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# Recommendations for Maximum Incorporation Rates of Whole Food in 90-Day Rat Feeding Studies

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**Abstract**

More than 25 years of 90-day rat feeding studies with GM crops have consistently shown that these studies provide no additional value to safety assessments in the absence of a testable hypothesis. However, some regulatory authorities continue to require these studies while also specifying that the test material should be relevant to the product to be consumed and tested at the maximum incorporation rate not causing nutritional imbalance. In the absence of known or suspected adverse effects, dose range-finding studies are not feasible, yet scientifically justified incorporation rates are needed to balance the nutritional requirements of the animals and to achieve the goal of observing adverse effects, should they occur. When 90-day rat feeding studies are required for GM crop safety assessments, the following maximum incorporation rates (w/w), are recommended: 50 percent maize, 30 percent soybean, 60 percent rice, 15 percent canola, and 10 percent cottonseed. These recommendations are based on empirical data regarding maximum exposure to test material and avoidance of nutritional imbalances and/or exposure to anti-nutrients or toxins naturally present in the whole food. Each recommended maximum incorporation rate provides test material consumption at levels substantially higher than the highest human worldwide chronic consumption and is fully sufficient to address regulatory requirements.

*Keywords:* incorporation rate, feeding studies, rat feeding studies, genetically modified

## 1. Introduction

Farmers have built upon millennia of human domestication and breeding improvements of a select suite of agricultural plants and animals to keep pace with the nutritional needs of a growing global population that is currently over 7.5 billion people. Cereals such as maize, wheat, and rice have shown steady increases in productivity over the past half century, although arable land per capita has declined 52 percent (1961 to 2016) [122]. The increases in agricultural productivity have largely occurred through performance improvements from both conventional breeding practices and, since the mid-1990s, the adoption of modern agricultural practices using biotechnology. Over the past two decades, genetically modified (GM) crops of predominantly maize, soybean, canola, and cotton, have been grown on an accumulated 2.5 billion hectares [65], and protected or increased yield by improving crop responses to both biotic (e.g., insect damage and weed pressure) and abiotic (e.g., drought and salinity) stressors [14].

The benefits of GM crops have been challenged by perceptions of risks to health, cultural heritage, environmental integrity, or moral values [66]. To address concerns regarding potential safety risks of GM crops, extensive comparative assessment approaches have been globally adopted in which both intended and unintended changes in GM crops relative to conventional controls are well characterized, and any identified differences are subjected to additional safety assessment [24, 32, 67]. Part of this safety assessment has included 90-day rat feeding studies, although for the majority of new GM varieties, extensive comparative assessment data have not identified any hazard or adverse effect to justify animal testing [9, 60, 97].

These 90-day rat feeding studies have typically followed traditional toxicology study designs used to assess chemical

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safety [33, 35, 38, 41, 81, 87]. However, whole foods are unlike defined chemical substances that can be administered at doses ranging several orders of magnitude above anticipated exposure levels [35, 38, 41]. Whole food or feed is a complex mixture of thousands of substances [35, 38] any one of which, if administered in excess, can lead to nutritional imbalance and secondary effects that confound study interpretation. Therefore, the dietary incorporation of processed GM crop material, unlike for chemicals, precludes testing over several orders of magnitude [9, 38]. In addition, 90-day rat feeding studies of chemical test substances are generally preceded by observations of adverse effects that provide testable hypotheses for further toxicological assessment (e.g., the need for longer-term testing or investigating specific target organ effects), which ultimately have an impact on the safety assessment. This situation is not the case for 90-day rat feeding studies that are testing GM crop material, which has regularly been characterized and found to be as safe and nutritious as its conventional counterpart prior to the feeding study. Consequently, such 90-day rat feeding studies confirm existing data, but do not bring new hazards to light, prompting some to question the ongoing propriety of requiring animal studies that do not provide additional value to safety assessments [9, 60, 97, 108, 113, 137].

Nonetheless, the EU Implementing Regulation (IR) [46] mandates the conduct of 90-day rat feeding studies, stating, "In the regulatory frame set by IR (EU) 530/2012, the 90-day feeding study in rodents on whole GM food/feed is among the toxicological tools for the identification of hazards related to the GM plant" [38]. This viewpoint prevails, despite the conclusion of the EU funded project GMO Risk Assessment and Communication of Evidence (GRACE), which states that a feeding study is needed only if a safety concern has been identified during molecular, compositional, phenotypic, and/or agronomic analyses [136, 137]. The EU IR [46] further requires that the GM food tested in 90-day rat feeding studies should be relevant to the product to be consumed [38]. In addition, the EU IR requires that the maximum incorporation rate not causing nutritional imbalance be used in 90 day rat feeding studies [46].

Currently, 90-day rat feeding studies using diets derived from GM crops are designed to maximize exposure via a high rate of incorporation in nutritionally-balanced diets. Higher incorporation levels are thought to be more likely to detect unintended adverse changes in the test animals that may be attributable to exposure to the GM crop material [8, 12, 20, 32, 34, 35, 38, 44, 63, 77, 80]. However, 90-day rat feeding studies, regardless of dietary incorporation levels, are comparatively insensitive relative to the sensitivity of the compositional analytical methods used to assess the levels of known anti-nutrients and toxicants in foods from GM crops. [22]. As requirements for increasing incorporation levels emerge, challenges are faced in formulating test and control diets that meet the nutritional specifications required for the test species, including determining the proper balance of protein and energy content and the appropriate levels of other essential nutrients. Moreover, it is important that the control diets used in such studies be nutritionally equivalent to the diets commonly used in relevant laboratory facilities to preserve the utility of baseline historical control data that define the normal ranges for measured parameters in the testing laboratory for comparable animals of the same age, sex, and strain.

The objective of this paper is to recommend, on a crop-by-crop basis, the maximum incorporation rates of GM crop materials for use in 90-day rat feeding studies. Five crop-specific sections present recommended maximum incorporation rates that allow the rat diet to be nutritionally balanced for the most commonly produced GM crops (i.e., maize, soybean, rice, canola, and cotton). For each crop, the scientific validity of the recommended maximum incorporation rate is supported by the margin of exposure (MOE) obtained when compared to the highest human consumption for the given crop. Normal dietary exposure to GM crop materials varies too extensively among livestock animals (e.g., large number of animal species and species types, geographic differences in daily intakes and body weights for a given animal species, variation in which parts of the crop are fed to livestock [48, 83]) to allow parallel comparisons to animal feed in this paper. Furthermore, the nutritional equivalence of GM crop material for feed use is already routinely performed (e.g., broiler chicken feeding study) and should be considered applicable to other livestock species.

