endpoint.

2.1.3. Sequence of the inserted DNA

It is important to sequence the inserted DNA, with special emphasis on the transgene(s) to ensure that the predicted protein(s) sequence would be produced. Through translation of the observed transgene nucleotide sequence, protein-based bioinformatics that address potential allergenicity or toxicity can be performed. To fully characterize the insert DNA, obtaining the genomic flanking sequence is also necessary to confirm the termini of the insertion.

Mutations such as single nucleotide polymorphisms (SNPs), truncations, and re-arrangements such as inversions, insertions/deletions, duplications, and translocations within the DNA insert, and between the insert and the integration site of the recipient genome (i.e., flanking genomic DNA or flanking site), have been reported in transgenic plants [6, 38, 62]. However, the genomes of plant species are dynamic and possess natural variability arising from events like single-nucleotide changes, transposon insertions, and horizontal gene transfer [37]. Moreover, conventional breeding techniques have a much larger impact on the plant genome compared with plant transformation [62]. Therefore, while sequencing of the inserted transgene DNA provides the most accurate information of integrated sequence(s) and variations that may have occurred, the studies assist in characterization of the GM event rather than providing data to inform the safety assessment.

2.1.4. Stability of inserted DNA across multiple generations

Trait stability is part of any successful breeding program regardless of the technique used, be it GM or conventional. Molecular stability of the inserted DNA from generation to generation can be affected by multiple factors (e.g., genetic recombination) [47]. Testing across a minimum of three generations should provide sufficient data to demonstrate generational stability of the introduced trait [55].

A transgenic insert located in the nuclear genome is expected to follow Mendelian segregation principles [44]. Although not unique to GM plant insertions, there are some instances when non-Mendelian inheritance of transgenes occurs in a variety of crops due to transgene deletion, duplication, or rearrangement [70]. Structural variations are also observed in conventional diploid and polyploid crops [76]. In this situation, the developer may choose to discard the transgenic event if it exhibits instability of the desired phenotype [76]. Transgenic inserts that are in organellar genomes, such as plastids, are expected to be inherited maternally, in which case Mendelian segregation principles would not apply [24]. Plants that are propagated through asexual reproduction (e.g., vegetatively) would also not follow Mendelian inheritance patterns. Non-Mendelian inheritance in these instances are not considered to be instability of the trait and demonstration of molecular stability is not necessary.

Further studies, routinely required to complete the molecular characterization requirements established by certain regulatory authorities, should not be considered core or supplementary studies, because they do not inform the safety assessment. These studies are discussed in Box 1.

Box 1: Bioinformatic assessments enabled by molecular characterization that do not provide additional value to the safety assessment

The assessments below are typically performed to meet current registration requirements in specific jurisdictions, but are not universally required and do not inform the safety assessment for GM plants.

Open Reading Frames (ORF) bioinformatic analysis: The bioinformatic assessment of ORFs in the insert and adjacent flanking genomic sequence is performed to determine if non-canonical transcription and/or translation can yield a novel protein sequence that is allergenic, toxic, or displays some other undesirable characteristic such as inhibition of proteases or nucleases found in animal digestive systems. Unlike the bioinformatic evaluation of the actual transgene-encoded protein itself (which is confirmed through protein characterization studies), the analysis of potential ORFs created due to transgenic insertion is theoretical and disregards basic biological processes. Theoretical ORF analysis provides no additional value to a safety assessment, unless the ORF contains a contextually correct initiation codon and is appropriately located relative to promoter and terminator (or gene expression) elements. Such detail would be uncovered through inspection of the organization of genetic elements in the transgenic insert sequence, which is included within the core molecular characterization studies. Furthermore, the potential for an unintended ORF generated through transgenic insertion is no greater than that for conventional breeding, and a safety issue arising from such random events is statistically negligible, consistent with the history of safety for new crop varieties (both conventional and GM) [62].

***Repeated bioinformatic analyses for endogenous genes*:** Although obtaining genomic flanking sequences for determining whether an endogenous gene was disrupted is required by some regulatory authorities, this analysis is not necessary to support the safety assessment of GM crops, as the risks associated with disrupting a gene by insertion of transgenic DNA is the same or less than that for conventional breeding [62]. When bioinformatic analyses are performed with well-annotated genome assemblies, definitive conclusions on the interruption or deletion of endogenous genes can be drawn. The database update of genomic assemblies including new information on gene annotation, function, etc., should not alter the conclusions made initially if the region of the recipient genome is not changed in the updates. Since gene disruption is not a unique risk of transgenesis, repeated bioinformatic analyses do not add value to safety assessments.

2.2. Protein Expression and Characterization

Protein characterization and *in planta* expression studies are part of the core characterization of GM plants in which NEPs are introduced (Figure 2). The objective of protein characterization studies is to confirm the identity of the NEP and to verify that the protein is expressed in the plant as intended. The objective of *in planta* protein expression studies is to quantify the levels of the NEP under representative growing conditions, to enable protein exposure assessments for humans and animals if there is uncertainty about protein hazard.

Since it is generally not feasible to isolate large amounts of NEP from the plant due to low concentrations, heterologous production of a surrogate protein test substance in another expression system is often necessary and aids to provide sufficient protein for both characterization and safety studies [57]. In this latter case, a further objective of protein characterization studies is to ensure that the protein test substance produced exogenously is a suitable surrogate and is sufficiently similar to the plant-produced protein for the purposes of safety assessment studies.

