Trends in Food Science & Technology 106 (2020) 469–484

Contents lists available at ScienceDirect

Trends in Food Science & Technology

journal homepage: www.elsevier.com/locate/tifs

Animal-free strategies in food safety & nutrition: What are we waiting for? Part I: Food safety

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ARTICLE INFO

Keywords:
Non-animal testing
Food safety
Legislation

ABSTRACT

Background: Methods and approaches that can be used in toxicology and safety assessment are changing at a faster pace than ever. Members of the International Life Sciences Institute (ILSI) Europe have formed an expert group to review possibilities, opportunities and challenges for the potential use of non-animal testing strategies in food safety and nutrition research, which can ultimately be used in support of regulatory submissions for pre-market authorisation.

Scope and approach: For the different areas of food improvement agents, genetically modified foods and novel foods, the acceptability of non-animal strategies is evaluated in comparison to legislative requirements in Europe. Current hazard and risk assessment tools that do not require additional animal testing are reviewed and emerging tools and methodologies considered, covering advanced *in vitro* methods, *in silico* and system biology approaches and high-throughput methods for mode-of-action assessment.

Conclusions: The paper highlights the great potential for research strategies to be developed that reduce or avoid the use of animal tests, with the generation of more human-relevant data from multiple sources. It also shows the discordance in current legislation: on one hand saying non-animal strategies should be used, but on the other hand not providing sufficient guidance, leading in practice to lack of use of these non-animal testing strategies. This emphasizes the need for scientific developments and acceptability to be more reflected in legislation (e.g. guidance). What are we waiting for?

1. Introduction

Methods and approaches that can be used in toxicology and safety assessment are changing at a faster pace than ever. Members of the International Life Sciences Institute (ILSI) Europe have formed an expert group to review possibilities, opportunities and challenges for the potential use of non-animal testing strategies in food safety research, which can ultimately be used in support of regulatory submissions for pre-market authorisation. In this paper, non-animal methods or approaches refer to the 3 R s concept (Replacement, Reduction and Refinement) (Russel & Burch, 1959), meaning the use of animal-free methods when and where possible, but any opportunity to reduce or refine would also be appropriate. (see Table 1).

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https://doi.org/10.1016/j.tifs.2020.10.034
Received 2 March 2020; Received in revised form 23 October 2020; Accepted 24 October 2020
Available online 5 November 2020

Table 1
Overview of applicable EU legislation, testing requirements and flexibility for non-animal approaches.

Regulation/Directive Nr	Regulation/Directive Title	Testing recommendations47	Flexibility for Alternatives
New nutrition sources			
Dir 46/2002/EC	Directive on food supplements	No requirements laid down in Directive. Guidance from European Commission (2001) requires biological and toxicological data EFSA Guidance (2018) specifies tiered approach (similar to the evaluation of novel foods and food additives): only when no data is available yet or when nutrients are absorbed unchanged from the GI tract, additional testing is required. Depending on Tier 1 tests (non-animal tests), higher level tests are required to be provided in the dossier. Minimum data set according to this guidance document includes modified 90-day toxicity test.	*In vitro* only possible for lowest tier genotoxicity assessment. When higher level tier tests are required, this includes animal tests. Flexibility on a case by cases, but seems limited.
Reg (EC) No 1925/2006	Regulation on the addition of vitamins and minerals and of certain other substances to foods	Similar to food supplements	Similar to food supplements.
Reg (EU) No 609/2013	Regulation on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control.	Similar to food supplements	Similar to food supplements.
Additives, enzymes & flavourings			
Reg (EC) No 1331/2008	Regulation establishing a common authorisation procedure for food additives, food enzymes and food flavourings	Lays down authorisation procedure. Does not specify data requirements.	
Commission Reg (EU) No 234/2011	Commission Regulation implementing Regulation (EC) No 1331/2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings	Further defines the authorisation procedure and data requirements for the risk assessment of a substance in general, as well as specific data requirements for food additives, flavourings and enzymes: core areas to cover include biological data of toxicokinetics and toxicity (subchronic, chronic, carcinogenic, genotoxic, reproductive and developmental toxicity). Detailed in corresponding EFSA guidances per substance group. Emphasise Tiered approach.	
Reg (EC) No 1332/2008	Regulation on food enzymes	Detailed in corresponding EFSA guidance: animal testing normally required for genotoxicity, sub-chronic toxicity and allergenicity.	
Reg (EC) No 1333/2008	Regulation on food additives	Detailed in corresponding EFSA guidance, which emphasizes the Tiered approach. Animal testing for genotoxicity, sub-chronic toxicity and allergenicity.	Tier 1 can be fulfilled with alternatives. Tier 2 and 3 are requiring animal studies. E.g. a positive result in 1 or 2 *in vitro* genotoxicity tests still needs confirmation *in vivo*. Animal studies still considered necessary, but EFSA considering alternative test methods case by case.
Reg (EC) No 1334/2008	Regulation on flavourings and certain food ingredients with flavouring properties for use in and on foods	EFSA guidance details testing requirements: Genotoxicity testing. 90-day repeated dose study if intake is above the TTC for the applicable flavouring group. More *in vivo* data might be requested if flavouring cannot be assigned to an existing group.	Tier 1 can be fulfilled with alternatives. Tier 2 and 3 are requiring animal studies. E.g a positive result in 1 or 2 *in vitro* genotoxicity tests still needs confirmation *in vivo*. Explicit acceptance of grouping substances, as well as the usage of TTC. Animal studies still considered necessary, but EFSA considering

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Table 1 (*continued*)

Regulation/Directive Nr	Regulation/Directive Title	Testing recommendations47	Flexibility for Alternatives
New nutrition sources			
			alternative test methods case by case.
Novel foods			
Reg (EU) No 2015/2283	Regulation on novel foods	Safety assessment for novel ingredients is described (Article 10) to revolve around comparability of a product to other food categories, (the safety of) the food composition, and ensuring that the product is not nutritionally disadvantageous. For traditional foods, Article 14 specifies that detailed composition, country of origin and documented data demonstrating history of safe use should be included in the notification. No testing requirements specified.	Animal studies still considered necessary, but EFSA considering alternative test methods case by case.
Commission Reg (EU) No 2017/2468	Commission Implementing Regulation laying down administrative and scientific requirements concerning traditional foods from third countries in accordance with Regulation (EU) 2015/2283	Article 6 defines that scientific data must include relevant studies related to assessing history of safe use. Details provided in corresponding EFSA Guidance: experience of continued food use need to be documented and the proposed conditions of use need to be described.	
Commission Reg (EU) No 2017/2469	Commission Implementing Regulation laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283	Article 5 describes that scientific data should enable a comprehensive risk assessment of the novel food, and the testing strategy should be specified for that purpose. Conducted tests need to comply with GLP standards. Article 8: next to compositional data and history of safe use, ADME data, nutritional information, toxicological information and allergenicity should be included in the opinion of EFSA. Dossier should thus include this type of information, as further detailed in corresponding EFSA guidance: ADME data Nutritional information Toxicity data (following tiered approach as for additive): 90-d sub-chronic tox study typically required. Allergenicity data: protein analysis and/or human testing.	Guidance suggests the use of specific animal models to study especially subchronic toxicity, but testing requirements are described to be determined on case-by-case approach. In case of negligible absorption, higher tier tox studies can be waived. Read-across accepted in principle. TTC accepted for contaminants & metabolites.
GM foods			
Dir 18/2001/EC	Directive on the deliberate release into the environment of genetically modified organisms	No testing requirements specified.	
Reg (EC) No 1829/2003	Regulation on genetically modified food and feed	No testing requirements specified.	
Implementing Reg (EU) No 503/2013	Commission Implementing Regulation on applications for authorisation of genetically modified food and feed in accordance with Regulation 1829/2003/EC	Provides required details on application, which includes a 90-day feeding study in rodents for studying markers related to sub-chronic toxicity.	None available yet.

1.1. Legislation

EU-food legislation aims to ensure that food products and their ingredients are safe for consumers as well as for the environment. In 2002, Regulation (EC) No 178/2002 laid down the general principles and requirements of food law (known as the General Food Law Regulation, GFL[1]). Based on the GFL and subsequent sectoral regulations and directives dealing with specific aspects of food safety, safety assessment or testing requirements of varying specificity are required that will be discussed in this publication. The food industry faces the challenge of assessing foodstuffs and food components for the general population, while using animal safety testing for extrapolation purposes can at times be of limited relevance for humans (Rovida et al., 2015). Therefore, there is a need to develop models and methods that better predict effects in humans.

Currently in Europe, approximately 45,000 animals/year[2,3] are used annually for scientific testing in the food sector. This number is mostly composed of rodents (more than 95%) and it is expected to reduce as

[1] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety.

[2] Average annual figure of 2015, 2016 and 2017 data reported from the Member States to the EU Commission of animal testing conducted to meet requirements of food legislation including food contact materials. More than 95% of the animals are rodents.