## 2. Crop-Specific Considerations Relevant to Recommendations for Maximum Incorporation Rates of Whole Food in 90-day Rat Feeding Studies

### *2.1. Maize*

#### *2.1.1. Maize Production and Use as Food and Feed*

Maize (Zea mays, subspecies Mays) is widely cultivated worldwide and the vast majority (>75 percent) [78] is used for ethanol production or as animal feed (e.g., cattle, pigs, and poultry) due to its high energy content and relatively low production cost [82]. However, maize has also been a staple in human diets for millennia. Sweet corn, popcorn, and select food-grade field maize varieties are consumed by humans in several foods. Maize used for food is often wet milled to produce ingredients such as starch and sweetener products. Corn starch is used in many foods such as ice cream and other processed dairy products, batters and breading, baked goods, soups, sauces and gravies, salad dressings, confections, drinks, and in processed meat and poultry products. Starch can also be converted to a variety of sweetener and fermentation products such as high-fructose corn syrup and ethanol [129]. Dry milling maize produces foods such as grits, corn meal, and flour [98]. Maize does not need special processing to make it safe for human consumption, although processing aids nutrient availability, palatability, and allows for a variety of consumable products with a stable shelf life.

Since human diets include whole maize kernels (i.e., grain), it is reasonable to use this test material in 90-day rat feeding studies, as recommended by EFSA [38, 41]. This test material contains the full constituency of the product that is being assessed for unintended changes in GM crops. Furthermore, from an experimental perspective, maize is a common ingredient in rat diets as a ground grain, meal, or flour, and thus extensive laboratory experience exists with feeding this substance to rats.

#### *2.1.2. Dietary Considerations for Maize in 90-day Rat Feeding Studies*

Grain-based diets used in 90-day rat feeding studies, including Purina LabDiet 5002, contain between 30 and 45 percent (w/w) ground maize. A number of published 90-day rat feeding studies in Sprague Dawley, Wistar Han, and BN rats have demonstrated no safety concerns at dietary incorporation rates of maize grain ranging from 11-40 percent (w/w) [3, 5, 52, 53, 59, 62, 72, 73, 137]. This range of dietary incorporation rates represents a significant portion of the animal diet and has historically been considered sufficient and appropriate for evaluation of any potential adverse effects in 90-day rat feeding studies resulting from intended or unintended changes in the food from a GM crop.

Nonetheless, maize incorporation at 50 percent (w/w) is recommended by EFSA [38, 41] to conform with EU IR [46]. Several published studies indicate that a 50 percent (w/w) maize dietary incorporation rate is tolerable for 90-day rat feeding studies [47, 57, 113, 118, 138]. However, from a scientific perspective, the 50 percent (w/w) incorporation rate for maize only corresponds to a 1.5-fold increase in the MOE for the test animal relative to the commonly used 33 percent (w/w) incorporation rate, such that this change would not materially change most safety assessments.

In addition, 90-day rat feeding studies with maize dietary incorporation rates as high as 70-76 percent (w/w) have also been conducted [57, 58]. These studies compared animals fed diets with high levels of maize incorporation rates (i.e., 50 percent and 70 percent [58] and 30 percent and 76 percent [57], w/w) (both GM and conventional maize) to commercial diets containing lower incorporation rates of maize flour (i.e., 43.3 percent [58] and 33.3 percent (w/w) [57]). When comparable dietary incorporation rates of maize were used, no adverse differences in assessed parameters (e.g., body weight, feed consumption/utilization, clinical chemistry, hematology, organ weights) were observed between animals fed GM and conventional maize [57, 58]. However, He et al. [58] noted some statistically significant differences in animals fed the diets with 70 percent (w/w) maize flour (both GM and conventional maize) compared to animals fed the commercial diet (43.3 percent maize flour) [58]. These authors also mentioned that differences in certain variables were noted as a result of nutritional differences between the animals fed diets with the highest maize concentrations and the animals fed the commercial diets [58]. Likewise, He et al. [57] noted differences between the animals fed 76 percent (w/w) GM maize (lysine-rich) and the corresponding control maize diets compared to the animals fed a commercial diet containing 33.3 percent (w/w) maize flour supplemented with casein and synthetic lysine [57]. A statement in a subsequent publication [140] by the authors of these two reports [57, 58] confirmed that diets containing the 70-76 percent (w/w) incorporation rates of maize caused changes in nutritional response variables [140].

An unpublished 90-day rat feeding study investigated the effect of incorporating grain from a conventional maize hybrid into rat diets (using Purina LabDiet 5002) at four rates: 33 percent, 50 percent, 66 percent, and 99 percent (w/w). This GLP study evaluated clinical observations, survival, body weight/changes, feed consumption, serum chemistry, hematology, clotting, urinalysis, necropsy, organ weights, and gross pathology. The unsuitability of the diet with 99 percent (w/w) maize was evidenced by numerous adverse findings, most notably significantly prolonged clotting times (30 percent higher prothrombin time and approximate doubling of activated partial thromboplastin time), reduced body weight gains, transient feed consumption reductions, hemorrhaging, cholestasis, impact to organ weights, and poor animal health/survival.

The observations of impaired clotting time endpoints are consistent with the fact that Purina LabDiet 5002 contains alfalfa and other plant ingredients that supply vitamin K precursors and lysine that are necessary for facilitation of blood clotting. Since maize is a poor source of both lysine and vitamin K, having 99 percent (w/w) maize as the highest incorporation rate in the diet means that the remaining 1 percent (w/w) of the diet was insufficient to adequately provide the nutritional factors needed for normal clotting parameters. Therefore, the 99 percent (w/w) maize incorporation rate is unsuitable to maintain healthy rats and, consequently, significantly confounds any observations at such a high incorporation rate.

It is noteworthy that some observations of animals fed diets containing 99 percent (w/w) maize were also made in animals fed diets containing 66 percent (w/w) maize. For example, males fed diets containing 66 percent (w/w) maize demonstrated a dose-related trend toward higher clotting time (about 10 percent higher prothrombin time and 40 percent higher activated partial thromboplastin time). However, no treatment-related effects were observed at 50 percent (w/w), indicating that this incorporation rate is suitable for use in 90 day rat feeding studies, a conclusion also supported by the previously published work summarized above [57, 140].

#### *2.1.3. Maize Maximum Incorporation Rate Recommendations/Conclusions*

Rat diets containing 50 percent (w/w) maize correspond to exposures of approximately 46 g maize/kg body weight bw/day for rats (based on a mean daily consumption of 92 g diet/kg bw/day in the Sprague Dawley rat) [121]. In humans, the highest chronic consumption of maize worldwide (116.66 g/person/day) from the WHO GEMS database [131] was reported for people in country cluster G13, which represents several African countries (Table 1). Based on a human body weight of 60 kg, this corresponds to a conservative estimated exposure of 1.94 g/kg bw/day. The lowest chronic consumption of maize worldwide was reported at 7.36 g/person/day or 0.12 g/kg bw/day. Thus, a 50 percent (w/w) maize incorporation rate in the rat diet represents a MOE of approximately 24-fold to 383-fold. It is important to note that maize incorporation rates of 33 to 50 percent (w/w) exceed anticipated exposures for even the highest maize consumption by humans, supporting the scientific validity to use maize levels within this range for 90-day rat feeding studies.