The sections below outline the core studies that are essential to characterize the NEP and describe the studies that are essential to establish that a surrogate protein test substance is sufficiently similar to the plant-produced protein for the purposes of safety assessment studies. To date, many of the proteins expressed in GM plants have been isolated and purified from either the plant or heterologous systems. However, intractable proteins - those that can be difficult to express or challenging to isolate in a functional form (e.g., membrane proteins, transcription-factors) - may require alternative approaches to establish protein safety [3, 39].

2.2.1. Core characterization of the NEP isolated from the GM plant

a) Molecular Weight: Determining the molecular weight of the protein expressed by the plant and comparing it with the theoretical mass calculated using the inserted DNA sequence and any known or intended proteolytic processing sites provide a key indication that the NEP is being expressed in the GM plant as intended. Knowledge of the molecular weight of the NEP also provides indications of any post-translational modifications (e.g., glycosylation, proteolytic processing, etc.) and may allow further insights into relevant characteristics of the protein, such as the formation of quaternary structures.

b) Amino Acid Sequence: The amino acid sequence of the NEP provides information about any protein processing that may occur in the plant, such as N-terminal methionine cleavage. While it is often not feasible to obtain com-

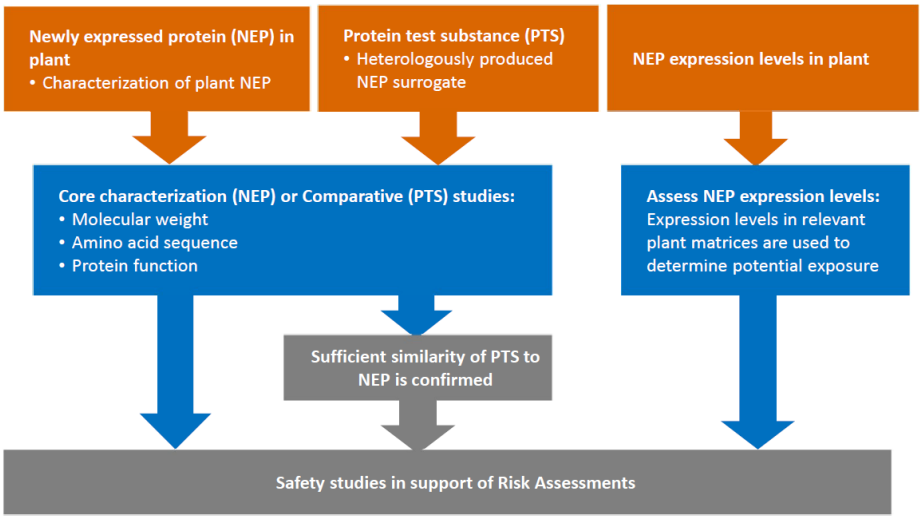

Figure 2: Schematic overview of the expression and characterization of newly expressed proteins (NEPs) in genetically modified (GM) plants

plete amino acid sequence coverage for the NEP isolated from the plant, determining a partial amino acid sequence and ensuring that it matches the complete inserted DNA sequencing results and molecular weight data will further determine protein identity. The adequacy of the level of amino acid sequence coverage should be assessed on a case-by-case basis, depending on the type of protein.

c) Protein Function: It is important to confirm that the NEP functions as expected. For NEPs that function as enzymes, functional activity analysis verifies that the NEP has the intended activity. For insecticidal proteins, an insect bioassay is usually conducted on a target organism to determine potency against target organisms. When the functional activity of a protein cannot be measured *in vitro* or in laboratory bioassays, the characterization and safety assessment must rely on alternative weight-of-evidence (WOE) information (e.g., field or trait performance data can provide important indirect evidence for the functional expression of the NEP in the newly designed GM plant).

2.2.2. *Determining that the surrogate protein test substance and the plant-produced protein are sufficiently similar: Core comparative studies*

A surrogate protein test substance of appropriate purity can often be produced in microbial organisms such as Gram-negative bacteria (e.g., *Escherichia coli*, *Pseudomonas fluorescens*), Gram-positive bacteria (e.g., *Bacillus* sp.), yeast, fungi, or in other cell culture systems such as insect cells or plant cells, as described in detail by Raybould et al. [57]. When an adequate amount of protein test substance is obtained, it is necessary to confirm that it is suitable as a surrogate for the plant-produced protein in subsequent characterization or safety assessment studies. The protein test substance and plant-produced protein need not be 100 percent identical in their characteristics if any observed differences do not impact functional or biochemical properties of the test protein, as described previously [57].