[3] Report from the Commission to the European Parliament and the Council. 2019 report on the statistics on the use of animals for scientific purposes in the Member States of the European Union in 2015–2017.

more and more alternative, non-animal testing methods or reduction and refinement methods as part of the 3Rs concept, are being employed. In fact, all testing carried out in Europe shall, based on Article 13 of the Treaty on the Functioning of the European Union (TFEU) on the Protection and Welfare of Animals, comply with the requirement to replace, reduce and refine the use of animals for scientific purposes in accordance with Directive 2010/63/EU[4] on the protection of animals used for scientific purposes. Compliance with this 3 R s principle is a legal obligation in the EU for research activities as well as regulatory testing using live animals since the Directive 2010/63/EU[4] came into force in 2013. This Directive is a horizontal piece of legislation and applies to testing conducted under sector legislation like the specific regulations dealing with food safety aspects: food additives, novel foods, genetically modified (GM) foods or foods with the opportunity of using nutrition and/or health claims. As Article 13 of the Directive[3] outlines, replacement approaches to an existing animal method must be used if "*another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognised under the legislation of the Union*". Concerning reduction or refinement of animal procedures, the Directive demands to select the method that "*uses the minimum number of animals; involves animals with the lowest capacity to experience pain, suffering, distress or lasting harm and causes the least pain, suffering, distress or lasting harm and is most likely to provide satisfactory results*".[3]

However, there is a great deal of uncertainty among both producers and regulators alike about which non-animal methods or approaches are useable for food safety and should hence be accepted for regulatory purposes. For example, in the context of current re-evaluations of food additives, the European Food Safety Authority (EFSA) still recommends through its guidance documentation a lot of animal tests[5,6], whereas their expert judgement might enable safety-assessment based on Weight of Evidence using existing animal data and information from non-animal strategies. As previous publications (EFSA & WHO, 2016) indicate, there are still concerns regarding the applicability of *in vitro* and *in silico* methods to predict food safety or to test complex foodstuffs and regarding the use of the Threshold of Toxicological Concern (TTC) concept (Kroes et al., 2007; Munro et al., 2008) for food safety assessment. So far, these methods have been used mainly for risk prioritization and impurity/contaminant assessment (de Boer & Bast, 2018). Initiatives like the launch of Databases and Scientific Data Warehousing,[7] such as OpenFoodTox, EFSA's chemical hazards database providing open source data (chemical and toxicological information) for individual substances. These initiatives should help to increase their acceptance in the near future. To obtain clarity about the acceptability of non-animal methods or approaches, sector legislation should ideally be updated frequently to reflect technical progress in the use of scientific evidence under food legislation. However, legislative updates tend to lag behind the scientific developments and hence there is a practical discordance or dilemma.

In other legislative areas, for example chemicals and biocides, the use of non-animal approaches is more actively encouraged and recognition of new non-animal methods in the EU works via their inclusion in the Test Methods Regulation[8] under REACH Regulation (EC) No 1907/2006.[9] At the international level, new methods are agreed upon within the Organisation for Economic Co-operation and Development (OECD). Reference to the current OECD testing protocols is also the basis of testing requirements in the food sector.

1.2. Transformation of toxicity testing

Over a decade ago, the US National Research Council (NRC) published 'Toxicity Testing in the 21st Century: a vision and a strategy' (Toxicity testing in the 21st Century: A vision and a strategy, 2007), which described an approach that "could transform toxicity testing from a system based on whole-animal testing to one founded primarily on *in vitro* methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin". At the core of this is an understanding of the key biological pathways, which if sufficiently perturbed by a chemical, would result in an adverse outcome for the individual.

In the context of safety assessment this concept has been built upon with the description of 'adverse outcome pathways' (AOPs[10]). Each AOP starts with a 'molecular initiating event' in which the chemical interacts with a biological target leading to a sequence of events resulting in an adverse outcome. The OECD have started to formalise a chemical risk assessment framework based on AOPs to capture the mechanistic understanding of specific toxic effects, and for the evaluation of non-animal methods that aim to predict key events in these pathways. Mechanistic understanding of toxicity pathways and AOPs may be the basis for establishing points of departure that could be used in risk assessment in the future. In a recent publication, Vinken et al. (2020) assessed the potential of applying AOPs in the safety evaluation of food additives and conclude that AOPs may be especially useful in the hazard identification of food additives by identifying the relevance of specific adverse effects observed in test animals.

Blaauboer et al. (2016) propose a stepwise roadmap using these new strategies for evaluating risk that can be applied for the evaluation of food and food ingredients. The authors recognise that the science of toxicology and safety assessment is changing, moving away from apical endpoints of toxicity in animal models to approaches that are more exposure driven and reliant on understanding the mechanism of toxicity in humans. The proposed roadmap consists of a stepwise evaluation of distinct aspects needed for a safety evaluation. These blocks of activities include for example, specification of chemical structures (QSARs–Quantitative Structure Activity Relationships), exposure scenarios, kinetics to evaluate internal exposure, methods to evaluate toxicity (including *in vitro* and computational models), mechanisms of toxicity, *in vitro/in vivo* extrapolations using physiologically based kinetic (PBK) modelling, ultimately leading to a risk assessment and the determination of safety levels. The emphasis of this roadmap is to recognise that models and methods should be used that provide relevant information for the mechanism of action in humans. Therewith the classical animal-based methods for food safety evaluations should be avoided, but the authors recognise that in some cases animal models may still be required

[4] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

[5] EFSA Call for technical and toxicological data on lecithins (E 322) for use as a food additive in foods for all population groups including infants below 16 weeks of age.

[6] EFSA Call for technical and toxicological data on locust bean gum (E 410) as a food additive for use in foods for all population groups including infants below 16 weeks of age.

[7] New tools to potentially reduce need for animal testing: https://www.efsa.europa.eu/en/press/news/170710.

[8] Council Regulation (EC) No 440/2008 of 30 Ma y 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH).

[9] Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC (Text with EEA relevance).

[10] OECD: Adverse Outcome Pathways, Molecular Screening and Toxicogenomics.

for addressing particular questions. In the case animal studies are needed, more data on AOP should be collected than only toxicological endpoints.

This article evaluates the current legislative requirements in Europe in the area of food safety in light of use of animal testing and opportunities for transformation. Furthermore, it informs about approaches and methods to contribute to this transformation, following the principles of the 3 R s that were developed over 50 years ago providing a framework for non-animal testing strategies for food safety or nutrition assessment, that are available now as well as discussing future opportunities arising from new, emerging technologies.

2. General food safety regulatory requirements

As the EU framework regulation on foods, the GFL[1] aims to guarantee the highest level of protection of human health and consumer interest, whilst ensuring that the internal European market is functioning effectively. It therefore defines general principles and procedures for food and feed safety legislation in Europe. One of the main requirements (Article 14) is that unsafe food (either injurious to health or unfit for human consumption) is not allowed on the European market. Due to this requirement, for products that are newly introduced on the European market, evidence should be provided to establish their safety. By requiring the use of risk analysis in all food related matters, the GFL (Article 6) clearly separates the scientific process of reviewing potential food risks (risk assessment) from the political decisions (risk management), which follow from this risk assessment interpretation. The GFL also is the founding regulation for EFSA and tasks it, as risk assessor, to provide scientific advice and scientific and technical support for all European legislation and policies related to food and feed safety matters. In various fields, EFSA is requested to, next to food and feed safety issues, also provide scientific opinions on matters related to animal health and welfare and plant health.

For specific food products, horizontal and vertical laws provide more details: horizontal regulations such as the Novel Food Regulation (Regulation (EU) No 2015/228,3[11]) provide rules for all food products that are newly brought to the market (either through new use of a food ingredient or products originating from third countries), whereas vertical rules deal with specific categories of foods, such as Foods for Specific Groups as regulated under Regulation (EU) No 609/2013. Various elements related to safety are described within these regulations as requirements to prove safety, including toxicological data on a product. The details on which toxicity tests should be conducted are further defined in corresponding EFSA guidance. Whilst most guidance documents describe the need to provide data from animal studies to show potential toxic effects of a food, EFSA does encourage the use of approaches that replace, reduce, or refine the use of animals in safety assessment and in 2009 published a review of approaches that could be applied in food and feed risk assessment in this respect (Opinion in EFSA Journal, 2009). This is largely based on the publication by the NRC (Toxicity testing in the 21st Century: A vision and a strategy, 2007) as mentioned in the introduction.

2.1. New nutrition sources

Article 29 (1) of the GFL describes that for every authorisation request that a food business operator makes, to bring a new product on the market or to use new claims on foods, EFSA can be requested to issue scientific opinions related to the safety of such a new product or the efficacy of the proposed claim. One group of products for which such scientific opinions may be issued are new nutrients or nutrition sources. When these new nutrients are used in food products, either in food supplements (in the context of Directive 2002/46/EC[12]), added to food, including vitamins and minerals (regulated under Regulation (EC) No 1925/200,6[13]), or as ingredient in Foods for Specific Groups (Regulation (EU) No 609/2013[14]), their safety needs to be established before such ingredients will be allowed for use in Europe. Although the GFL does not provide any specific details how to study and prove the safety of a product, applications to authorise new nutrients follow Article 29 (1) for this procedure. This Article describes that EFSA can be consulted to review the scientific dossiers that are submitted with these authorisation requests but does not specify the content of the dossier. Further guidance on this content is provided by EFSA on what elements are of interest for these applications (EFSA ANS Panel, 2018c).