Although the weight of evidence from the published literature and unpublished data show that 50 percent (w/w) maize is tolerable in rat diets, the fractional increase in dosage gained with this incorporation rate [38, 41] compared to a 33 percent (w/w) maize diet, for example, does not provide an advantage that balances the loss of existing historical control data at slightly lower maize incorporation rates. Therefore, a rat diet with 50 percent (w/w) maize incorporation provides a recommended maximum incorporation rate for 90-day rat feeding studies intended to identify unintended adverse effects. However, rat diets containing 33 to 50 percent (w/w) maize should be considered as suitably high dietary incorporation rates for use in 90-day rat feeding studies.

### *2.2. Soybean*

#### *2.2.1. Soybean Production and Use as Food and Feed*

Soybean (Glycine max) is primarily grown as a broad acre crop in more than 35 countries, with soybean sprouts and edamame being a relatively low acreage specialty vegetable crop. Although the majority of the value of soybean production (50-75 percent) is the use of field soybeans to produce key feed ingredients such as soybean meal for livestock, soybean is a staple of the human diet [85]. Soybean and soybean-derived fractions are consumed in a variety of foods, including soybean oil, protein isolate, tofu, soy sauce, soymilk, energy bars, and meat products, although soybean meal is not a significant food source for humans [85]. Vegetable soybean varieties (e.g., edamame) generally differ from field varieties in terms of size, texture, flavor, and other physical characteristics that allow these soybeans to be more easily cooked than field soybeans. Most field soybeans are a blended commodity that is highly processed to inactivate anti-nutrient factors such as trypsin inhibitors that interfere with protein digestion and lectins (hemagglutinins that causes severe GI tract upset), to make it safe for consumption by humans and non-ruminants [43, 54, 70]. Processing results in minimal exposure to functionally active proteins in processed fractions [54].

EFSA considers toasted defatted (or full-fat) dehulled meal as the suitable test material for oilseed crops, including soybean, but not the oil alone [38]. However, soybean oil makes up 94 percent of the soybean food ingredients consumed by humans, with the remainder being mainly highly refined protein isolates [85]. In the absence of a testable hypothesis for these highly processed fractions such as with a nutritionally-enhanced oil [27, 55], no scientific justification exists to conduct 90-day rat feeding studies for soybean. In addition, 90-day rat feeding studies are conducted as a surrogate for humans, but if humans do not consume the test material, then the animal studies cannot be scientifically or ethically justified, even though such a test material would include the full constituency of the product that is being assessed for unintended changes in GM crops.

#### *2.2.2. Dietary Considerations for Soybean in 90-day Rat Feeding Studies*

Several 90-day rat feeding studies in Sprague Dawley, Wistar Han, and BN rats conducted using toasted/defatted soybean meal have been published at dietary incorporation rates ranging from 7.5-33 percent (w/w) without any adverse effects [4, 23, 27, 56, 89, 117]. Similarly, incorporation of full-fat soybean flour, the standard soybean feed ingredient used in 90-day rat feeding studies by the Chinese Ministry of Agriculture, has not produced any adverse effects at incorporation rates ranging from 7.5-30 percent (w/w). On occasion, some studies, in addition to testing soybean meal, have also substituted soybean oil from GM/non-GM soybeans for the conventional soybean oil in the standard rat diet [27, 89, 90]. Unprocessed soybean seeds are not suitable for food and their use for animal feed is limited because they contain anti-nutritional factors such as trypsin inhibitors and lectins, which are inactivated only by adequate heat processing [88]. Therefore, toasted defatted (or full-fat) dehulled (the partial or complete removal of the outer shell, hull, or seed coat) meal is recommended by EFSA as the appropriate test material for soybean, but not the oil alone [38].

A 90-day rat feeding study was conducted with escalating incorporation rates (0, 30, 60, and 90 percent, w/w) of meal from Roundup Ready® soybeans [139]. Three of the four diets were adjusted to have the same nutrient levels and contained 60 percent (w/w) soybean meal, either as conventional soybean meal (0 percent GM), a 50:50 mix (30 percent GM), or all from Roundup Ready® soybeans (60 percent GM). The fourth group was fed a diet containing 90 percent (w/w) Roundup Ready® soybean meal. At the 90 percent incorporation rate, it was not possible to balance nutrients to be the same as the other diets in this study. Although analysis of diet composition was not reported, the lack of an added fat source (e.g., corn oil) in the diet formulations suggests that they were lower in total fat than standard rat diets. The diet with 90 percent (w/w) soybean meal was also higher in total protein, due to the higher-than-normal levels of protein-rich soybean meal in the diets compared to those in typical standard rat diets. It is noteworthy that no differences in body weight or feed intake responses over the 90-day study were noted, except during the first week for the rats fed the 90 percent (w/w) GM soybean meal. Furthermore, no treatment-related deaths occurred during the study, and no meaningful differences were noted between the rats fed control or GM soybean meals, including gross necropsy findings, hematological and urinalysis values, and clinical serum parameters. However, because all the animals in the study were fed diets containing at least 60 percent (w/w) soybean meal, the data do not support the author's conclusion that diets with 90 percent (w/w) soybean meal had no adverse effects on the rats, since all animals may have experienced the same adverse consequences of soybean meal incorporation rates that exceed standard nutritional guidelines for rat diets. Furthermore, as described below, these results do not align with results from an unpublished 90-day rat feeding study performed under GLP in which animals fed diets with 60 percent (w/w) soybean meal showed adverse effects compared with rats fed standard rat diets.

An unpublished 90-day rat feeding study performed under GLP involved feeding groups of 20 rats (10 per sex) diets containing one of three incorporation rates of toasted/defatted meal from conventional non-GM soybeans. Three diets were prepared according to Purina LabDiet 5002 specifications. The control diet contained 15 percent (w/w) soybean meal (to approximate the level in Purina LabDiet 5002), while the other two non-GM diets contained either 30 or 60 percent (w/w) soybean meal. The test diets with 30 and 60 percent (w/w/) soybean meal contained 4-4.5 percent (w/w) fat, whereas the control diet contained approximately 6 percent (w/w) fat. In addition, the 60 percent (w/w) soybean meal diet contained approximately 32 percent (w/w) protein, relative to the approximately 2 percent (w/w) protein in the other two soybean meal diets. Study endpoints included clinical observations, survival, body weight/changes, feed consumption, serum chemistry, hematology, clotting, urinalysis, necropsy, organ weights, gross pathology, and histopathology.

Cumulative female body weights in the groups fed diets with 30 and 60 percent (w/w) soybean meal were approximately 17 and 33 percent lower than groups fed control diets, respectively, and cumulative body weight gains in male rats fed diets with 60 percent (w/w) soybean meal were approximately 15 percent lower than controls in study weeks 12 and 13. A number of measured endpoints were different in rats fed diets with 60 percent (w/w) soybean meal compared with controls, including clotting times (prothrombin time and APTT), absolute and percent reticulocyte counts, albumin, total protein, globulin, cholesterol, triglycerides, blood urea nitrogen, absolute adrenal weights, absolute and relative epididymis weights, absolute thymus weights, and findings of mixed inflammatory cells and crypt hyperplasia in cecal mucosa. No other biologically-relevant or apparent treatment-related differences in other measured parameters were observed. Furthermore, none of the observed differences in hematology or clinical chemistry parameters were considered to be toxicologically relevant due to all values falling within the laboratory historical control data ranges and the absence of any correlated toxicological findings. However, based on the weight of the evidence from the studies reviewed above and these unpublished study results, it is clear that soybean meal rates of up to 30 percent (w/w) are acceptable for 90-day rat feeding studies and that incorporation rates above 30 percent (w/w) may confound interpretation of any study observations. The suitability of 30 percent (w/w) incorporation is further supported by the absence of adverse effects in chronic rodent feeding studies with diets containing 30 percent (w/w) soybean flour (heat treated) when consumed for one or two years [100, 101].