The determination of sufficient similarity is based on a weight-of-evidence approach, following comparisons of properties of the protein test substance and plant-produced protein, to confirm suitability for use in protein safety assessment studies [57]. The comparative studies are discussed below. Similarity in these pertinent attributes of the proteins derived from both sources allows them to be used interchangeably in protein characterization and safety studies.

a) Molecular Weight: Molecular weight of the protein test substance and plant-produced protein should be compared to assess similarity. If differences exist, it will be necessary to understand whether the differences arise because of changes in the amino acid sequence or post-translational modifications.

b) Amino Acid Sequence: Amino acid sequence comparison of the protein test substance and the plant-produced protein is important in establishing sufficient similarity. The amino acid sequences do not necessarily need to be complete, nor do the sequences need to be identical, for the protein test substance to be considered suitable for use in safety studies, as described in detail by Raybould at al. [57]. For example, minor changes to the plant-produced protein (e.g., single amino acid substitutions, N-terminal modification, affinity tags added to aid purification, or differential cleavage of N-terminal targeting peptides) may be considered acceptable when there is evidence indicating that the changes do not impact biochemical and functional properties relevant to the safety assessment.

c) Protein Function: If the NEP has a measurable functional activity (e.g., enzyme, receptor, insect-toxin, etc.), determination of the functional activity of the protein test

substance and the plant-produced protein contributes to the weight-of-evidence assessment of sufficient similarity, even when the activity levels are not quantitatively equivalent [57]. In instances where the functional activity of a protein cannot be measured (e.g., protein cannot be isolated in a functional form) or an *in vitro* assay does not exist, the comparative studies must rely on alternative weight-of-evidence information to confirm the suitability of the protein test substance as a surrogate, as suggested, for example, by Bushey et al. and Delaney et al. [3, 14]. For proteins with no activity, or where activity is not readily measurable (e.g., transcription factors, storage proteins, or plant resistance proteins [R-proteins]), a protein functionality comparison is not applicable.

2.2.3. Quantification of NEP expression levels in planta

When an exogenous protein is being expressed in the GM plant, or expression of an endogenous protein has been intentionally altered, quantitative information about protein expression levels in GM plants provides important information in support of the risk assessment. Protein expression data enable an accurate assessment of human and animal exposure and would form the basis for certain safety studies and an exposure-led safety assessment. For example, if abundance or dietary exposure assessment studies are deemed necessary for the food safety assessment (Section 3), protein expression levels enable assessment of exposure. In cases when a hazard is identified and hazard characterization is necessary, determination of NEP expression levels in relevant plant matrices is important. Expression data are also needed for calculating safety margins in certain toxicology studies performed for hazard characterization [60]. In cases where novel plant traits are enabled without NEPs, e.g., by the silencing or over-expression of an endogenous plant protein, the expression level of the impacted endogenous protein (or an appropriate surrogate endpoint) should still be measured to understand the potential impact on safety.

The levels of NEP in plants can be influenced by environmental factors. Therefore, analyzing plants grown in field trials is desirable for determining expression levels under commercially relevant conditions.

2.3. Protein Safety

Following molecular and protein characterization, hazard identification encompassing toxicity and allergenicity should be conducted, and the outcome of this step and other core studies will determine the need for additional supplementary studies. A brief background discussion of toxicity and allergenicity assessments that may supplement the core studies on a case-by-case basis is provided below. Further detail on these assessments can be found in Roper et al. [60] and McClain et al. [42].

2.3.1. Toxicological Assessment

As a result of the acidic conditions and digestive enzymes of the gastrointestinal tract, dietary proteins are typically rapidly degraded into small peptides and individual amino acids before absorption and metabolic use by the body. Some biological barriers may restrict the oral bioavailability of intact proteins after dietary consumption. Several factors may affect protein such as ionic charge and lipophilicity. Additionally, protein size may be a consideration as systemic absorption of any orally consumed substance is typically inversely proportional to its molecule size [21]. Proteins resistant to degradation by digestive enzymes may have limited systemic uptake due to their large molecular weight (e.g., lectin proteins). The effectiveness of these biological barriers has been demonstrated through the unsuccessful attempts to orally administer proteins for therapeutic purposes [23, 25, 46, 63]. Therefore, as also concluded in Roper et al., consumption of proteins is not normally associated with adverse effects, and additional studies to confirm the dietary safety of a protein should only be conducted on a case-by-case basis where there is an identified hazard (see Section 3.4) [60].

Applying a weight-of-evidence approach, key hazard identification studies for toxicity are required to assess the safety of all NEPs [14]. Hazard identification can be built by evaluating the four elements described below.

(1) History of safe use of the NEP: Probable dietary safety of the NEP can be established through a history of safe consumption of closely related proteins (considering both structure and function) by humans and/or animals [1, 9, 14]. To demonstrate history of safe use, evidence of structural and/or functional similarity and exposure to other endogenous proteins found in foods or other species expressing these proteins or similar proteins is necessary [26, 42, 60]. However, the absence of a clear history of safe use does not automatically indicate a hazard, only that some further evidence and analysis is needed for the safety assessment.

(2) History of safe use of the source organism: The history of safe use in the food or feed chain of the source organism for the gene encoding the NEP provides additional evidence about the safety of the protein. The safe consumption of the source organism indicates that the NEP should also have limited potential for allergenicity, toxicity or other anti-nutrient for animals or humans [13, 42, 60].

(3) Bioinformatics for sequence comparison: Bioinformatic screens are an excellent tool for placing a protein within the context of related proteins based on recognizing localized homologies, common domains, and larger protein families or super-families. This screen should be done early in the hazard identification phase and can be useful in providing the preliminary protein and protein family context that will help determine the scientific rationale for conducting supplementary toxicology studies. However, bioinformatics results should not be regarded as necessarily indicative of toxicity, and any hazard prediction based upon bioinformatic results must subsequently be examined in conjunction with other data from core studies when assessing risk.