The European Food Supplement Directive, Directive 2002/46/EC[10], defines a supplement as a foodstuff that is sold in a dose form, which has the purpose to supplement the normal diet and is a concentrated source of one or multiple nutrients (vitamins or minerals) or other substances with nutritional or physiological effects. Only vitamins and mineral listed in Annex I of the Directive,[11] in the forms listed in Annex II, can be used for the manufacturing of food supplements in the EU. The Directive also defines that purity criteria follow either Community legislation or recommendations of international bodies. National rules from Member States regulate minimum and maximum levels, until such rules are set by the European Commission. Since a supplement is regulated as a food product, for new substances to be used for food supplements, evidence of their safety is required. Whereas no details are provided on how safety needs to be demonstrated in the Directive itself (EC Scientific Committee on Food, 2001a), these details can be found in the European Commission's guidance document on submissions for safety evaluations of sources of nutrients (EC Scientific Committee on Food, 2001b). This 2001 guidance document describes that biological and toxicological data must be provided and depending on the extent of already available data, specific additional toxicological data may be necessary to be provided in the application. EFSA's recently adopted guidance document (EFSA ANS Panel, 2018c) highlights that a tiered approach is followed in the evaluation of sources of nutrients, similar to safety evaluations of food additives and novel foods: existing data is the basis for the assessment and additional testing is only required when no data is available yet, or when nutrients are absorbed unchanged from the GI tract lumen. Depending on the results from Tier 1 tests (non-animal tests); higher level tests (including animal tests) are required to be provided in the dossier. The minimum dataset required to be presented for evaluating the safety of a nutrient includes a bacterial reverse mutation assay (following the OECD TG 471 standard) and an *in vitro* mammalian cell micronucleus test (OECD TG 487), as well as a modified 90-day toxicity test in rats (OECD TG 408). The decision tree provided in the adopted guidance document (EFSA ANS Panel, 2018c) could aid in the decision-making process to conduct further studies.

Also, the safety requirements of nutrient sources used in foods under Regulation (EC) No 1925/2006[12] and in Foods for Specific groups under

[11] Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (Text with EEA relevance).

[12] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements.

[13] Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods.

[14] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009.

Regulation (EU) No 609/2013[12] follow from Article 29 (1) of the GFL and therefore, the same guidance documents are referred to for their safety requirements. Regulation (EC) No 1925/2006 specifies (in Annex I) which vitamins; minerals and certain other substances (that have nutritional or physiological effects) may be added to foods, in the forms listed in Annex II. The specifications of this Regulation explicitly (Article 1) do not apply to food supplements, since these are dealt with in Directive 2002/46/EC[11], but do apply to all other food products. Ingredients allowed for use in specific categories of foods for specific groups (including *i.e.* infant formulae and diet replacement products) are described in the Annex of Regulation 609/2013.[13]

3. Food improvement agents

Rules regarding food additives, food flavourings and food enzymes are laid down in separate legislative acts: Regulation (EC) No 1333/2008,[15] Regulation (EC) No 1334/2008[16] and Regulation (EC) No 1332/2008[17] respectively. A common authorisation procedure for these ingredients is established in Regulation (EC) No 1331/2008.[18] This authorisation procedure is further defined in Commission Implementing Regulation (EU) No 234/2011[19] that lays down which type of data is requested for the risk assessment of a substance in general (Article 5) and specifies in separate articles the specific data that should be provided for food additives (Article 6), food flavourings (Article 10) and food enzymes (Article 8). Whilst Implementing Regulation (EU) No 234/2011[18] focusses on which core areas should be covered, including biological data of toxicokinetics and toxicity (sub chronic, chronic, carcinogenic, genotoxic, reproductive and developmental toxicity), specific EFSA guidance documents define what types of tests are recommended to be included in applications for the different ingredients.

3.1. Food additives

The EFSA guidance for submission for food additive evaluations (EFSA ANS Panel, 2012; EFSA; Scientific Committee, 2017a) talks about toxicological studies being based on a tiered approach, which balances data requirements against risk: based on the expected risks (originating from other studies), further testing is required. In the first step, Tier I, animal testing is explicitly described but may be avoided. Subsequent testing in Tier II and III would typically require animal testing specifically to address questions on toxicokinetics, genotoxicity, toxicity (sub-chronic, chronic, carcinogenicity) and reproductive/developmental toxicity. As a special case, botanical food additives derived from conventional food sources with a long-term history of food use, may benefit from a 'presumption of safety' under certain circumstances when an adequate body of knowledge exists, and which has to be evaluated on a case-by-case basis.

In the EFSA guidance, explicit reference is made to Directive 2010/63/EU[2] on the protection of animals used for experimental and other scientific purposes, requiring that care is taken to avoid unnecessary use of animals. The EFSA Panel on Food Additives and Nutrient Sources also mentions that as adequate human data are unlikely to be available, *in vivo* studies are still needed in order to assess possible risk from ingestion of food additives. Importantly however, EFSA states in its guidance that studies submitted using non-animal testing methods will be considered by the panel on a case-by-case basis. Specifically, the EFSA guidance addresses:

- Toxicokinetics: *In vitro* studies are recognised as being able to provide useful information for the investigation of absorption, distribution, metabolism and excretion (ADME), as well as there being established models for absorption studies.
- Genotoxicity: EFSA acknowledges that a battery of *in vitro* tests to evaluate gene mutation, structural and numerical chromosomal alteration are available, recommending for tier 1 a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus test. They also recommend the consideration of structure activity relationships and 'read-across' from other similar molecules, which can reduce use of animal testing. Also, the TTC concept is mentioned as possibly being helpful when assessing the genotoxicity of low exposure substances e.g. impurities and metabolites. The TTC approach is a pragmatic risk assessment tool for which thresholds are derived related to the chemical structure of a substance (Kroes et al., 2005). TTC thresholds can be used in risk assessment in case no hazard data for a substance is available. Based on the molecular structure of the substance, a threshold can be assigned below which there is a very low probability of an appreciable risk to human health.
- Toxicity testing: Essentially animal testing is proposed with no alternatives suggested. The 90-day rat study being the typical starting point for establishing the point of departure that carries through into the risk assessment. More specialised areas of toxicology (e.g. reproductive and developmental toxicity, immunotoxicology, carcinogenicity, neurotoxicity) are flagged as a potential requirement after conducting the 90-day rat study when effects of potential concern for further follow up may be seen.

Hartung (2018) suggests a way for updating the GRAS evaluation process for food additives (the regulatory framework applicable in the USA), to bring testing requirements up to date and consider the new toxicological approaches that are being developed. He points out that the 3 R s principle of replacing, reducing and refining animal use (Russel & Burch, 1959) is the cornerstone of any toxicological test guidance, but these principles are not specifically mentioned in the 2000 FDA Redbook[20]. The proposal by Hartung (2018) for food additives in GRAS evaluations makes use of available toxicological data and proposes a strategy for evaluation based on *in silico* and *in vitro* approaches.

3.2. Food flavourings

Similar to food additives, the specific Regulation dealing with food flavourings[21] does not specify how to establish the safety of such products. Article 10 of Commission Implementing Regulation (EU) No 234/2011[19] defines that with regard to the biological and toxicological data, structural and metabolomic similarities to flavouring substances in

[15] Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives.

[16] Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC.

[17] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97.

[18] Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

[19] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings.

[20] https://www.fda.gov/regulatory-information/search-fda-guidance-documents/redbook-2000-i-introduction.

[21] Commission Regulation (EU) No 234/2011 of 10 March 2011 implementing Regulation (EC) No 1331/2008 of the European Parliament and of the Council establishing a common authorisation procedure for food additives, food enzymes and food flavourings Text with EEA relevance.

an existing flavouring group evaluation should be studied, as well as genotoxicity, and where applicable (sub-) chronic and developmental toxicity and carcinogenicity data. The EFSA guidance document on data requirements for food flavourings risk assessment (EFSA CEP Panel, 2010) further specifies what type of studies are recommended in the dossier, including 90-day feeding studies in rodents when sub-chronic toxicity needs to be studied. Regarding the latter, EFSA refers to the TTC concept. If the exposure to the flavouring is below its TTC level, then the flavouring is considered as safe and no additional (animal) safety testing is needed. Moreover, also for food flavouring safety assessment grouping approaches are being used.

In the Guidance document, a distinction is made between flavouring substances (chemically defined substances with flavouring properties) and flavourings other than flavouring substances (certain food ingredients with flavouring properties for use in and on foods), which can also be used for other purposes such as food additives. The status of the ingredient depends on the intended functional effect in the final food. Similar data requirements are described for both flavouring substances and ingredients that have flavouring properties, although the source of the ingredient might influence whether additional tests are required.

