Review

Considering new methodologies in strategies for safety assessment of foods and food ingredients

Bas J. Blaauboer [a], Alan R. Boobis [b], Bobbie Bradford [c], Andrew Cockburn [d], Anne Constable [e], Mardas Daneshian [f], Gareth Edwards [g], Jossie A. Garthoff [h], Brett Jeffery [i], Cyrille Krul [j, l], Jeroen Schuermans [k, *]

[a] Utrecht University, Division of Toxicology, Institute for Risk Assessment Sciences, PO Box 80.177, 3508 TD, Utrecht, The Netherlands
[b] Imperial College London, Department of Medicine, Centre for Pharmacology & Therapeutics, London, W12 0NN, United Kingdom
[c] Unilever, Safety & Environmental Assurance Centre, London, EC4Y 0DY, United Kingdom
[d] University of Newcastle, Toxico-Logical Consulting Ltd, The Old Boiler House, Moor Place Park, Kettle Green Lane, Much Hadham, Hertfordshire, SG10 6AA, United Kingdom
[e] Nestlé Research Centre, Vers-Chez-les-Blanc, 1000, Lausanne 26, Switzerland
[f] University of Konstanz, Center for Alternatives to Animal Testing-Europe CAAT-Europe, 78457, Konstanz, Germany
[g] Consultant, 63 Woodlands Road., Sonning Common, Reading, Berkshire, RG4 9TD, United Kingdom
[h] Danone Food Safety Centre, Utrecht, 3584 CT, The Netherlands
[i] Mars, Global Chemical Food Safety Group, Slough, SL1 4JX, United Kingdom
[j] University of Applied Sciences, Research Centre Technology & Innovation, Dept. Innovative Testing in Life Sciences & Chemistry, PO Box 12011, 3501 AA, Utrecht, The Netherlands
[k] ILSI Europe, Avenue E. Mounier 83, Box 6, 1200, Brussels, Belgium
[l] TNO Healthy Living, PO box 360, 3700 AJ Zeist, The Netherlands

ARTICLE INFO

ABSTRACT

Toxicology and safety assessment are changing and require new strategies for evaluating risk that are less depending on apical toxicity endpoints in animal models and relying more on knowledge of the mechanism of toxicity. This manuscript describes a number of developments that could contribute to this change and implement this in a stepwise roadmap that can be applied for the evaluation of food and food ingredients.

The roadmap was evaluated in four case studies by using literature and existing data. This preliminary evaluation was shown to be useful. However, this experience should be extended by including examples where experimental work needs to be included. To further implement these new insights in toxicology and safety assessment for the area of food and food ingredients, the recommendation is that stakeholders take action in addressing gaps in our knowledge, e.g. with regard to the applicability of the roadmap for mixtures and food matrices. Further development of the threshold of toxicological concern is needed, as well as cooperation with other sectors where similar schemes are under development. Moreover, a more comprehensive evaluation of the roadmap, also including the identification of the need for in vitro experimental work is recommended.

© 2016 ILSI Europe. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Contents

* Corresponding author. ILSI Europe, Avenue E. Mounier 83, Box 6, B-1200, Brussels, Belgium.
  E-mail address: publications@ilsieurope.be (J. Schuermans).

1. Introduction: food safety assessment and testing

Foods and food ingredients encompass a broad spectrum of food materials, ranging from the relatively simple chemical compounds at one end of the spectrum to complex whole foods and ingredients at the other. Food additives and flavourings are largely, but not exclusively, chemically defined substances that lend themselves to traditional approaches used in a toxicological assessment. Across the world, food additives and flavourings are governed by legislation that includes well-established requirements for the demonstration of safety in order for them to be used as ingredients in food products. Other ingredients used for technological purposes, such as solvents and enzymes, also have their own legislation or conform to internationally agreed standards. Many whole foods and characterised food ingredients are deemed safe by way of their, often traditional, history of use in the human diet. Where such a history of safe use is absent, the foods or ingredients in question are seen as 'novel' and subject to control under novel foods legislation, now in place in a growing number of countries around the world. It is noteworthy that the vast majority of novel foods are used in the form of ingredients. Although there are differences of detail between novel foods legislation in different countries, they are relatively consistent in their approach to the evidence required to demonstrate safety. To this end, guidance on safety assessment of novel foods, including genetically modified organisms (GMOs) has been published by various international authorities, e.g. The United States Food and Drug Administration (1992), OECD (1993), Health Canada (1994), FAO/WHO (1996), European Commission (1997), Howlett et al. (2003).