(4) Mode of action and functional specificity: The potential of the NEP as an allergen, toxin, or anti-nutrient can also be established by understanding the mode of action and

functional specificity of the protein. If the mode of action and functional specificity of the NEP are well understood and have been shown to have low relevance to humans or animals, this provides confidence that it is unlikely to cause harm when consumed.

2.3.2. Allergenicity Assessment

Since the initial Codex guidance documents for allergenicity assessment of GM plants were published, improved tools have been developed to more accurately and precisely identify allergens [42]. With current knowledge of molecular biology, genomics and bioinformatic techniques, a revised approach for assessing the allergenic potential of NEPs is warranted, hinging on the standard risk equation (Risk = Hazard x Exposure). Since there is no single test or predictive assessment to establish whether a protein will act as an allergen, hazard identification and exposure characterization require measurement of several physiochemical properties. In *Allergy risk assessment for newly expressed proteins (NEPs) in genetically modified (GM) plants*, a stepwise approach is recommended where hazard identification is first performed for all NEPs [42]. If a hazard is identified, exposure characterization should be done (supplementary study). Fundamental to this allergenicity assessment is the degree of similarity of the NEP to known allergens.

a) History of safe use of the NEP and familiarity with the source organism: These concepts are one of the fundamental and initial elements in the overall safety assessment and are used to evaluate potential for allergenicity in a manner similar to the evaluation of toxicity (see Section 2.3.1).

b) Amino Acid Sequence Similarity and Bioinformatics: The best use of bioinformatics for protein safety assessments is the combination of a thorough understanding of existing allergens with a coordinated review of putative allergens and their placement into a qualified database [11]. To enhance the accuracy and reliability of bioinformatic assessments for allergenic potential of NEPs, a stepwise approach is recommended as below; conclusions from step 1 would determine the necessity for further analyses described in steps 2 and 3:

   1. *Sequence level consideration*: Bioinformatic algorithms evaluate sequence identity and similarity, and the probability that two sequences share structure and common evolutionary origin. Such relationships also provide a measure of likely physicochemical similarity among proteins that might reflect immunoglobulinE (IgE) cross-reactivity between a NEP and known allergens. Conventional linear sequence-based algorithms Fast All (FASTA) and Basic Local Alignment Search Tool (BLAST) are used for these analyses and expectation value (E-value or E-score) is the typical statistical measure of relatedness.
   2. *Structural relatedness*: The potential of cross reactivity can be assessed by determining if a NEP shares structural features with known allergens. The degree to which structure is compared can include determination as to whether the NEP is in the same protein family as known allergens, if it shares a domain with known allergens or, at the finest level of granularity, if the NEP contains known IgE-binding epitopes. Such structural comparisons then contribute to a weight-of-evidence conclusion.
   3. *Structural considerations*: Three-dimensional modelling offers a more sophisticated measure of similarity between a NEP and an allergen, but it would need to be performed based on the results of sequence level analyses. The knowledge of any specific allergens and their associated epitopes and other clinically relevant sequence mapping is a key to understanding similarity with the NEP.

2.4. Outcome of Core Studies

If no hazard is identified after conducting core studies, further hazard and exposure characterization for GM plants should not be required according to established principles for risk analysis. In this case, core studies alone would be sufficient to conclude that the GM plant has negligible risk and is as safe as its conventional counterpart. It is noteworthy that food and feed safety assessments of many diverse GM plants over the past 25 years have not identified unique hazards associated with GM plants [19, 33, 45].

3. Supplementary Studies

If the weight-of-evidence from core studies is not sufficient to determine negligible hazard, further hazard and exposure characterization are needed to support the safety assessment. Alternately, depending on the nature of the NEP, case-by-case studies may be required for complete characterization even when hazard is absent. As mentioned in the introduction, the choice of supplementary study or studies would depend on the introduced GM trait and intended use.

Hypothesis-driven studies identified by problem formulation can be used for the characterization of hazard and exposure [61]. Hazard characterization expands beyond the hazard identification step to more fully understand the conditions under which the hazard may be present [68]. The appropriate supplementary hazard characterization studies needed should be determined based on the results of the core studies and an understanding of the nature of the identified hazard, and may include toxicological studies with the NEP or IgE binding studies, as examples. Expression levels, likely protein degradation during processing (e.g., heat stability), resistance to digestion, and dietary exposure assessments are some studies that can be considered that are relevant to exposure characterization.

Examples of hypothesis-driven and case-by-case supplementary data studies are discussed below.

3.1. Protein abundance in food and feed

While protein expression data in plant tissues may be helpful as part of the environmental risk assessment for specific traits, exposure estimates related to consumption by humans and animals are less relevant for proteins for which no hazard has been identified [1]. The abundance of a protein has historically been recognized as supportive information for allergy safety assessment. However, if the NEP is not allergenic or cross reactive, abundance is not relevant to safety [7, 9, 42]. While low abundance does suggest a lower probability of allergy relevant exposure, if there is not an identified hazard, greater or lower abundance is not a contributing factor in an allergy risk assessment for an NEP. This topic is further discussed in *Allergy risk assessment for newly expressed proteins (NEPs) in genetically modified (GM) plants* [42].