3.3. Food enzymes

Regulation (EC) No 1332/2008,[22] and Implementing Regulation (EU) No 234/2011[19] lay down the rules regarding food enzymes, whereas the corresponding EFSA guidance on submission of a dossier on food enzymes (EFSA, 2009) describe the data requirements for food enzymes. Again, specific tests are not defined in the Regulations, only the requirement to present specific data on for example toxicity is put forward in Article 8 of Regulation (EU) No 234/2011.[19]

The key component of evaluating safety of a food enzyme from microbial sources is the safety assessment of the production strain, in particular its pathogenic and toxigenic potential (Pariza & Johnson, 2001) and the fermentation process, to understand what the chemical products of the enzymatic process are. The default assumption is that toxicological testing in animals is necessary for enzymes and should address genotoxicity, sub-chronic toxicity as well as potential allergenicity (EFSA, 2009). The assessment of genotoxicity should start with two *in vitro* tests (gene mutation in bacteria and detection of chromosomal aberration). A positive result would need confirmation in an *in vivo* study. The standard assessment of systemic toxicity is performance of a 90-day sub-chronic oral toxicity study in rats.

EFSA's guidance document on food enzyme applications does address several scenarios when toxicological testing may not be needed. The toxicology data may be reduced or waived, if:

- There is a documented history on the safety of the source of the food enzymes, supported by existing toxicological studies;
- Food enzymes are produced by microorganisms that have a status of qualified presumption of safety (QPS) and there are no concerns with the total production process;
- A food enzyme from a specific strain has been thoroughly tested and the manufacturing process does not differ significantly from other food enzymes from the same strain, the full testing battery may be waived for these food enzymes. EFSA will consider on a case-by-case basis.

[22] Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and Regulation (EC) No 258/97 (Text with EEA relevance).

4. Novel foods

Before a novel food product can be authorised by the European Commission to be placed on the market, its safety needs to be established. The Novel Food Regulation (NFR), Regulation (EU) 2015/2283,[23] deals with all new foods and food ingredients, either being newly developed products or being traditional foods from third countries. The NFR establishes the authorisation procedure for novel foods and defines a food as 'novel' when it firstly has not been consumed to a significant degree in Europe before May 15, 1997 and secondly falls into one of the ten predefined categories in the Regulation. These predefined categories include foods (a) 'with a new or intentionally modified molecular structure', (b) 'consisting of, isolated from or produced from cell culture or tissue culture derived from animals, plants, microorganisms, fungi or algae', and (c) 'food exclusively used in supplements before May 15, 1997'. Article 2 of the NFR explicitly defines that these rules do not apply to genetically modified foods, as well as when foods are or are used as food enzymes, food additives, food flavourings or extraction solvents.

Similar to other European food laws dealing with authorisation requests, an individual applicant should submit a dossier for a novel food application, containing details on the safety aspects of the product. Upon request of the Commission, this scientific dossier is assessed by EFSA and their opinion forms the basis for the final authorisation decision.

Depending on whether the authorisation request concerns a completely new ingredient (a novel food) or whether it addresses a traditional food from a third country, the safety testing requirements differ. For traditional foods from third countries, a simplified procedure is in place, which is specified in EFSA guidance (EFSA NDA Panel, 2016a) and Implementing Regulation (EU) 2017/2468.[24] This procedure consists of a notification procedure that should include data upon the composition of the product as well as the country of origin, together with information demonstrating the history of safe use (EFSA NDA Panel, 2016a).

For novel ingredients, Article 10 of the NFR describes the requirements for such a safety assessment, which are further specified in Implementing Regulation (EU) 2017/2469[25] lying down administrative and scientific requirements for novel food applications without prescribing which tests should be presented in the dossier. When assessing the safety of a novel food, it is considered whether:

- The novel food is as safe as foods from a comparable category already placed on the market;
- The composition of the novel food and its conditions of use do not pose a safety risk to human health;
- The novel food (when intended to replace another food) will not be nutritionally disadvantageous for the consumer.

For food safety reasons, the Commission may impose post market monitoring requirements as a condition of a novel food approval.

The EFSA guidance (EFSA NDA Panel, 2016a) for authorisation of a

[23] Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (Text with EEA relevance).

[24] Commission Implementing Regulation (EU) 2017/2468 of 20 December 2017 laying down administrative and scientific requirements concerning traditional foods from third countries in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods (Text with EEA relevance).

[25] Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods.

novel food outlines the requirements for the type of information that needs to be presented, which includes compositional data, production process, specification, history of use, proposed uses, use levels and anticipated intake, ADME and nutrition data as well as toxicological information. In considering the history of use of the novel food, a comprehensive literature search on studies with specific and safety-relevant components, not only on the novel food itself, but also on similar foods from the same or other closely related sources is advised to be conducted. The purpose of this is presumably to ensure that everything on the novel food related to safety is captured, which will also help minimise any duplication of animal studies.

ADME is flagged by EFSA as important, with the acknowledgement that the demonstration of negligible absorption may provide a scientific justification for not undertaking higher tier toxicological studies. A broad range of toxicology studies are recommended to be considered, with the tiered toxicity testing approach proposed for food additives being the default approach. In line with guidance for food additives, a sub-chronic toxicity study should normally be submitted, the results of which may trigger additional studies e.g. chronic toxicity, reproductive toxicity, which may not be required if argued on a case-by-case basis. In the case of genotoxicity, a basic battery of *in vitro* tests is recommended as a first step. In addition, toxicological data on structurally related substances ('read-across') should be considered, and the TTC approach is suggested as helpful when assessing the risk of low exposure to substances such as contaminants and metabolites (see chapter VI for further elaboration). Although avoiding the unnecessary use of animal studies is explicitly mentioned in the EFSA guidance, *in vivo* animal data is thus still required to assess potential human risks, with the acknowledgement that the panel will only assess the use of non-animal testing in a case-by-case basis. Even though suitable alternatives are under development (see more details in chapter VI), this guidance does not seem to stimulate the use of non-animal methods by emphasising the need for substantial amounts of *in vivo* data (de Boer & Bast, 2018).

EFSA's opinion regarding the scientific evidence underlying the novel food authorisation request for egg membrane hydrolysate shows that the requirements to conduct specific tests are flexible (EFSA NDA Panel, 2018) and that there may be scope to avoid animal tests. The dossier contains information related to the production, composition and specifications (including physicochemical parameters and microbiological specifications), which provide information upon which is decided to not conduct further safety tests. This is supported by the positive opinion of EFSA's Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel) on the safety of using egg membrane hydrolysate as food supplement, resulting in the authorisation by the European Commission as novel food.[26]

Also for traditional foods from third countries authorisation needs to be requested before these products are allowed on the market. History of safe use should be presented in a notification document (Article 14 of Regulation (EU) 2015/22,8[27]). The administrative and scientific requirements for such a notification are further specified in Implementing Regulation 2017/246,8[28] and details on what type of data should be presented are described in EFSA's guidance on traditional foods (EFSA NDA Panel, 2016b). Detailed compositional data as well as documented data demonstrating the history of safe food use in a third country is required for the dossier. This is a simplified process compared with other novel foods, and does not require any additional testing (including animal testing).

5. Genetically modified organisms

Genetically modified (GM) foods and crop products are regulated under a regulatory scheme consisting mainly of three legislative documents: Directive 2001/18/EC[29], Regulation (EC) No 1829/2003[30], and Commission Implementing Regulation (EU) No 503/2013[31] as well as the specific guidance documents from EFSA which define how to address specific scientific and administrative requirements for a new GM crop product.

Prior to the commercialisation of a GM crop product, a comprehensive safety assessment is carried out, which includes the performance of:

1) A compositional comparison and an assessment of the phenotypic and agronomic characteristics of the GM crop compared with its non-GM conventional counterpart;
2) A molecular characterisation of the inserted gene;
3) An extensive characterisation of the expressed protein(s) and assessment for toxicity/allergenicity potential;
4) An assessment of the dietary exposure to the expressed protein(s) from consumption of foods and feed derived from the GM crop; and
5) A rigorous science-based environmental risk assessment (Codex Alimentarius Commission, 2003; Nair et al., 2002).

Although to date no comparative assessment or toxicity study for any Genetically Modified Organism (GMO) has indicated significant unintended effects on the plant (e.g. compositional differences outside of intentional compositional changes to the crop), assessments of potential impacts on animal nutrition and potential for toxicity would be appropriate if such effects were observed. Whole food feeding studies for evaluation of wholesomeness (e.g. livestock feeding studies) and toxicity studies have been conducted to address potential concerns pertaining to putative unintended effects on animal performance and health and are required by some regulatory authorities, sometimes on a case-by-case basis. In the EU, the 90-day sub-chronic study is a mandatory component of the regulatory dossier for GM products consisting of single events in accordance with Implementing Regulation (EU) No 503/2013[27] and these studies are to be conducted in compliance with the EFSA guidance documents (EFSA, 2011)[32]. However, when the safety of food or feed from GM crops has been demonstrated through comparative assessment between the GM crop product and its conventional counterpart and by assessing the safety of the introduced protein(s), sub-chronic toxicity studies are not scientifically justified as there is no testable risk hypothesis and, in these cases, such studies are therefore not aligned with the EU policy on animal welfare (Devos et al., 2016; Koch et al., 2015).