Although foods and food ingredients make up the most significant part of the daily diet it should be remembered that none are without some level of risk. Some ingredients may be hazardous in high concentrations, they may elicit allergic responses in some individuals, they may accumulate in the body, and they may include chemicals (Dreisig et al., 2013) that could be the cause of adverse effects due to long-term low-concentration exposure. Further, they may modulate adaptive processes, or they may interact with compounds from other sources and routes leading to adverse health conditions. For risk assessment of foods and food ingredients, it is desirable to identify possible toxic compounds also

List of abbreviations

ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism, and Excretion
AOP	Adverse Outcome Pathway
CFAs	Cetylated fatty acids
CMC	Cetyl Myristoleate Complex
CWD	Cold Water Dispersion
DSSTox	Distributed Structure-Searchable Toxicity
ECM	Extra Cellular Matrix
EDI	Estimated Daily Intake
GI	Gastro-Intestinal tract
HACCP	Hazard Analysis of Critical Control Points
hESC	Human Embryonic Stem Cells
HPLC	High Performance Liquid Chromatography
iPSC	Induced Pluripotency Stem Cells
mES	Mouse Embryonic Stem Cells
MoA	Modes of Action
MoS	Margin of Safety
MoE	Margin of Exposure
NCGC	US National Clinical Guideline Center
NGF	Neural Growth Factor
NOAEL	No Observable Adverse Effect Level
PBBK	Physiologically-Based Biokinetics Models
PAHs	Polycyclic Aromatic Hydrocarbons
PCBs	Polychlorobiphenyls
PoD	Point of Departure
PoT	Pathways of Toxicity
QD	Quantum Dots
(Q)IVIVE	(Quantitative) *in vitro-in vivo* Extrapolation
(Q)SAR	(Quantitative) Structure-Activity Relationship
(Q)SPR	(Quantitative) Structure-Property Relationships
RDI	Reference Daily Intake
SCFE	Supercritical Carbon-dioxide Fluid Extraction
TNF	Tumor Necrosis Factor
TT21C	Toxicity Testing in the 21st Century
TTC	Threshold of Toxicological Concern

Table 1
A global description of the current practices in food safety assessment comprise the following elements (Howlett et al. 2003).

1. Depending on the purpose of the assessment and depending on the source of food different categories of food can be recognised:
   - single ingredients, ranging from simple to complex
   - whole foods
   - GM foods (plant or animal origin)
   - irradiated foods
   - etc.
2. Steps taken in the collection of data (i.e. before any testing):
   - purpose of assessment: how is food used?, etc.
   - interrogation of data bases
   - description of food: origin, composition, nutritional characteristics; chemical analysis as required.
   - known potential hazards, e.g. by comparing with similar foods
   - for novel foods, GM foods: is there a traditional comparator?
   - identify differences from comparator
3. Testing: initial screening of key endpoints:
   - *in silico* and *in vitro* methods
   - for single ingredients: QSAR/read across methods
   - protein allergenicity checks
   - protein stability
   - mutagenicity (Ames test), clastogenicity test
4. Toxicity tolerance tests, animal tests:
   - palatability (determine max % that can be incorporated)
   - diet formulation to balance for nutrient content
   - ADME/PBPK
   - single and repeated dosing
   - sub-acute toxicity (if needed for target population)
   - for GMO: 28 day study in mouse
   - 90 day sentinel study in rat
   - case by case: Specific studies addressing specific endpoints and target populations/target organs/evidence for adverse or nutritional effect, dose-effect relationship?
5. Human studies (target population):
   - volunteer study to confirm tolerance
   - any unexpected or unintended effects: adjust intended exposure?
6. Hazard characterisation:
   - understanding toxicity/nutritional effect: mode-of action, Adverse Outcome Pathway (AOP), dose-effect
   - is there a reliable NOAEL
7. Exposure assessment:
   - use of EU data bases and product patterns to estimate mean and 97.5 percentile
   - take into account sensitive populations: need for labelling requirements?
8. Risk/safety assessment:
   - use MOS in context of intended use
   - need for post launch monitoring?

on the basis of their chemical structure and mechanism of action (Daneshian et al., 2013). Moreover, it is desirable to describe concentration-dependent effects, long-term low-concentration exposure effects as well as a proper risk-benefit analysis.