3.2. Processing

Processing is another factor that can be considered in exposure characterization when a hazard has been identified. Processing has typically referred to the assessment of how stable a NEP may be when the grain in which it is contained is processed, using methods that would be typical for turning grains into food and feed fractions. Measuring NEP functional intactness after heat treatment(s) that mimics food processing conditions could contribute to an exposure assessment but does not otherwise characterize allergy or toxin hazard for NEPs. Although exposure assessments are required by some regulatory agencies, they provide no quantitative value for risk assessment if negligible hazard has been determined [54].

3.3. Resistance to digestion

The *in vitro* degradation of a protein using simulated gastric fluid (SGF) and/or simulated intestinal fluid (SIF) assays can also be used as part of the WOE safety assessment [42, 60]. SGF/SIF studies aid in the understanding about the potential digestive fate of a NEP in food and feed and also inform about potential human and animal exposure to NEPs. When there is a known hazard, SGF/SIF assays help to understand and assess internal exposure.

Traditionally, stability of a NEP in SGF was used as a distinguishing feature of food allergens, resulting in the wide adoption of this criterion as part of the WOE approach supporting the allergenic risk assessment of NEPs [73]. However, follow-up studies showed the SGF assay to be an inconsistent predictor of impact on the immune system (allergenicity), and modifications of digestion studies to include more physiological gastric conditions and SIF were explored, without any notable improvement in the contribution to the WOE for assessing the allergenic risk of NEPs [28]. As discussed recently, there is poor correlation between digestion results and the allergenic status of proteins [29, 31]. SGF stability provides value only when there is a known hazard, as digestion characteristics would contribute to exposure considerations in the risk assessment.

3.4. Toxicity studies

The toxicological evaluation of all NEPs as a default assessment is not hypothesis-driven, nor supported by the current weight-of-evidence. As discussed in Roper et al., "*defaulting to in vivo toxicology studies, as is often required for regulatory approvals, does not reflect ethical use of animals in scientific research and testing as outlined by the 3R's of responsible animal use (Replacement, Reduction and Refinement) that have been increasingly incorporated into regulatory in vivo studies*" [60, 66].

Acute oral toxicology studies with proteins should only be conducted if deemed necessary to address specific hazard hypotheses arrived at through problem formulation [60]. When toxicity studies are deemed necessary, acute toxicity studies are generally sufficient given the observation that, while most proteins do not present a hazard, most protein toxins elicit their toxicity through acute mechanisms of action [64].

Evidence to date for NEPs in GM crops indicates that when no hazard is identified, no evidence of adverse effects is observed in acute oral toxicology studies [4, 14, 36, 40, 65, 75]. Nevertheless, acute toxicology studies are still required by many regulatory authorities regardless of the nature of the protein [43].

The routine requirement for repeated dose toxicity studies with proteins in the safety assessment of GM plants is also not scientifically justified, as discussed in Box 2. No evidence exists to suggest that protein digestion is altered as a result of repeated exposure or consumption of proteins [14]. Furthermore, most protein toxins act acutely, and therefore, do not have repeated dose or cumulative toxicity [50].

3.5. Compositional assessment

Currently, extensive assessment of the nutritional composition of a new GM crop is a requirement by many government regulatory authorities around the world. The main purpose of these compositional assessments has been to determine whether introduction of the GM trait(s) has altered the nutritional profile in a way that would have a meaningful impact on the food or feed use of the GM crop. These compositional studies do not attempt to show that the GM crop and the conventional crop are identical, but merely that one crop could be substituted for the other in the diet without any meaningful impact. Any noted changes in nutritional component levels in the GM crop are evaluated against the breadth of component variability in the conventional crop.

The risk assessment of GM crops according to Codex guidelines includes, "*an assessment of a whole food or a component thereof relative to the appropriate conventional counterpart: A. taking into account both intended and unintended effects; B. identifying new or altered hazards; C. identifying changes relevant to human health and key nutrients*" [7]. Although the assessment has included intended effects due to the GM trait(s) of interest, much of the assessment continues to be focused on uncovering possible unintended effects due to the trait insertion process. However, Codex recognized that, "*many unintended effects are largely predictable based on knowledge of*

Box 2: Routine 28-day and 90-day repeated dose toxicity studies are scientifically unjustified

If the NEP is related to a family of proteins that has a history of safe use based on bioinformatics and literature review, and is not homologous to known protein toxins, then any supplementary toxicology study is not necessary. Furthermore, if an acute oral toxicology study has been performed with no observed adverse effects, then a 28-day repeated-dose toxicity study with the protein is unlikely to contribute any additional valuable information to the protein safety assessment [3,14,15,26].

The routine requirement for a 90-day toxicity study with whole foods (e.g., grains) in the safety assessment of GM plants is also not scientifically justified. However, these studies are required in some countries to supplement the molecular and compositional data included in the risk assessment, even in the absence of a plausible risk hypothesis [10]. Studies to date have shown that unintended effects on the composition of GM plants occur less frequently and are of a lower magnitude as a result of the process of genetic modification or transformation compared with their occurrence in traditionally-bred crop varieties [30].