In the area of GMO risk assessment, the most relevant component of

[26] Commission Implementing Regulation (EU) 2018/1647 of 31 October 2018 authorising the placing on the market of egg membrane hydrolysate as a novel food under Regulation (EU) 2015/2283 of the European Parliament and of the Council and amending Commission Implementing Regulation (EU) 2017/2470.

[27] Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (Text with EEA relevance).

[28] Commission Implementing Regulation (EU) 2017/2468 of 20 December 2017 laying down administrative and scientific requirements concerning traditional foods from third countries in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods.

[29] Directive 2001/18/EC of the European Parliament and of the Council of 12 March 2001 on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC.

[30] Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed.

[31] Commission Implementing Regulation (EU) No 503/2013 of 3 April 2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006.

[32] Recommendations based on the required safety endpoints that are described in legislation and in EFSA guidance documents.

the 3 R s principle and recommended action is a reduction in the use of animals through the deletion of the requirement for the 90-day study. Whole food toxicity studies such as the 90-day feeding studies in rodents are not scientifically justified as there is proven history of safe use and sufficient weight of evidence to indicate that the conduct of whole food toxicity studies should only be required on a case-by-case basis (where a potential for adverse effects is identified in comparative assessment studies). This testing is not warranted in the absence of a testable hypothesis as recommended by EFSA and as highlighted within the results of several EU-funded projects[33][34][35]. In over 20 years of GMO risk assessment, additional animal toxicology studies with whole foods have been considered by international projects, including EU-funded projects, unnecessary to confirm safety.

In the EU, there has been a long-standing debate on the necessity, value and ethics of whole food animal studies, and it is still ongoing. Over the past decade, the EU has funded over 50 projects to address the question, involving 400 research groups and costing €300 million under the EC's Framework Programmes for research, technological development and demonstration activities. The two most recent long-term animal feeding projects are the GMO Risk Assessment and Communication of Evidence (GRACE)[28] and Genetically modified plants Two Year Safety Testing (G-TwYST)[29] projects, which consisted in 90-day and 1- or 2-year combined chronic/carcinogenicity studies respectively, in addition to the GMO90+[30] project, co-funded by the French government (Coumoul et al., 2018; Steinberg et al., 2019; Zeljenkova et al., 2014). These three studies did not show any adverse effects in independent GM crop feeding studies and have demonstrated that "*the added scientific value of animal feeding studies without a targeted hypothesis is very limited and does not significantly reduce remaining uncertainties*"[36] and that the mandatory requirement to conduct rodent tests for safety assessment lacks any scientific basis.[37] These combined GRACE, G-TwYST and GMO^{90+} studies required the use of 2200 laboratory animals and accounted for €15 million.

There is currently a lack of consensus on the need for a 90-day feeding study in rodents for the risk assessment of GMOs among various stakeholders in the EU, including academia, industry, citizens, non-governmental organisations (NGOs) and risk assessment bodies (Devos et al., 2016), impacting global harmonisation of data requirements for GM crop risk assessment.

6. Current hazard and risk assessment tools that do not require additional animal testing

In the previous chapters current legislative requirements and recommendations on food safety in light of animal testing were described. It can be concluded that much of the current legislation requires animal testing, but also offers opportunities for toxicity testing and risk assessment tools that do not require additional animal testing. In this chapter, methods and approaches are described, which are ready for application in this respect.

6.1. Exposure assessment

The first part of a risk assessment is to develop an understanding of the exposure. In the case of a food or beverage, this would relate to the level of ingredient in a food and how much of the food is consumed (food supply data, data from household food expenditure/consumption surveys, results of surveys of individual consumers). If the exposure is low, as in the case of some contaminants, approaches such as threshold of toxicological concern (TTC) can be applied – see later.

6.2. Importance of food or ingredient characterisation

Before starting a hazard assessment, the food product or ingredient should be well characterised including potential impurities. By doing so relevant knowledge can be gained on the potential hazard of the food product/ingredient as well as it helps in deciding on the hazard assessment approach. For certain substances or structurally related substances already sufficient data might be available in public literature, and additional testing would not be required. Also substance characteristics can provide information on the mechanism of action, which can be linked to AOPs. It can then be decided if specific *in vitro* tests or *in silico* approaches can be helpful in the hazard characterisation.

6.3. Integrated Approaches to testing and assessment (IATA)

In its guidance on food additives (EFSA ANS Panel, 2012), EFSA welcomes the development of integrated testing strategies (ITS). These are a systematic combination of several information sources to build up a picture of the safety that a single test alone wouldn't be able to give (Hartung et al., 2013), and are anticipated to support the 3 R s in current toxicological approaches. ITS approaches comprise methods that can efficiently generate toxicological data for both the hazard identification and risk assessment, aiming to reduce costs and minimise the need for experimental animals. ITS also represent the way of combining pathway-based tests as suggested in the 'Toxicology for the 21st Century strategy' (Toxicity testing in the 21st Century: A vision and a strategy, 2007).

ITS is an example of an integrated approach to testing and assessment (IATA) in which the overall weight of evidence is used for hazard characterisation. IATA are science-based approaches for hazard characterisation that rely on an integrated analysis of existing information coupled with generation of new information using testing strategies.[38] The OECD has a programme on IATA to which, amongst others, the Joint Research Council (JRC) of the European Commission also contributes.[39] Increasingly, IATA is based on methods that measure or predict key events in AOPs relevant for the biological effect of interest. There is a range of IATA from more flexible, non-formalised judgement-based approaches (e.g. grouping and read across) to more structured prescriptive, rule-based approaches (ITS). On the OECD website[34] (considerations from) case studies on IATA are published from which can be concluded that so far mostly grouping (read-across) approaches are proposed for multiple toxicological endpoints.

6.4. Read-across and grouping

Read-across is a technique for predicting endpoint information for one substance (target substance), by using data on the same endpoint from (an) other substance(s) (source substance(s)). Prerequisite for applying read-across is performing a similarity analysis on the substances. A scientific justification should be given on the choice for read across. A choice based on similarity of molecular structure and chemical properties is in most cases not sufficient to justify read across prediction. Further scientific justification might be required to justify a read across prediction like considerations on bioavailability, metabolism and biological/mechanistic plausibility (Schultz et al., 2015). Also it is important to assess potential uncertainties in this respect. Considerations on

[33] http://www.grace-fp7.eu/.
[34] https://www.g-twyst.eu/.
[35] http://www.recherche-riskogm.fr/sites/default/files/projets/2015_02_13_gmo90plus_en_ligne.pdf.
[36] https://www.g-twyst.eu/files/Conclusions-Recommendations/G-TwYSTandGRACEPolicyBrief-Def.pdf.
[37] http://www.europabio.org/sites/default/files/G-TwYST%20results%20Press%20Release-Final-.pdf.
[38] OECD: Integrated Approaches to Testing and Assessment (IATA).
[39] https://ec.europa.eu/jrc/en/eurl/ecvam/alternative-methods-toxicity-testing/iata.

the acceptability of a read-across candidate can therefore be arbitrary. The European Chemicals Agency (ECHA) in this respect has released the ECHA's Read Across Assessment Framework (RAAF)[40] in order to evaluate read-across in a consistent manner. With applying read-across, experimental toxicity testing can be reduced as the endpoint information from one or multiple other substances is being used for the target substance. However, it should be noted that the use of read-across to confirm the absence of toxicological effects could be challenging, as it is commonly used to identify the hazard properties of a substance rather than the lack of them.

Additional evidence to substantiate the read across and justify data gap filling may be obtained through conducting bridging studies e.g. *in vitro* tests, *in chemico* analysis or reliable predictions from validated (Quantitative) Structure Activity Relationships ((Q)SAR) models[41]. Different software tools are available for molecular modelling and are able to estimate, in a cost and time efficient way, the binding affinity of small molecules to three-dimensional structure targeted protein or the time-dependent stability of ligand-protein complexes, known as QSAR (Eid et al., 2013). QSAR have been applied in food sciences as computational modelling to predict the theoretical binding affinity to target proteins or receptors, in the following examples:

- Natural bioactive compounds (curcumin, retinoic acid, genistein, apigenin, cyanidin, kaempfenol and docosahexaenoic acid) with known anti-inflammatory activities as inhibitory binding cyclo-oxygenase (COX-2) action utilizing AutoDock Vina, GOLD and Surflex-Dock (SYBYL) as docking protocols (Maldonado-Rojas & Olivero-Verbel, 2011).
- Molecular docking studies to investigate the binding interactions between active ginger components and various anti-Alzheimer drug targets using AutoDock 4.2 program (Azam et al., 2014).
- Comparison of quercetin glycosides as ligands for angiotensin-converting enzyme (ACE) with Drug Discovery Studio version 3.0 software.