#### *2.2.3. Soybean Study Maximum Incorporation Rate Recommendations/Conclusions*

Rat diets containing 30 percent (w/w) soybean meal correspond to approximately 27.6 g soybean/kg bw/day for rats (based on a mean daily consumption of 92 g diet/kg bw/day in the Sprague Dawley rat) [121]. In humans, the highest chronic consumption of soybean worldwide (222.52 g/person/day) from the WHO GEMS database [131] was reported for people in country cluster diet G11, which contains Belgium and the Netherlands (Table 1). Based on a human body weight of 60 kg, this corresponds to conservative estimated exposure of 3.71 g/kg bw/day. The lowest chronic consumption of soybean worldwide was reported at 14.29 g/person/day or 0.24 g/kg bw/day. Thus, a 30 percent (w/w) soybean incorporation rate in the rat diet represents a MOE for humans of approximately 7-fold to 115-fold.

Setting aside the caveat that humans only routinely consume highly-refined fractions (oil and protein isolate) from soybean, the following recommendations are relevant. The collective weight of evidence of all of the studies reviewed above provide strong scientific support to using 30 percent (w/w) dehulled, defatted soybean meal as the maximum incorporation rate in 90-day rat feeding studies, a conclusion aligned with EFSA recommendations [38, 108]. This incorporation rate ensures that diets meet the nutritional requirements of the test system and the specifications of the standard rat diet (e.g., soybean meal at greater than 30 percent (w/w) results in total dietary protein levels greater than available in standard rat diets). Therefore, a rat diet with 30 percent (w/w) defatted, dehulled soybean meal incorporation provides a recommended maximum incorporation rate for 90-day rat feeding studies conducted to identify unintended adverse effects. For GM crops in which the oil content is relevant for a 90-day rat feeding study, EFSA recommends 20 percent (w/w) full-fat, dehulled, soybean meal [38]. Thus, rat diets containing less than 30 percent (w/w) defatted or less than 20 percent (w/w) full-fatted soybean meal should be considered as suitably high dietary incorporation rates for use in 90-day rat feeding studies.

### *2.3. Rice*

#### *2.3.1. Rice Production and Use as Food and Feed*

Two types of rice (Oryza sativa), Indica and Japonica, are cultivated in more than 100 countries and consumed by about half of the world's population [112]. The majority of global rice production is consumed by humans, with a small amount of poor grade paddy rice and by products of processing used as animal feed. Brown rice, which is high in energy and low in fiber content [86], is excellent for animal feed, but it is not routinely used because of its cost. Rice is consumed by humans in various forms, including brown rice, milled rice, or parboiled rice, after being cooked in the grain form. Brown rice is produced from paddy rice by dehulling (the partial or complete removal of the outer shell, husk, hull, or seed coat). Milled rice, also known as white rice, is derived from brown rice by milling to remove all or most of the bran, which primarily consists of seed coat, aleurone layer, and germ. Parboiled rice is prepared by soaking in water, draining, heating (most often steaming; sometimes under pressure), then drying, followed by dehulling and milling. Rice flour is a pulverized product of the outer part or the whole milled rice [86]. A small amount of rice is processed into prepared products such as noodles, cakes, crackers, sweets, and alcoholic beverages. Rice does not need special processing to make it safe for human or animal consumption, although processing aids nutrient availability and palatability and allows for a variety of consumable products with a stable shelf life.

Since human diets include dehulled rice, it is reasonable to use this test material in 90-day rat feeding studies, as recommended by EFSA [38]. This test material contains the full constituency of the product that is being assessed for unintended changes in GM crops.

### *2.3.2. Dietary Considerations for Rice in 90-day Rat Feeding Studies*

Several 90-day rat feeding studies have included rice-based diets with incorporation rates ranging from 20-70 percent (w/w) [18, 92, 93, 104, 107, 109, 115, 116, 127, 129, 132, 134, 135, 138, 141]. Three 90-day rat feeding studies were performed as a part of the SAFOTEST project (EU-project titled "New methods for the safety testing of transgenic food"), a project that used rat diets containing brown rice flour [92, 93, 104]. In the SAFOTEST project studies, a 60 percent (w/w) brown rice flour incorporation rate was selected after testing different incorporation rates in a preliminary 28-day study (data not published), which suggested that this incorporation rate met the nutritional and palatability needs for rat diets [92]. The results from these studies demonstrated that a 60 percent (w/w) incorporation rate did not induce any nutritional imbalances or any other adverse effects in the animals. Although some published 90-day rat feeding studies have used diets with 70 percent (w/w) incorporation, it was unclear whether white or brown rice flour was used [109, 134, 141]. In another study [138], white (milled) rice flour was incorporated at 70 percent (w/w) in the diet. Although no significant differences in body weight gain were observed in this study between the groups (non-GM and GM) fed rice-incorporated diets compared to the group fed the standard rat diet, feed consumption was significantly decreased in females of the GM group compared to the standard rat diet group. The feed utilization rate was also significantly higher in female rats fed the 70 percent (w/w) non-GM and GM rice-incorporated diets compared to the standard rat diet. The authors suggested that these differences were likely due to the reduced palatability of diets with the high level of rice flour incorporation. In another study with 70 percent (w/w) white (milled) rice flour incorporation, no significant change in feed consumption was noted, although body weight gain and feed efficiency were significantly higher for both males and females in the groups fed the diets with a 70 percent (w/w) non-GM and GM rice incorporation compared to the control group fed the standard rat diet [134].

Although it is possible to formulate rat diets with 70 percent (w/w) rice and maintain the same major nutritional content (e.g., protein, carbohydrate and fat) as standard rat diets, the above-mentioned publications indicate that the use of a different nutritional source (i.e., rice instead of corn, bean pulp, starch) at a very high incorporation rate can impact feed consumption and feed efficiency. Therefore, it follows that very high levels of rice in the rat diet could present nutritional imbalances or deficiencies that may impact experimental response variables and animal performance independent of the non-GM or GM characteristics of the rice source. It is also important to note that several of the studies cited above did not evaluate many of the key endpoints outlined in the OECD test guideline 408 [81] that are required by EFSA (e.g., missing several histopathology and clotting parameters) [35, 38].