When a 90-day study is performed to meet individual country requirements, it is performed by feeding whole food/feed material, ostensibly to identify potential adverse effects of consumption of edible fractions from GM plants. The methods are principally based on existing guidance for the identification and characterization of potential hazards of chemicals from repeated oral administration during a critical period of animal growth and development, with necessary adaptations for evaluation of whole food and feed [49]. Although the study design provides satisfactory estimates of no-effect and no-adverse-effect levels, the incorporation of whole food or feed fractions into these studies has inherent limitations for exposure due to nutritional and satiety concerns that impact animal performance irrespective of the GM crop fraction included in the diet. Additionally, feeding studies conducted in the absence of a risk hypothesis are generally considered to lack sufficient sensitivity to yield meaningful results relevant to the safety assessment of GM plants, create challenges for study design due to difficulties in determination of adequate sample size for appropriate statistical power, place undue emphasis on feeding study results in comparison with other components of the safety assessment, and are inconsistent with the principles of reduction, refinement, and replacement of animals in research [16]. These conclusions have been reinforced by the recent completion of two European Commission work programs, GMO Risk Assessment and Communication of Evidence and GM Plants Two Year Safety Testing [20]. The findings of these European projects are consistent with those presented above that 90-day feeding studies with GM food/feed materials are scientifically superfluous to the overall risk assessment.

the inserted trait and its metabolic connections or of the site of insertion", and that unintended effects also result from use of conventional breeding. Likewise, more than ten years ago, the European Commission [19] stated that, "*The main conclusion to be drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research, and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not per se more risky than e.g., conventional plant breeding technologies*".

Variability in nutritional components occurs naturally in conventionally bred crops due to the influence of both genotype and growing environment [12, 59]. Genotype differences arise spontaneously in plants (e.g., transposon movement, mutation, chromosome crossing-over, etc.), and transgenic modification leads to molecular changes in the genome similar to insertions and disruptions that occur naturally in plant genomes [37]. Researchers have demonstrated that conventional breeding methods contribute more to compositional variability than the process of transgene insertion [62, 69, 72]. Human and livestock animal populations have been exposed to the full breadth of variability of components within crop commodities (within recommended dietary intake levels) without evidence of harm. Therefore, variability in levels of nutritional components does not in itself indicate an impact on safe consumption.

Within the context of this inherent variability in composition, the accumulated experience in evaluation of compositional data has revealed the lack of biologically meaningful differences between GM crops and their conventional comparators [22, 27]. To date, compositional studies have not documented evidence of notable consequences attributed to the process of developing a new GM plant [30]. Just as it is done for conventional breeding, extensive evaluation prior to selection of the GM line for commercial development greatly reduces chances of unintended impacts of the GM process on the commercialized crop variety [22]. When biologically relevant compositional changes have been observed, these changes can be predicted from the mode of action of the introduced trait. Additionally, in a 2010 review of transgenic safety assessments, Parrott et al. noted that, "*results emphasize that the GM and non-GM comparators are of similar composition*" and that, when considering other expression products (such as RNAi or transcription factors), "*there is no scientific rationale to justify new or more*

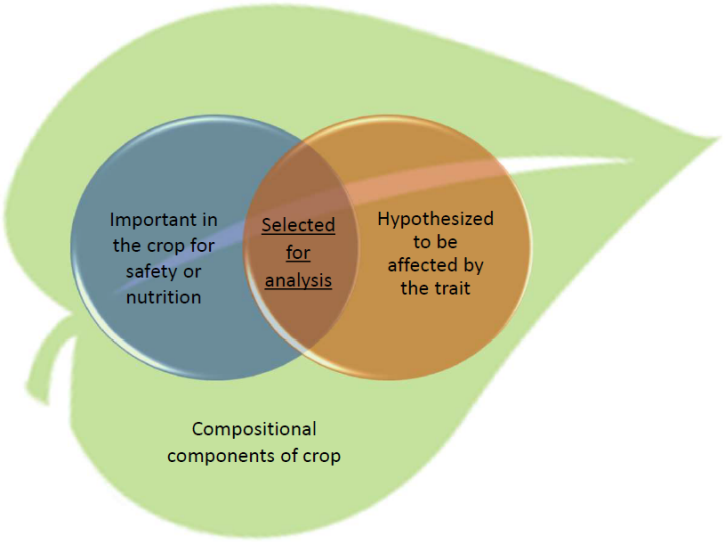

Figure 3: Component selection for genetically modified (GM) plant composition analyses in support of food and feed safety assessment

complex safety assessments" [51].

Based on the compelling body of evidence collected since Codex guidelines were developed [8], it is recommended that a compositional assessment of a new GM plant should follow a stepwise approach to determine if further data generation is necessary, and if so, what data should be collected. The goal of this approach is to focus the compositional assessment on the key components that are critical to the nutritional and/or safety considerations for the crop and also have potential to be altered by the introduced trait(s) (Figure 3), since not all compositional changes are an indicator of a hazard [51].

Our proposal is to first formulate sound hypotheses, based on the trait mode of action, to further refine the list of components to be targeted for analyses, and then use a stepwise approach to evaluate known information and decide what additional information is necessary to inform the safety assessment as detailed in Box 3.