QSAR requires the prior knowledge of the three-dimensional target protein structure, the chemical structure of the ligand to evaluate and computational expertise.

In a grouping approach, the safety of a category of chemical is assessed for the whole group at once. In this case there is no need for a full toxicological package per chemical, as toxicological information for one/several of the members of a given chemical category are used to determine toxicity/safety limits for all the substances in the chemical category. This approach is well-known as read-across to multiple substances. A chemical category is defined by the OECD as a group of chemicals whose physicochemical and human health and/or ecotoxicological properties are likely to be similar or follow a regular pattern usually as a result of structural similarity.[42] EFSA has used this approach for the assessment of food additives, an example of which is the group of microcrystalline, powdered and modified celluloses (consisting of 10 food additives). In this respect, EFSA notifies that not for all concerned celluloses toxicity information was available (on specific end points). Given their structural, physicochemical and biological similarities, the EFSA panel considered it possible to read across between the different celluloses (EFSA ANS Panel, 2018a) and has set an acceptable daily intake (ADI) for the whole group.

6.5. *threshold of toxicological concern*

The health risk upon exposure to a substance depends on its toxicity and the exposure. In case exposure to a substance is low, it should be determined whether it is necessary to perform additional toxicity testing for safety assessment. The TTC concept (Kroes et al., 2004) is based on the assumption that there is a level of exposure to a given substance below which no significant risk is expected to occur. It is widely used in case toxicological information on a substance is lacking. The TTC is a decision-tree based approach for which substances are grouped based on their chemical structure and related hazard.

Based on the decision tree an exposure level is determined for the substance below which there would be no appreciable risk to human health and hence no need for further toxicological testing. Once the threshold exposure level has been established, one simply compares the actual/predicted exposure level with the threshold value to determine if relevant risk is acceptable or not. The TTC approach has been used by regulatory authorities to assess the risks of flavouring substances, impurities in food and pesticide metabolites.

The TTC concept can be applied via the software tool Toxtree,[43] which has been developed under the umbrella of the Joint Research Centre (JRC) of the European Commission.

6.6. *Adaptation of animal study protocols*

Besides methods and approaches that avoid animal testing, animal study protocols can be adapted to reduce the amount of animals needed for the experiment or to improve the animal welfare conditions. An illustrative example for this is the adaptation of the one generation study for reproductive and developmental toxicity testing by which a two-generation study can be avoided. This Extended One Generation Reproduction Toxicity Study (EOGRTS) has been approved by the OECD (OECD guideline 443) and is recommended by EFSA for the assessment of food additives in tier II. Instead of assessing the effects within two generations of animals (circa 2600 animals), the effects are assessed within one-generation in a more extensive and accurate way (circa 1400 animals). With the EOGRTS, multiple toxicological endpoints are combined and assessed among different life stages. The toxicological endpoints included are reproductive toxicity, developmental neurotoxicity, developmental immunotoxicity, endocrine disruption and systemic toxicity. The development of the EOGRTS protocol is an illustrative example of a study protocol in which the number of animals used is significantly reduced (40% compared to the two-generation toxicity study) and yet it generates more information by combining multiple endpoints within the study.

6.7. *Human/clinical studies & post launch monitoring*

In the development of foods and food ingredients for human consumption, also data from well-designed scientific studies in humans is going to provide valuable information that can be used in the safety assessment. However, there are a number of considerations, most notably that there should be sufficient confidence in the safety of the food/ingredient before conducting human studies and a risk assessment must be conducted to determine whether there is a risk to human health. It is obviously not appropriate to investigate potential toxicity, but there is scope to investigate digestibility, tolerance, allergenicity and acceptability/palatability. Other considerations include ethics, informed consent, and adequacy of the study design.

There is scope ultimately for well-designed human studies to be part of the safety package of foods and food ingredients and avoid or reduce animal usage. For example, human studies have been used to investigate the high dose tolerability of phytosterol esters where specifically the potential effects on gut microflora, bile acid formation and level of sex hormones was investigated, and shown to be well tolerated (Ayesh et al., 1999; Weststrate et al., 1999). In addition, human studies in combination with *in vitro* studies were a critical element of the investigations of

[40] ECHA Read Across Assessment Framework (RAAF).
[41] OECD: Quantitative Structure-Activity Relationships Project [(Q)SARs.
[42] OECD: Grouping of Chemicals: Chemical Categories and Read-Across.
[43] Toxtree Tool.

potential allergenicity of ice structuring protein where animal models were not appropriate (Crevel et al., 2007).

A sub-set of human studies is post launch monitoring (PLM), extensively reviewed by Hepburn et al. (Hepburn et al., 2008), as a tool which is available as a complement to, but not a substitute for, the premarket risk assessment of specific cases of foods/food ingredients. It can be used as part of the safety assessment of foods and food ingredients, specifically in order to confirm that the assumptions used in the pre-market risk assessment were correct. For example, confirming the extent to which the product is being consumed by the target group, exposure in non-target groups as well as confirming that no unexpected effects were seen in the exposed population. PLM has notably been used as part of the safety assessment of aspartame (Butchko et al., 2002; Hepburn et al., 2008), olestra (Allgood et al., 2001; Hepburn et al., 2008) and phytosterol-esters (Hepburn et al., 2008; Lea & Hepburn, 2006). The approach has the potential to be used as part of the strategy to avoid animal testing, but only after a risk assessment has been conducted and there is confidence that the product is safe to be put on the market, albeit that may be a limited test market.

6.8. History of Safe Use

History of safe use (HoSU) is an additional approach which can be used to avoid the use of additional animal testing. HoSU is the knowledge accumulated from the use of a food/ingredient in one region, which establishes it as safe. The criteria for HoSU have been defined by Constable et al. (2007), and includes considerations such as conditions of use e.g. preparation/cooking, limitations and restrictions for sensitive populations (e.g. anti-nutrients and allergens). Thus, it can be used to determine whether additional safety questions exist and helps direct any subsequent safety evaluation. Various databases can be used to establish whether a food has a HoSU, including national food surveys and novel food directories. Conducting an integrated safety review to compile all relevant preclinical toxicological studies and to combine them with substantial evidence gathered from clinical paediatric use, can be part of the weight of evidence supporting the safety and tolerability of food and additives in a specific age-category, which may prevent the need for any additional (animal) testing (Meunier et al., 2014).

7. Emerging tools and methodologies

In the previous chapters for food specific categories of legislation, it was shown what safety testing is required according to the corresponding guidelines as well as what methods and models are being accepted by authorities that contribute to the 3 R s. *In vitro* genotoxicity tests, read-across/grouping approaches and the use of the TTC concept are accepted under certain conditions. Also use of existing literature and a tiered approach in toxicity testing are more and more required. This shows an improvement in the use of 3 R methods compared with a few decades ago. However, there is still much room for further improvement. On the one hand, this improvement is encouraged from an animal ethical perspective, but on the other hand recent technological developments enable more detailed information to be obtained which is relevant for the human situation. With these new methods, the effects might be better studied and predicted. In this chapter, illustrative new tools and methodologies are shown.

In vitro methods, whether simple 2D or complex models, like the organo-typic models described below, need to meet quality standards to be able to give qualitative, reliable and reproducible results. Several methods that are currently applied involve practices that could be improved. Recently, the OECD has adopted and published its "Guidance Document on Good *In Vitro* Method Practices".[44] Among others, the document describes issues with regard to quality control of test systems, consumables and reagents, the cell model (identification of cells), the culture media and its components (also ethical and scientific concerns associated with the use of foetal bovine serum), contaminants screening, etc.

7.1. Advanced in vitro models

In vitro cell based assays come in all shapes and sizes. The US Toxicology in the 21st Century programme has focused predominantly on applying *in vitro* assays for high throughput screening of chemicals. Integrating readouts from the hundreds of assays with toxicokinetic modelling to derive human bioequivalent effective doses has been shown to be an intuitive approach to prioritizing chemicals for further testing (Sipes et al., 2017). As discussed later, the application of such approach to food safety assessment is promising. However, as Kramer et al. (2019) note, adverse outcomes associated in test animals exposed to high doses of food additives, specifically, are often chronic, systemic and complex, involving the kidney, liver, intestines, immune and developing organ systems. The relevance for these effects for humans is in many cases unclear and not necessarily covered by the Tox21 *in vitro* test battery. This suggests that mimicking these effects *in vitro* benefits from the current development of complex human organotypic *in vitro* models.

Complex *in vitro* organotypic models include three-dimensional (3D) tissue models. They are regularly generated by seeding multiple cell types either in low-adhesion culture conditions to promote cell self-aggregation into spheroid structures or into culture inserts or porous 3D scaffolds. This enables crosstalk between different cell types and extracellular matrices. In so doing, they have the potential to emulate tissue characteristics, like the expression of organ-specific transporters and biotransformation enzymes, better and for longer time periods than standard 2D cultures (Weinhart et al., 2019). Although yet to be used for this purpose, this has great potential for assessing food safety testing to assess long-term human-relevant health effects. For example, monocultures of the human colon cell line Caco-2 cells are commonly used to assess permeability and toxicity to the intestine *in vitro*. However, a number of studies have shown that integrating other cell types into Caco-2 *in vitro* models improves the barrier integrity, viability and cytokine secretion of these intestinal models (Georgantzopoulou et al., 2016; Martínez-Maqueda et al., 2015; Ude et al., 2019; Yuan et al., 2013).