An unpublished 90-day rat feeding study performed under GLP was conducted with seven groups of 12 animals (6 per sex) following the OECD test guideline 408 (except that it excluded the neurobehavioral assessment). This study was conducted to assess the suitability of using 60 percent (w/w) brown rice flour in rat diets compared with animals fed standard rat diets and to generate historical control data at the test facility, as rice is not a typical ingredient in rat diets. The control group was fed standard rat diet and the other six groups were fed diets containing 60 percent (w/w) brown rice flour from one of six conventional (non-GM) rice varieties. In this study, rice at 60 percent (w/w) dietary incorporation was well tolerated with feed consumption levels and animal growth rates comparable with the animal group fed the standard rat diet. No adverse effects were observed in animals fed diets containing 60 percent (w/w) rice incorporation rates for any of the six non-GM rice varieties.

### *2.3.3. Rice Study Maximum Incorporation Rate Recommendations/Conclusions*

Rat diets containing 60 percent (w/w) rice correspond to approximately 55.2 g rice/kg bw/day for rats (based on a mean daily consumption of 92 g diet/kg bw/day in the Sprague Dawley rat) [121]. In humans, the highest chronic consumption of rice worldwide (339.67 g/person/day) from the WHO GEMS database [131] was reported for people in country cluster diet G09, which contains several Asian countries (Table 1). Based on a human body weight of 55 kg, this corresponds to conservative estimated exposure of 6.18 g/kg bw/day. The lowest chronic consumption of rice worldwide was reported at 14.99 g/person/day or 0.27 g/kg bw/day. Thus, a 60 percent (w/w) rice incorporation rate in the rat diet represents a MOE for human food consumption of approximately 9-fold to 204-fold.

Numerous publications containing rice-based diets substantiate the scientific validity of feeding Sprague Dawley and Wistar Han rats diets with 20-70 percent (w/w) rice incorporation rates over 90 days [18, 92, 93, 104, 107, 109, 115, 116, 127, 129, 132, 134, 135, 138, 141]. However, diets containing 70 percent (w/w) brown rice flour incorporation reported diet palatability and animal performance issues that can confound study results and interpretation. By comparison, the 90 day rat feeding studies that were part of SAFOTEST, as well as results from an unpublished study, showed that the growth performance and other measured endpoints of rats fed diets with 60 percent (w/w) brown rice flour were comparable with those of rats fed standard diets. Therefore, a rat diet with 60 percent (w/w) rice incorporation provides a recommended maximum incorporation rate for 90-day rat feeding studies conducted to identify unintended adverse effects. This conclusion is supported by the EFSA recommendation of 60 percent (w/w) for dehulled rice, based on Wang et al. [127, 38]. However, rat diets containing 20 to 60 percent (w/w) rice should be considered as suitably high dietary incorporation rates for use in 90-day rat feeding studies.

### *2.4. Canola*

#### *2.4.1. Canola Production and Use as Food and Feed*

Global rapeseed/canola (Brassica species napus, rapa, juncea, or campestris) production accounts for 10-15 percent of the world's oilseed crop, second only to soybean, generating products for both human and livestock consumption after processing [79, 122, 123]. However, in rapeseed, the presence of anti-nutritional factors (e.g., high concentrations of erucic acid, a cardiotoxic fatty acid, and glucosinolates, whose breakdown products are goitrogenic) render it unsuitable for food use. Thus, Canadian breeders collaborated with end-users (e.g., chemists, animal physiologists and pathologists) to develop agronomically competitive varieties (mostly B. napus) called canola. Varieties that carry the name, canola, must meet internationally-regulated quality standards for maximum erucic acid content below 2 percent (w/w) in oil and less than 30 micromoles of total glucosinolates per gram of oil-free meal [7, 16, 26, 120]. Development of canola enabled utilization of the oil as a healthy oil for direct human consumption, containing the least amount of saturated fat of any common edible oil [17]. With regard to canola meal, only highly-processed protein products, such as concentrates and isolates, have been considered for development as food ingredients [6, 13, 36, 37, 71, 84, 114, 126]; however, canola protein isolate products have not established a significant commercial presence to date.

EFSA considers toasted defatted (or full-fat) dehulled meal as the suitable test material for oilseed crops, including canola, but not the oil alone [38]. However, only highly refined canola oil is routinely consumed by humans. In the absence of a testable hypothesis for canola oil, no scientific justification exists to conduct 90-day rat feeding studies for canola. In addition, 90 day rat feeding studies are conducted as a surrogate for humans, but if humans do not consume the test material, then the animal studies cannot be scientifically or ethically justified, even though such a test material would include the full constituency of the product that is being assessed for unintended changes in GM crops.

#### *2.4.2. Dietary Considerations for Canola in 90-day Rat Feeding Studies*

Fiber, glucosinolates, lignin, condensed tannins, and other polyphenolic compounds are all anti-nutrients concentrated in the seed coat (hull) of the canola plant. Other edible oilseeds such as soybean, cottonseed, and sunflower are dehulled (the partial or complete removal of the outer shell, hull, or seed coat) prior to processing for food and feed applications. The small seed size of canola and its tightly-bound seed coat, cause oil-containing kernel fragments to adhere to the hull during separation, precluding industrial development and deployment of efficient large-scale pre-press dehulling procedures [19]. For this reason, dehulling is not a commercial process for canola [84]. Since canola seeds are typically processed without dehulling, the anti-nutrients from the seed coat reduce the feed value of canola meal for non-ruminants, including rats, by impairing palatability, decreasing digestibility, and interfering with protein utilization and mineral and nutrient absorption [15, 29, 42, 76, 84, 102, 106, 127]. Due to fiber and anti-nutrient content, the recommended limit for conventional canola meal incorporation in diets for monogastrics (e.g., rats) is 15 percent (w/w) [84]. Many considerations were taken into account when establishing this maximum incorporation rate for canola, including anti-nutrient content, available energy content and protein/amino acid content, and digestibility. Additionally, as defined by OECD [84], glucosinolates are anti-nutrients found in canola meal that possibly impact the palatability of the diet; therefore their concentration should be taken into account [38].

Given the published literature, it is reasonable to anticipate no adverse health effects when rat diets are formulated with rapeseed/canola meal at low levels [10]. The first published 90-day rat feeding study of canola meal (partially dehulled) that was based on OECD test guideline 408 [81] was a complete functional replacement of soybean meal (24 percent, w/w) in the rat diet [28]. Prior to this study, no historical precedent existed to predict how well rats would perform when fed canola meal as a full replacement of the soybean meal protein equivalent in 90-day rat feeding studies. Furthermore, it should be noted that even this study was not fully relevant to commercially available canola meal since it did not employ meal derived from a commercially-relevant process that uses non-dehulled canola seed.

Additional precedent for 90-day rat feeding studies with canola meal is available in an EFSA Scientific Opinion that summarized results of three 28-day rat feeding studies on GM canola meal EFSA [31] and was also reviewed by Australia New Zealand Food Authority (ANZFA) in 2000 [2]. Significantly higher liver weight parameters were observed in rats fed diets containing 15 percent (w/w), but not up to 10 percent (w/w), commercially-processed canola meal. These observed liver weight differences were considered not biologically relevant and non-adverse due to lack of corroborative findings following necropsy. However, when combined with other published results by Delaney et al. [28], 15 percent (w/w) commercially-processed canola meal provides a recommended maximum incorporation rate for 90-day rat feeding studies conducted to identify potential unintended hazards associated with GM canola.