As an example of implementation of the proposed approach, consider the tyrosine catabolic pathway and a trait that affects levels of hydroxyphenylpyruvate (HPP). HPP is dehydrogenated into homogentisate, which is upstream from the tocochromanols (tocopherols and tocotrienols). (α-Tocopherol is the most biologically active form of vitamin E in the diet). All tocopherols and tocotrienols act as antioxidants when present in vegetable oils, preventing development of rancidity. The null hypothesis to test is that there are no differences in levels of tocopherols and tocotrienols between the GM crop and its non-GM comparator. There is no reasonable expectation, and thus no sound hypothesis, that the trait, based on its mode of action, would affect other crop components outside of this pathway (e.g., levels of minerals, crude protein, dietary fibers) any more than is possible with conventional breeding. Therefore, the components to be measured and compared should only be those hypothesized to be impacted as a result of the trait mode of action and impacting health or safety. Generation of other data for unrelated components is superfluous and would be detracting from the safety assessment of the novel GM crop. This hypothesis-driven approach is also in line with the problem formulation approach described by Raybould and MacDonald [58] for environmental risk assessment of GM crops, who emphasized that there should be movement, "*toward hypotheses that help decision-making and realization of policy objectives*".

The automatic requirement of in-depth, multi-component compositional studies within the set of safety evaluations of a new GM crop has been called into question by the increasing body of knowledge regarding the extent of natural variation in crop composition, the innate variability and plasticity in plant genomes, and the empirical evidence supporting a negligible impact of the transgenesis on composition [30]. The hypothesis-driven approach to compositional studies described here serves to characterize the impact of the trait(s) on the levels of the targeted components. This focused approach is consistent with the established practices of conventional variety registration and meets food and feed product standards.

3.6. Dietary exposure assessment

Dietary exposure assessments are recommended to be hypothesis-driven studies. If, during core safety assessment, the weight-of-evidence points to negligible hazard, a formal dietary exposure assessment is unnecessary for the overall risk assessment [41]. Conversely, problem formulation may demonstrate that there is minimal or no exposure to a NEP, precluding the necessity for additional hazard assessment. This holds true for NEPs and also for other expression products such as RNA-based mechanisms for gene regulation [53].

In the case where a dietary exposure assessment is needed, e.g., if hazard is not negligible or is uncertain, a stepwise approach should be taken, using the most straight-forward di-

Box 3: Recommended stepwise approach for compositional assessment

At each step, a decision is made whether the available information is sufficient to assess possible risk or whether additional information may be needed (e.g., further information concerning mode of action, generation of appropriate data to address the hypothesized risk).

Step 1: Based on knowledge of the* mode of action *or function of the introduced GM trait, determine whether a supplementary compositional study will be useful for informing the overall risk assessment

GM plants possess specific phenotypic traits determined by the mode of action of the introduced genetic material. The expected functional or biological activity of the intended genetic modification is studied prior to commercialization of the new GM plant (information gained from core studies). A compositional study is not necessary if there is no scientifically reasonable hypothesis that the GM trait introduction will compromise crop composition in a manner that could lead to a safety or nutritional concern. For some GM traits, there is a reasonable hypothesis, based on the mode of action or function of the introduced trait, to justify a compositional assessment [32]. If the outcome of Step 1 concludes that a targeted compositional assessment is necessary to address hypothesized changes in composition, then the assessment proceeds to Step 2.

Step 2: Determine which components are relevant to include in a composition study

In cases where a composition study will provide informative data that are meaningful to the safety assessment, the decision on which components to include are limited to those components that are predicted to be both affected by the introduction of the trait and relevant to the safety or nutritional properties of the crop (Figure 3). If levels of the selected components are within what is considered typical for the crop, no further assessment is necessary. If the introduced trait is predicted to potentially result in the production of a metabolite novel to the crop, then levels of this metabolite are to be evaluated as well. If the assessed components are present at levels outside the natural range for the crop commodity, or if further evaluation of a novel metabolite is deemed necessary, then Step 3 is performed.

Step 3: Evaluate the safety and nutritional relevance of altered component levels

The focused compositional analysis may indicate that the level of one or more components falls outside the range of values previously observed for the crop commodity. However, such a result does not necessarily signify that the new GM plant is less safe, but that further assessment of the implications of the change may be necessary. The scope of the additional assessment would depend on the nature of the change and on the intended use(s) of the crop. Particular component changes could mandate a change in the use of, or the level of inclusion in, downstream products (e.g., processed food/feed). For example, the level of inclusion of cottonseed meal in livestock diets can be influenced by the level of the anti-nutrient gossypol, and the functionality of soybean oil used in food service or processed food could be impacted by intentional alterations in its fatty acid profile. Novel metabolites would be similarly assessed for possible impacts to safety and nutrition: history of safe use of the metabolite, levels of exposure from other food sources, etc.

etary exposure assessment method, starting with an unrefined, conservative assessment. Strengths and limitations of available databases should be considered, and the exposure duration selected should be relevant to the NEP. In cases where the unrefined assessment does not allow for acceptable risk, a refined dietary exposure assessment may then be leveraged to provide more realistic quantitative exposure estimates. Refinement factors include market share, food processing effects, variety-specific NEP data, NEP digestibility, and probabilistic modelling. Assessment of human dietary exposure to NEPs in GM plants have been described recently [41].

The Codex guidance on biotechnology-derived plants does not address the safety assessment for animals fed with feed produced from GM plants. Dietary exposure assessments for animal species should only be performed for a NEP expressed in GM plants if deemed necessary during the risk assessment process. Such an assessment can follow a similar stepwise approach as proposed for a human dietary exposure assessment, but should consider both the relevant animal species and the crop fractions that they consume. The major livestock species should be sufficient, as crop products are traditionally the main ingredient sources for livestock feed and animals are fed at high inclusion levels. The Organization for Economic Cooperation and Development (OECD) provides a single, international source of body weight, feed intake, and dietary feed inclusion data for livestock species [48]. Feed consumption databases are lacking for other animal species, in particular companion animal species, where animal protein sources are becoming more common ingredients. Crop fractions should be relevant and justified for the application; for example, seed im-

port applications should not require a dietary exposure assessment for forage.