To improve the long-term culture of *in vitro* models, an immature stem cell population is needed to replenish senescent cells. This is where organoids come into the picture. They are derived from populations of adult or induced pluripotent stem cells. Their long-term viability is optimized by the addition of extracellular matrices such as Matrigel® and a selection of agonists (e.g. Wnt and tyrosine kinase receptor) and inhibitors (e.g. bone morphogenetic protein/transforming growth factor-β) (Fatehullah et al., 2016). For example, culture methods have allowed the maintenance and continual differentiation of intestinal stem cells into the epithelial cell types resident in the intestine. They self-assemble into microtissues with *in vivo*-like architecture and have been validated for their predictivity for diarrhoea-inducing drugs (Peters et al., 2019).

One level up from 3D tissue models including organoids is the advent of organ-on-a-chip technologies. Here, cell models of different tissues are linked by microfluidic flow (Bhatia & Ingber, 2014). Organotypic cells are cultured within continuously perfused microchannels running through an AA-battery size chip to recreate the physiological forces that cells normally experience *in vivo*. They have been proposed as models to test the ADME of as well as toxicity of bioactivated drugs (reviewed in Ishida, 2018). For food safety assessment, the gut-on-a-chip model may prove particularly useful (Lee et al., 2019). Santbergen et al. (2020) developed a gut-on-a-chip model that consisted of a dynamic transwell system with co-cultures of Caco-2 and HT29-MTX-E12 cells coupled to an ultra-performance liquid chromatography quadrupole time-of-flight

[44] OECD: Guidance Document on Good In Vitro Method Practices (GIVIMP).

mass spectrometer (UPLC-QTOF-MS), allowing for alternating analytical measurements of the apical and basolateral concentrations of ergotamine epimers, natural toxins in food. As a first, results showed epimer-specific transport across gut epithelium for ergotamine *in vitro*.

7.2. In silico tools

In silico models have been developed to simulate the digestion and absorption of different drug molecules, and this type of studies have also been applied to lipophilic micronutrients. Two subsequent *in silico* models were able to predict bioaccessibility kinetics of lipophilic vitamins considering the parameters as food matrix (triglyceride composition, vitamin A form and localization) or digestion conditions (gastric step or mixed micelle formation) (Marze, 2014). Both simulators were built and run using NetLogo 4.1.3 using the Logo programming language, which is able to operate multiple agents independently and works in both 2D and 3D.

Computational models (DIANA-mirPathv3 software (Vlachos et al., 2015)) allow a holistic integration of interrelated factors, such as dietary components, metabolic pathways and physiological and pathological processes, providing the base for hypothesis to design experimental studies in new therapies or applications (Carotenuto et al., 2016). Also, the development of predicting computational models are key to understand and predict the complex human-microbial co-metabolism interaction and environmental factors involved (Heyde & Ruder, 2015).

Machine learning and artificial intelligence combined with toxicological human big data are promising technologies in the near future for safety assessment. A recent publication from Luechtefeld et al. (2018) proposes the development, and further training, of a read-across structure activity relationship, using a database of ECHA. The authors proposed that this model had a better reproducibility than animal tests.

7.3. High-throughput methods for mode-of-action assessment

All chemical toxicological adverse effects are related to the interaction of a chemical (or a food ingredient) with the biological system. The above novel methodologies relate to novel test methods that will likely change the way we will evaluate chemical safety (including that of food ingredients) in the coming decades. Typically, in these test systems one may focus on the overall perturbations of the cell biology leading to adverse apical endpoints associated with differentiated cell function, e.g. loss of barrier function of the gut epithelia, transport function of the renal epithelial cells, hepatic inflammation, etc. However, there is also a need to further define the mode-of-action of such a chemical-biological interaction in the context of these adverse effects.

Over the past decade, a large panel of high throughput assays has been established under the ToxCast and Tox21 programs at the US Environmental Protection Agency (EPA) and US National Institute of Environmental Health Sciences and National Toxicology Program (NIEHS/NTP) (Juberg et al., 2017). These assays include various reporter assays for activation of e.g. nuclear hormone receptors (ERalpha, AR, PXR, AhR, etc), cellular stress response reporter assays, kinase activity assays and others. Many of these assays represent molecular initiation events (MIEs) that are part of AOPs. Integration of large datasets from the ~10,000 different compounds tested with cheminformatics strategies has allowed definition of QSARs for various receptor interactions (Attene-Ramos et al., 2013; Pradeep et al., 2017; Zang et al., 2013). Similar type of high throughput reporter assays have been established in the framework of the EU FP7 ChemScreen project and are currently applied in various case studies in the H2020 EU-ToxRisk project to test the validity and the applicability of these assays for mode-of-action identification (van der Burg et al., 2015). While these reporter assays have largely been applied in assessing mode-of-action of environmental chemicals, the systematic application and relevance in the food safety area needs further evaluation.

In parallel to the MIE reporter assays, high throughput imaging-based methods have been established to evaluate specific molecular and biochemical perturbations that occur in the cells prior to onset of cytotoxicity, including e.g. oxidative stress, mitochondrial functioning, GSH depletion and lipid accumulation (Antonica et al., 2012; Xia et al., 2018). Such assays can successfully predict the liability of chemical-induced liver toxicity. More recently, fluorescent protein-based reporters have been integrated in cellular systems that allow the live-cell monitoring of cellular stress response pathway activation that are critical for the onset of cytotoxicity, including oxidative stress responses, unfolded protein responses, DNA damage responses and inflammatory signalling responses (Wink et al., 2017). The integration with high throughput imaging platforms allowed for the assessment of liver injury liabilities of chemicals (Wink et al., 2018). Novel fluorescent protein biosensor probes are based on fluorescence resonance energy transfer (FRET) approaches and can quantitatively monitor the activity of various signalling pathways (Zhou et al., 2012). A next challenge will be the integration of these various fluorescent imaging-based approaches in the novel test methods, thus maximizing on both state-of-the-art developments.

The assays described above only cover a limited amount of biological pathways that can be modified in biological systems. Moreover, since signalling pathways in neuronal cells are likely differently wired than in hepatocytes, the mode-of-action might need evaluation in different cell systems. In the past 15 years much attention has focused on toxicogenomics to evaluate the consequences of chemical exposure on biological systems. Moreover, toxicogenomics allowed the comparative evaluation of the differentiation status of various test systems, e.g. iPSC-derived hepatocytes versus primary human hepatocytes (Godoy et al., 2016). Limitations have been the cost effectiveness of the transcriptomics, which hampered thorough concentration-response evaluation to gain insight in pharmacological mode-of-action versus toxicological mode-of-action. Novel high throughput transcriptomics analysis making advantage of targeted array (Subramanian et al., 2017) or targeted RNA sequencing (House et al., 2017; Mav et al., 2018) approaches now allow the cost-effective detailed concentration-response evaluation of thousands of compounds. The related large transcriptomics-based compound mode-of-action databases are referred as the Connectivity Map and can serve as a reference for uncovering mode-of-action of novel food components. This should then involve the transcriptome analysis of food components in the novel test methods to connect ultimate apical endpoints of these assays to upstream cellular perturbations. The integration of transcriptomics to assess mode-of-action of food ingredients may also lead to hypothesis formulations and identification of candidate AOPs that may possibly be implicated in adversity liabilities. This could next lead to targeted testing in relevant test systems, rather than testing substances in all novel test systems. This would in particular be critical to maintain the cost-effectiveness of safety testing, since novel testing strategies should not be an accumulation of various expensive organoid models and/or organ-on-chip systems.

8. Next generation risk assessment

Toxicology and risk assessment is undergoing a paradigm shift at the moment with a move away from the use of apical toxicity data in animals such as organ pathology, to an approach based on understanding the mechanism of action underlying the adverse effect (Toxicity testing in the 21st Century: A vision and a strategy, 2007). This has led to the concept of Adverse Outcome Pathways (AOPs) with adverse events being seen as a cascade of molecular and cellular events (Willett et al., 2018). These developments occur alongside the advancement of new technologies and methodologies, such as *in vitro*, -omics, computer modelling and concepts in systems biology (Leist et al., 2012).

A roadmap for the implementation of these new toxicological approaches as they apply to foods and food ingredients has been developed by Blaauboer et al. (2016). Their approach provides an opportunity for

integrating data from studies on food substances, from *in vitro*, *in silico* and human studies, and developing a mechanistic understanding that can be applied to risk assessment. The steps in the roadmap consider the different aspects needed for a safety evaluation, including exposure scenarios, kinetics to understand the internal exposure, target specific toxicities, mechanism of action, and *in vitro* to *in vivo* evaluations. Through the use of a number of case studies they were able to demonstrate that classical animal toxicology studies could be avoided, but acknowledged that animal models may still be needed for particular questions such as developmental toxicity.