#### *2.4.3. Canola Study Maximum Incorporation Rate Recommendations/Conclusions*

Rat diets containing 15 percent (w/w) canola meal correspond to approximately 13.8 g canola/kg bw/day for rats (based on a mean daily consumption of 92 g diet/kg bw/day in the Sprague Dawley rat) [121]. Humans consume canola oil, but not foods made from canola meal. Therefore, the amount of canola meal incorporated into these rat diets provides a MOE many orders of magnitude greater than can be actually calculated because the human consumption estimates used for comparison only represent the intake of rapeseed/canola oil. In humans, the highest chronic consumption of rapeseed/canola (oil) worldwide (32.68 g/person/day) from the WHO GEMS database [131] was reported for people in country cluster diet G07, which includes Australia and several European countries (Table 1). Based on a human body weight of 60 kg, this corresponds to a conservative estimated exposure of 0.54 g/kg bw/day. The lowest chronic consumption of canola oil worldwide was reported at 0.10 g/person/day or 0.002 g/kg bw/day. Thus, a 15 percent (w/w) canola meal incorporation rate in the rat diet represents, at a minimum, a MOE of approximately 26-fold to 6,900-fold relative to human consumption of canola oil. Of note, three country clusters reported no consumption of rapeseed/canola oil.

Setting aside the caveat that humans only routinely consume highly-refined canola oil, the following recommendations are relevant. Commercially processed (non-dehulled) canola meal presents unique and significant challenges for rat diet formulation. On a protein equivalency basis, full substitution of soybean meal with partially dehulled and gently processed canola meal (41 percent w/w protein) was achieved with dietary incorporation of 24 percent (w/w) [28]. Based on this single publication with dehulled canola meal, EFSA recommends a dietary incorporation of 25 percent (w/w) for 90-day rat feeding studies to support regulatory safety assessment of new GM canola varieties [38]. It is important to note that commercially-processed (non-dehulled) canola meal contains anti-nutrients (especially fiber, glucosinolates, lignin, and tannins) that could negatively impact monogastric animals such as rats, if a 25 percent (w/w) level of incorporation of non-dehulled canola meal is included in study diets.

For example, using lignin as a representative anti-nutrient in canola meal, the lignin content in commercially-processed (non-dehulled) canola meal is expected to be around 7 percent (w/w, unpublished data). Meal from mechanically-dehulled canola was estimated to contain approximately 3.7 percent (w/w) residual lignin [28]. When taking into account the various plant-based ingredients used to prepare the rat diet in this study, the total dietary lignin content is estimated to be approximately 1.5 percent (w/w). To achieve a similar total dietary lignin content of 1.5 percent that was well-tolerated [29], the maximum incorporation rate of commercially-processed (non-dehulled) canola meal should not exceed 15 percent (w/w) of the diet. This level of canola meal incorporation is consistent with results EFSA summarized from three 28-day rat feeding studies with canola meal from a GM canola variety (EFSA [31] and was also reviewed by ANZFA [2]. Therefore, a rat diet with 15 percent (w/w) canola meal incorporation provides a recommended maximum incorporation rate for 90-day rat feeding studies conducted to identify unintended adverse effects.

## 3. Cottonseed

### *3.0.1. Cottonseed Production and Use as Food and Feed*

Cotton (mostly Gossypium hirsutum) is generally cultivated as a source of high-cellulose fiber that is used primarily in textiles. Nonetheless, approximately 15 percent of the value of cotton is derived from the products of the seed and associated linters (young cotton fibers closest to the seed) for food uses (e.g., cottonseed oil and sometimes as additional dietary fiber) and for animal feed uses as cottonseed meal, a good source of protein, energy, and fiber [105]. It is noteworthy that cottonseed meal is not consumed by humans. Cottonseed contains several anti-nutritional compounds, most importantly gossypol [83]. Gossypol is concentrated in the cottonseed but can also be found in the hulls, leaves, and stems of the cotton plant. Gossypol levels vary considerably with variety, climate conditions, and processing methodology, and thus, its concentration may be different between preparations. Gossypol has several known toxicological effects including cardiotoxicity, hepatotoxicity, renal toxicity, and reproductive toxicity. Due to the toxicity of gossypol, it is highly encouraged to limit the consumption of cottonseed in the human diet [32]. Additionally, it is recommended that the level of gossypol be taken into account when formulating the rat diet, to avoid toxicity [36]. Gossypol must be removed from cottonseed by solvent extraction and toasting prior to use in most feed diets [11, 61, 75]. This extensive processing is also known to remove any traces of protein from the oil [51]. Of note, a GM cotton variety with low gossypol levels in its cottonseed has recently been developed. The use of this low-gossypol cottonseed as a protein source in both food and feed may prove to be a significant contribution to human and animal nutrition in the future [1, 96].

EFSA considers toasted defatted (or full-fat) dehulled meal as the suitable test material for oilseed crops, including cottonseed, but not the oil alone [38]. However, only highly-refined cottonseed oil (and sometimes fiber) is routinely consumed by humans. In the absence of a testable hypothesis for these highly-processed fractions, no scientific justification exists to conduct 90-day rat feeding studies for cottonseed. In addition, 90-day rat feeding studies are conducted as a surrogate test for humans, but if humans do not consume the test material, then the animal studies cannot be scientifically or ethically justified, even though such a test material would include the full constituency of the product that is being assessed for unintended changes in GM crops.

### *3.0.2. Dietary Considerations for Cottonseed in 90-day Rat Feeding Studies*

Various forms of cottonseed (ground whole seed, whole flour, and processed meal) have been incorporated into diets and fed to rats at different incorporation rates. In a 90-day rat feeding study performed with ground whole cottonseed and reviewed in an EFSA Scientific Opinion [39], groups of 20 Sprague Dawley rats were fed diets containing 2 or 5 percent (w/w) ground GM cottonseed. Six additional groups were fed diets containing 5 percent (w/w) ground cottonseed from commercial non-GM cotton varieties. It was concluded that there were no adverse effects in rats consuming diets containing ground GM cottonseed up to the 5 percent (w/w) incorporation rate.

In a 90-day rat feeding study with lightly processed conventional whole cottonseed flour, a 12 percent (w/w) incorporation rate was associated with adverse reproductive effects in rats after four weeks of feeding [113]. As reviewed above, this observation is likely due to gossypol, which can still be present after defatting.