3.7. Case-by-case protein characterization studies

In addition to the core studies performed for protein characterization (see Section 2.2), studies may be needed on a case-by-case basis for complete characterization of certain NEPs.

3.7.1. Case-by-case studies with the plant-produced protein or protein test substance

a) Post-Translational Modifications: Post-translational modifications can affect the activity, tertiary structure, and biophysical properties of the NEP. If there are indications that the plant-derived NEP is post-translationally modified, this should be confirmed through analytical methods specific to the potential modification. One common post-translational modification of plant proteins is glycosylation, which can change physicochemical properties of the protein [57].

b) Mode of Action: The mode of action is a mechanistic understanding of how the NEP functions to produce the desired trait. An understanding of the mode of action can support establishing the design of safety studies for a particular trait product. However, the requirement for more complex or detailed understanding of the mode of action, in addition to what is done for hazard identification (Section 2.3.1), is supplementary and only required in cases in which an impact on safety is identified through, for example, the problem formulation process.

c) Substrate Specificity: For a NEP which is an enzyme that adds a new capability to the plant, assessment of the substrate specificity of the NEP may be necessary. Knowledge of how an enzyme acts on a substrate can help identify the range of substrates on which it might act. This may provide information about potential impact on existing metabolic pathways or on the potential to produce newly formed metabolites. The safety implications of such changes would need to be addressed, possibly through a compositional assessment.

3.7.2. Comparative studies of the protein test substance and plant-produced protein

In addition to the core comparative studies to demonstrate sufficient similarity between the protein test substance and the plant-produced protein, additional studies may be required in some cases to demonstrate the suitability of the protein test substance for use in a safety assessment.

Post-Translational Modifications: If a NEP isolated from the GM plant is found to be modified, the impact of that modification on safety should be assessed. If this modification impacts the function or biochemical properties of the protein, it will be necessary to produce a protein test substance modified in a similar manner for conducting safety assessment studies.

3.8. Nutritional assessment

Nutritional assessments of GM plants are based on a comparative assessment of the composition of food and feed derived from the GM plant. Extensive nutritional analysis should only be performed on a case-by-case basis when compositional assessment demonstrates that analytes critical to the nutritional value of the diet are altered, i.e., when Step 3 of Compositional assessment (see Section 3.5) is performed. In fact, in studies where compositional analyses demonstrated no meaningful differences between the GM plant and comparator or commercial varieties, no differences in intake, digestibility or other parameters have been found [67].

However, numerous nutritional studies with fast growing animal species such as broiler chickens have historically been required by regulatory authorities to assess the nutritional value (or "wholesomeness") of GM plant products compared with those from conventional plants, even in cases where compositional equivalence had already been established. These historical data do not support the standard requirement of more extensive nutritional analysis without a hypothesis for nutritional change.

3.9. Immunoglobulin E binding

Traditionally [7], the need to perform IgE binding studies for an assessment of the allergenic potential of a NEP was conducted only in the case of significant similarity identified through bioinformatics. With the advent of more sophisticated bioinformatic techniques and in using the proposed problem formulation approach described herein, the application of IgE binding would be considered a case-by-case study performed to evaluate the potential allergy risk identified through bioinformatics [42].

4. Summary and Conclusion

Earlier guidelines and recommendations for the safety assessment of GM plants containing NEPs still provide a valid resource for the risk assessment of GM plants. However, given the history of safety and familiarity after many years of experience with these products, it is time to reconsider the approach to safety assessments for GM plants.

Despite the accumulated knowledge and familiarity of developers, academic scientists, and regulators with GM plants, regulatory reviews of their safety for food and feed use continue to be inconsistent internationally. In some cases, the safety assessment data required has continued to increase without adding value to the risk assessment. In this paper, a systematic approach for the safety review of GM plants used as food or feed is presented. A set of core studies is recommended, including characterization and protein safety assessment. It is important to perform hazard identification in core studies, and if hazard is determined to be negligible, then core studies should be sufficient to conclude that the GM plant is as safe as its conventional comparator. Rather than making additional assessments a routine requirement, these additional assessments would only be needed if, given the trait mode of action, the hazard and

exposure assessments from the core studies were not conclusive. Only when the information from the core studies is clearly not adequate to conclude on risk may supplementary studies be necessary.

Waters et al. [71] present a compelling rationale and concepts for the adoption of science-based approaches to GM plant safety assessment, and the present paper details a systematic approach to evaluate the safety of GM plants. The approach for safety assessment discussed in these papers, if implemented, could provide a first step towards standardizing requirements across regulatory systems based on current scientific knowledge and 25+ years of experience in the development and food/feed safety assessment of GM plants. Examples of case studies that use problem formulation and hypothesis-driven studies will be explored in future articles.

5. Declaration of Conflicting Interest

All the authors of this paper are currently employed by, or have been employed by, the agricultural biotechnology industry.

6. Disclaimer

The findings and conclusions in this publication are those of the author(s) and should not be construed to represent any official USDA or U.S. government determination or policy.

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