An essential element if these new approaches are going to be applicable in risk assessment for foods and food ingredients is whether a point of departure (POD) can be established as a basis for establishing health-based guidance values. In the absence of animal data, and if next generation risk assessment approaches are going to be applicable, then a mechanistic exposure driven approach should be developed in which the approach mimics the human physiology as good as possible. In this respect, Desprez et al. proposed the use of a harmonised ontology for repeated dose toxicity in a reproducible and consistent manner. This ontology consists of 4 pillars (in chronological order): kinetics and systemic exposure, chemistry, triggered molecular initiating event (AOP and mode of action) and toxicity (Desprez et al., 2019). The identification of POD from mechanistic data is a critical element to these new approaches being accepted by the scientific community and consequently a key challenge which has been reviewed by Levorato et al. (2019). Opportunities for establishing a POD may come from -omics technologies, to help define the mechanism of action, and the application of QIVIVE (quantitative *in vitro* to *in vivo* extrapolation). The knowledge on physico-chemical properties (chemistry pillar in ontology model) might be very valuable for extrapolation (Desprez et al., 2019). Also consideration needs to be given to the application of appropriate uncertainty factors to PODs developed in this way, in order that health-based guidance values can be established.

9. Conclusions and future recommendations

Toxicology has been undergoing a rapid period of change following the publication of "Toxicity Testing in the 21st Century: a vision and a strategy" (Toxicity testing in the 21st Century: A vision and a strategy, 2007), and made possible by the development of new scientific approaches, in particular computational toxicology, genomics and informatics. There is great potential for strategies to be developed that avoid the use of animal tests, with the generation of more human-relevant data from multiple sources replacing the approach where one animal test is replaced with one alternative non-animal test (see roadmap for food safety assessment proposed by Blaauboer et al. (2016)).

Whilst there is a legal obligation in Europe to replace, refine or reduce the use of animals for scientific purposes (Directive 2010/63/EU[45]), the guidance provided by EFSA is still largely based on a requirement for animal data, and large numbers of animals are still being used to support the food and beverages sector. The majority of this animal use is for safety testing purposes, the remainder being for claim support and efficacy testing.

In order to allow that these new alternative strategies and approaches can be used in risk assessment, the identification of points of departure from *in vitro* data is a critical area that is receiving a lot of attention (Levorato et al., 2019). As a consequence also attention should be paid into the extrapolation of the POD to the *in vivo* situation. Using a standardized ontology as proposed by Desprez et al. (Desprez et al., 2019) might help in this respect. This is an area of active research and will help in the establishment of health-based guidance values, such as the acceptable daily intake (ADI) that can be used in risk assessment and ultimately risk management.

However, some relatively straightforward integration approaches can be used in risk assessment already now, such as read-across, weight of evidence and HoSU. Dedicated guidance has for example been developed for the use of quantitative and qualitative weight of evidence approaches in scientific assessments by EFSA (EFSA Scientific Committee, 2017b). These approaches which can be used to avoid animal tests are rarely reported as accepted in regulatory approvals, as shown in the 2016 study by Agerstrand & Beronius (Agerstrand & Beronius, 2016). However, they are probably used more than is actually published in the open literature. One recent exception is the case of the novel food ingredient "egg membrane hydrolysate" (EFSA NDA Panel, 2018) where the assessment was essentially based on the history of safe use and *in vitro* genotoxicity. An acute rat oral toxicity study was also conducted on this material, which EFSA concluded did not add anything to the scientific risk assessment. History of safe use was also the main tool used in the risk assessment by EFSA of the novel food ingredient orthosilicic acid-vanillin complex (EFSA ANS Panel, 2018b). In this case, the applicant also submitted acute toxicity data as well as a subchronic toxicity study, which the EFSA panel considered of very limited suitability for use in the risk assessment due to the solubility issues of the test material. Other examples of food ingredients where animal testing was conducted but where it is actually debatable whether the animal studies are actually adding anything to the scientific assessment are mung bean protein isolate and soy leghemoglobin safety assessments (FDA GRAS notices 684[46] and 737[47]).

Producers and regulators need to be bolder in considering the use of non-animal approaches for food safety risk assessment. The legislation is at discordance: on one hand saying alternatives should be used, but on the other hand not providing sufficient guidance, whereby even relatively simple tools can be used. The scientific developments and acceptability of approaches need to be more reflected in legislation and a willingness to engage on the acceptability of new approaches needs to be promoted by the regulatory bodies as well as producers. What are we waiting for?

Author Contribution Statement

Alie de Boer: Writing - original draft, Writing - review & editing. Lisette Krul: Conceptualization, Writing - original draft, Writing - review & editing. Markus Fehr: Writing - review & editing. Lucie Geurts: Conceptualization, Writing - review & editing, Supervision, Project administration. Nynke Kramer: Writing - review & editing. Maria Tabernero Urbieta: Conceptualization, Writing - original draft . Johanneke van der Harst: Conceptualization, Writing - original draft, Writing - review & editing. Bob van de Water: Writing - original draft. Koen Venema: Writing - original draft. Katrin Schütte: Conceptualization, Writing - original draft, Writing - review & editing. Paul A. Hepburn: Conceptualization, Writing - original draft, Writing - review & editing.

Funding

This work was conducted by an expert group of the European branch of the International Life Sciences Institute, ILSI Europe. This publication was coordinated by the Alternatives to Animal Testing Task Force. Industry members of this task force are listed on the ILSI Europe website at http://www.ilsi.eu. Experts are not paid for the time spent on this work; however, the non-industry members within the expert group received a small compensatory sum (honoraria) and travel support from the Alternatives to Animal Testing Task Force to attend meetings to discuss the review. For further information about ILSI Europe, please email info@i

[45] Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

[46] FDA GRASS Notice (GRN) No. 684 for Mung Bean Protein Isolate.

[47] FDA GRASS Notice (GRN) No. 737 for Soy Leghemoglobin for protein preparation derived from pich/a pastoris.

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Acknowledgements

The authors would like to acknowledge Ms Laura Caneva for her in-depth input and feedback to earlier draft versions of the review. This paper has been shared and debated during a workshop organised on 6–7 September 2018 in Brussels by the European branch of the International Life Sciences Institute, ILSI Europe, through the financial support from ILSI Europe's Alternatives to Animal Testing Task Force. The authors wish to thank all workshop participants for their valuable comments. The authors carried out the work that is, collecting/analysing data/information and writing the scientific paper separate to other activities of the task force. The research reported is the result of a scientific evaluation in line with ILSI Europe's framework to provide a precompetitive setting for public–private partnership (PPP). ILSI Europe facilitated scientific meetings and coordinated the overall project management and administrative tasks relating to the completion of this work. The opinions expressed herein and the conclusions of this publication are those of the authors and do not necessarily represent the views of ILSI Europe or those of its member companies.

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Abbreviations

ADME: Absorption, Distribution, Metabolism and Excretion
ADI: Acceptable Daily Intake
AOPs: Adverse Outcome Pathways
ACE: Angiotensin-Converting Enzyme
RAAF: ECHA's Read Across Assessment Framework
EOGRTS: Extended One Generation Reproduction Toxicity Study
FRET: Fluorescence Resonance Energy Transfer
GRAS: Generally Recognised As Safe
GM: Genetically Modified
G-TwYST: Genetically modified plants Two Year Safety Testing
GSH: Gluthatione
GRACE: GMO Risk Assessment and Communication of Evidence
HoSU: History of Safe Use
IATA: Integrated Approaches to Testing and Assessment
COX-2: Inhibitory Binding Cyclooxygenase
ITS: Integrated Testing Strategies
MIEs: Molecular Initiation Events
NGOs: Non-Governmental Organisations
POD: Point of departure
QPS: Qualified Presumption of Safety
QSAR: Quantitative Structure-Activity Relationship
3R: Replacement, Reduction and Refinement
TTC: Threshold of Toxicological Concern
UPLC-QTOF-MS: Ultra-Performance Liquid Chromatography Quadrupole Time-Of-Flight Mass Spectrometer
List of Organisations, Agencies and Committees, International
ILSI: International Life Sciences Institute
OECD: Organisation for Economic Co-operation and Development
WHO: World Health Organisation
European Union (EU)
ECHA: European Chemicals Agency
JRC: European Commission Joint Research Centre
SCF: European Scientific Committee on Food
EFSA: European Food Safety Authority
ANS: EFSA Panel on Additives and Nutrient Sources added to Food
NDA: EFSA Panel on Nutrition, Novel Foods and Foods Allergens
TFEU: Treaty on the Functioning of the European Union
United States (US)
EPA: US Environmental Protection Agency
FDA: US Food and Drug Administration
NIEHS: US National Institute of Environmental Health Sciences
NRC: US National Research Council
NTP: US National Toxicology Program