In other published 90-day rat feeding studies, when cottonseed is processed to meal to inactivate the anti-nutrients, a 10 percent (w/w) incorporation rate has been tolerated without adverse effects on the animals. For example, a 90-day rat feeding study in Sprague Dawley rats with diets containing 10 percent (w/w) cottonseed meal from a non-GM and GM cotton variety and three commercial non-GM varieties were all well tolerated [30]. An EFSA GMO panel reviewed other similar 90-day rat feeding studies in which rats were fed diets containing approximately 5 or 10 percent (w/w) toasted cottonseed meal from one of three GM cotton varieties, or 10 percent (w/w) toasted cottonseed meal from the non-GM control, or 10 percent (w/w) toasted cottonseed meal from a commercial non-GM cotton variety [40]. For each study, the EFSA GMO Panel concluded that no adverse effects were observed after 90 days of consumption of diets containing 5 and 10 percent (w/w) toasted cottonseed meal from the respective GM cotton variety, compared to groups fed diets with similar levels of toasted cottonseed meal from non-GM control or commercial cotton varieties.

Taken together, regardless of the level of processing, the maximum incorporation rate of cottonseed in rat diets should be correlated with the concentration of anti-nutrients, especially gossypol, in the specific preparation being used because variety, climate conditions and processing methodology can all impact concentrations. This recommendation is consistent with other documents that suggest gossypol concentration should be taken into account to properly formulate diets to avoid gossypol toxicity [34, 38, 83].

#### *3.0.3. Cottonseed Study Maximum Incorporation Rate Recommendations/Conclusions*

Rat diets containing 10 percent (w/w) cottonseed correspond to approximately 9.2 g cottonseed/kg bw/day for rats (based on a mean daily consumption of 92 g diet/kg bw/day in the Sprague Dawley rat) [121]. Humans consume cottonseed oil and cotton linters, but not foods made from cottonseed meal. Therefore, the amount of cottonseed meal incorporated into these rat diets provides a MOE many orders of magnitude greater than can be actually calculated because the human consumption estimates used for comparison only represent the intake of cottonseed oil. The highest amount of chronic human consumption of cottonseed oil worldwide (20.53 g/person/day) from the WHO GEMS database [131] was reported for people in country cluster diet G01, which includes mostly Middle Eastern countries (Table 1). Based on a human body weight of 60 kg, this corresponds to conservative estimated exposure of 0.34 g/kg bw/day. The lowest chronic consumption of cottonseed oil worldwide was reported at 0.32 g/person/day or 0.0005 g/kg bw/day. Thus, a 10 percent (w/w) cottonseed incorporation rate in the rat diet represents, at a minimum, a MOE of approximately 27-fold to 1840-fold relative to human consumption of cottonseed oil.

Setting aside the caveat that humans only routinely consume highly refined fractions (oil and sometimes fiber) from cotton, the following recommendations are relevant. Cottonseed contains the anti-nutrient gossypol making rat diet formulation challenging. Consequently, cottonseed should be processed into meal using standard processing practices known to reduce gossypol content (defatting and toasting) for incorporation into rat diets. Therefore, with the exception of cases in which gossypol levels would be too high to be permissible, a rat diet with 10 percent (w/w) cottonseed meal incorporation provides a recommended maximum incorporation rate for 90-day rat feeding studies conducted to identify unintended adverse effects. However, rat diets containing lower dietary incorporation rates of toasted cottonseed meal may also be considered appropriate.

## 4. Discussion

Ninety-day rat feeding studies based on OECD test guideline 408 [83] are required by some regulatory authorities [32, 33, 38, 41, 46] for providing added safety assurance for foods and feeds derived from GM crops. However, unlike traditional toxicology studies that assess the effect of chemicals at levels several orders of magnitude above anticipated human or animal intake, whole food is a complex mixture of largely nutritional components (e.g., carbohydrates, lipids, proteins, vitamins) that can only be incorporated into the rat diet at marginally higher levels than the anticipated exposure rates [9, 38]. Therefore, to avoid confounding adverse observations unrelated to the purpose of the study, the maximum incorporation rate of a whole food from a GM crop in 90-day rat feeding studies is scientifically limited for one or more of the following reasons: 1) avoiding nutritional imbalances related to overfeeding a prevalent nutrient in the whole food (e.g., high protein content in meal from soybean); 2) limiting the available space for other dietary ingredients that provide critical nutrients but are decreased proportionately as the percentage of the whole food is increased (for example, high rates of incorporation of maize in rat diets limits the ability to include sufficient nutritional factors); and 3) limiting the exposure of animals to anti-nutrients or toxins naturally present in the whole food (e.g., the presence of gossypol in cottonseed).

If 90-day rat feeding studies continue to be a mandatory requirement (as opposed to being considered on a case-by-case basis when a specific hypothesis exists for testing), then it is important that the control diets be nutritionally equivalent to the diets commonly used by the testing facility to avoid usage of additional animals to produce sufficient baseline data. Using dietary incorporation rates that are the same as the historical control data incorporation rates enables a reduction in the

Table 1: Overview of currently available consumption databases.

| **Crop** | **Commodity** [a] **(food groups included)** | **Human Consumption Range (g/day)** | **Country Cluster with Highest Consumption** [b] | **Human Body Weight** [c] **(bw, kg)** | **Human Consumption Range (g/kg bw/day)** | **Recommended Maximum Incorporation Rate (%, w/w)** | **Rat Consumption** [d] **(g/kg bw/day)** | **MOE (rodent/ human)** |
|---|---|---|---|---|---|---|---|---|
| Maize | Maize, raw (glucose, high-fructose syrup, flour, oil, beer, germ, starch) | 7.36 - 116.66 | G13 | 60 | 0.12 - 1.94 | 50% | 46.0 | 23.7 - 383 |
| Soybean | Soya bean, dry, raw (paste, curd, oil, sauce | 14.29 - 222.52 | G11 | 60 | 0.24 - 3.71 | 30% | 27.6 | 7.4 - 115 |
| Rice | Rice, husked, dry (polished, flour, starch, oil, beverages) | 14.99 - 339.67 | G09 | 55 | 0.27 - 6.18 | 60% | 55.2 | 8.9 - 204 |
| Canola | Rapeseed, raw (oil) | 0.10 [e] - 32.68 | G07 | 60 | 0.002 - 0.54 | 15% | 13.8 | 25.6 - 6,900 |
| Cotton | Cottonseed, raw (oil) | 0.32 - 20.53 | G01 | 60 | 0.005 - 0.34 | 10% | 9.2 | 27.1 - 1,840 |

[a]From WHO GEMS International Estimated Daily Intake (IEDI) template [131].

[b]G13 = Botswana, Burkina Faso, Central African Republic, Chad, Ethiopia, Gambia, Haiti, Kenya, Malawi, Mali, Namibia, Niger, Nigeria, Senegal, Somalia, Sudan, Swaziland, United Republic of Tanzania, Zimbabwe;
G11 = Belgium, Netherlands;
G09 = Bangladesh, Cambodia, China, Democratic People's Republic of Korea, Guinea Bissau, Indonesia, Lao Peoples Democratic Republic, Myanmar, Nepal, Philippines, Sierra Leone, Thailand, Timor Leste, Viet Nam;
G07 = Australia, Bermuda, Finland, France, Iceland, Luxembourg, Norway, Switzerland, United Kingdom, Uruguay;
G01 = Afghanistan, Algeria, Azerbaijan, Iraq, Jordan, Libya, Mauritania, Mongolia, Morocco, Occupied Palestinian Territory, Pakistan, Syrian Arab Republic, Tunisia, Turkmenistan, Uzbekistan, Yemen

[c]For most populations in the world, an average body weight of 60 kg is assumed. For the Asian population, an average body weight of 55 kg is assumed [133].

[d]Based on mean daily consumption of 92 g/kg bw/day [124].

[e]Three country cluster reported no consumption of rapeseed, raw (including oil).

number of animals needed to produce interpretable results per study. Additional reference groups, and consequently more animals, are needed if appropriate historical control data are not available at the testing facility. This practice is consistent with the principles of humane animal experimentation [45, 49, 64], that were first defined as the "3 R's" of reduce, refine, and replace, in 1959 [99]. Historical control data provide context in the determination of whether any differences observed between treatment groups are the result of natural biological variability or related to treatment. This determination is especially important when a control group falls at the extreme of the historical control data range (triggering a statistical difference) or when testing substances for which there is no testable hypothesis regarding putative adverse effects (i.e., the majority of 90-day rat feeding studies with GM crops in which Codex-prescribed studies have shown the GM crop to be substantially equivalent to the conventional counterpart prior to testing).

All of the recommended maximum incorporation rates (see Table 1) are based on both published and unpublished data and reflect experiences from conducting and analyzing 90-day rat feeding studies to meet regulatory requirements for GM products intended for use in food or feed. Although in the absence of testable hypotheses, arguments against conducting these studies have been put forth [9, 60, 108, 113, 137]; if the studies are to be conducted, then resources including time and animal lives must be used wisely [45, 49, 64].

The recommended maximum incorporation rates of GM crop materials for use in 90-day rat feeding studies are compared to the highest human consumption for the given food in this paper for several reasons. First, comparing rat consumption to the highest human consumption enables calculation of a MOE. It is noteworthy, therefore, that for each crop, the recommended maximum incorporation rate for use in 90-day rat feeding studies provides consumption at levels substantially higher than the highest human worldwide chronic consumption and is fully sufficient to address regulatory requirements. Second, although EU (IR) 503/2013 requires that the incorporation rate for 90-day rat feeding studies should allow for extrapolation of data to target animals, normal dietary exposures to GM crop materials varies extensively among livestock animals. The variation in exposures is due to many factors: 1) large number of animal species and species types; 2) geographic differences in daily intakes and body weights for a given animal species; and 3) variation in which part(s) of the crop plant are fed to livestock [48]. Additionally, a large number of animal feeding studies, including beef cattle and swine, have been well summarized previously and do not need further discussion in this publication [48]. The nutritional equivalence of GM crop material for feed use is already routinely performed (e.g., broiler chicken feeding study) and should be considered applicable to other livestock species.

The scientific justification and ethical considerations related to 90-day rat feeding studies for GM crops should be revisited. Since 1996 when the first GM crop completed regulatory safety assessments, more than 29 crops with nearly 500 different GM traits have been reviewed by global regulatory authorities. More than 3300 reviews have been completed, with each reaching a conclusion along the lines that the new GM crop was found to be as safe as the conventional varieties for that crop that have a history of safe consumption [64]. These regulatory assessments have reviewed extensive data packages prior to the commercialization of each new GM crop [24, 32, 88, 94, 95]. Importantly, the data from extensive crop composition studies have repeatedly shown that the GM crop selected for commercialization are compositionally equivalent to conventional crops, except in those few cases where the desired trait is an intentional change in composition [21]. These results provide evidence that the GM crop production process, including molecular and phenotypic screening during event selection, results in products free from harmful unintended effects [25, 124, 133, 142]. Similarly, a review of animal feeding studies, including livestock and poultry studies and short-term (21 to 30-day) and 90-day rat feeding studies (38 studies), concluded that no evidence exists to suggest that GM crops produce unintended compositional effects that result in adverse effects [9].

Significant advances in DNA sequencing technologies have expanded the available information on the genomic histories of plants during crop domestication and evolution. Many of the features of modern biotechnology that were conjectured to have potentially unique effects on plant biology [71] have, in fact, been shown to have parallel occurrences in nature. Numerous studies have shown that the insertional effects on the plant's genome caused by the transformation process are small compared with naturally-occurring genomic changes [50, 69, 103]. Additionally, potential for unintended differences in composition or other plant characteristics is more likely to be the result of the conventional breeding process and/or the environment, than usage of GM transformation to modify a specific crop [60, 97, 125]. Modern genome sequencing has shown that the plant genome is very dynamic and that mechanisms for, and examples of, natural genetic change are common in plants [69, 103]. Even examples of natural inter-species exchange of DNA (sometimes referred to as hybridization events) have been documented in every studied genome (i.e., humans, plants, insects, animals, [74, 91]. Moreover, horizontal gene transfer across phylogenetic boundaries are now well documented in higher order organisms, like plants [68, 111].

In addition, more than 139 projects have been reviewed 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. The overall conclusion of these reviews was that GM technologies are no more risky than conventional plant breeding approaches [45]. Additional toxicology studies, such as 90-day rat feeding studies conducted within the GRACE Project [137] and a two-year carcinogenicity study conducted under the Genetically Modified Plants Two Year Safety Testing (G TwYST) initiative [113], produced similar results showing lack of adverse effects of foods from GM crops. It has recently been concluded that the EU sponsored GRACE and G TwYST studies substantiated the claim that untargeted, extended feeding studies with rats provide no added value relative to the available non-animal studies for the risk assessment of GM crops [113].

## 5. Conclusions

The recommended maximum incorporation rates of GM crop materials for use in 90-day rat feeding studies are presented. These recommendations endeavor to balance the desire for feeding high concentrations of whole food to optimize the chance of detecting unintended adverse effects (which are unlikely to occur given the extensive comparative assessment data for each GM crop that has yet to identify any hazard or adverse effect to justify animal testing [19, 60, 97]) with the need to ensure that the use of animals in 90-day rat feeding studies generates interpretable data, free from artifactual effects of dietary imbalances. These recommended maximum incorporation rates (all w/w based) are 50 percent ground maize grain, 30 percent toasted defatted dehulled soybean meal, 60 percent rice flour, 15 percent non-dehulled canola meal, and 10 percent toasted defatted dehulled cottonseed meal. With the exception of canola meal, these rates are consistent with the rates proposed in the explanatory guidance provided by the EFSA Scientific Committee for EU IR [46]: 50 percent maize [139], 30 percent soybean [108], 60 percent rice [127], and 25 percent canola meal [28, 38]. No maximum incorporation rate recommendations were provided for cottonseed by EFSA. Importantly, many 90-day rat feeding studies have been performed with incorporation rates below the maximum incorporation rates recommended in this paper and by EFSA. Given that these incorporation rates provide a MOE several fold higher than the highest human chronic consumption data, these studies are scientifically valid in terms of dietary incorporation rates and fully appropriate for the assessment of human safety.

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## 7. Conflict of interest statement

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

## 8. Article Information

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