In Table 1 an overview is presented of the elements taken into account in the current common practices applied to the safety assessment of foods and food ingredients. Whereas the safety assessment of food additives, for example, follows a well-established pattern, the assessments of complex foods and ingredients have to be approached on a case-by-case basis, depending very much on the nature and intended use of the food in question and the specific questions to be answered. While the important first step is a thorough characterisation and detailed chemical and nutritional analysis of the food, from Table 1 it is evident that thereafter there is strong reliance on the interpretation of animal toxicology studies in the absence of other relevant models or methods. Other elements consist of methods based on comparison with existing data on similar compounds or products making use of the read-across techniques, a number of *in vitro* methods, e.g. genotoxicity (where practical), protein stability (GMOs), etc. Where these are available additional data on the safety or toxicity after human exposure should also be considered. The end result is based on an evaluation of the Margin of Safety (MoS) or Margin of Exposure (MoE) for the intended use in a product.

The safety assessment of foods and ingredients is clearly much more complicated than for well-characterised, non-food substances, e.g. industrial chemicals, plant protection products or medicines. Food additives and flavourings may have more similarities to the aforementioned examples, but the majority of food ingredients, including GMOs, present a greater challenge, e.g. because of the complexity of the food composition (Palafox-Carlos et al., 2011). This diversity of foods is recognised in the legislative approach adopted internationally and the principle that safety assessment should be approached on a case-by-case basis is embodied in the guidelines published by different expert groups. Furthermore, it is widely recognised that traditional approaches to hazard and risk assessment applied to defined chemicals, including food additives, cannot be applied directly to novel foods that are more complex in nature. The more complex the food, the more challenging this becomes. For example, the traditional toxicological approach of setting an acceptable daily intake (ADI), that typically includes a one hundredfold safety margin when compared with the lowest no observable adverse effect level (NOAEL) seen in toxicology studies, is not feasible for the majority of novel foods. Complete freedom from risk is an unattainable goal and the circumstances and degree of exposure to the food in question is an important consideration. The OECD (1993) proposed that 'safety' equates to 'a reasonable certainty that no harm will result from intended uses under the anticipated conditions of consumption'.

In all cases, the first step is establishing thorough characterisation of the food. For a novel food this includes its source, method of production, compositional analysis, including nutrients and possible contaminants, known toxins or anti-nutritional compounds. This information should permit direct comparison with a

traditional counterpart where this is available, e.g. genetically modified crops. Intended mode of use as human food and any history of previous human consumption also form part of this preliminary set of information. In some cases, e.g. the introduction of a new food which has a history of traditional consumption elsewhere in the world, the information outlined above may be sufficient to complete the safety assessment. The existence of a known counterpart gives the most important reference point with which to compare the novel food, both in terms of composition and when testing in animals may be required. It is particularly relevant in the case of foods from GM crops and may also apply in other cases, e.g. a novel process applied to improve the production of an existing food. Where no traditional counterpart exists and when toxicological testing in animals is deemed necessary, an existing food with a similar, though rarely identical, nutritional profile is normally chosen as a point of comparison. For example, a novel food expected to be a source of protein in the human diet may be compared with a protein such as casein (milk protein) or with another appropriate dietary source of protein.

Undertaking toxicology studies in laboratory animals as part of the safety assessment of complex novel ingredients or whole foods may be challenging (Paparella et al., 2013). Whether such studies are necessary and relevant has to be judged on a case-by-case basis and is dependent on a number of factors, including information derived from its characterisation and whether the novel food or its source have any history of use in the human diet.

The traditional approach when testing chemicals in animal studies is to add a range of concentrations of the test substance to an already complete diet appropriate for the test animal in question. Because the amounts of test substance added are physically small, they should have little or no significant impact on the nutritional balance of the diet, thereby affording a simple comparison between the control and test diets employed. In other words, differences in response between the test groups are likely to be of toxicological origin rather than being due inadvertent nutritional differences, with the exception of effects on food intake caused by palatability of the test substance. This approach may not be appropriate for safety testing of complex foods where adding them to the test diets at high enough levels relevant for desired human exposure, could both upset the range and balance of nutrients provided by the diet. It is vital to eliminate, as far as possible, such unwanted nutritional differences, so that observed effects can be interpreted from a toxicological perspective rather than being confounded, or even hidden, by nutritional effects. With a detailed knowledge of the nutritional analysis of the novel food and of the other dietary ingredients to be used, control and test diets can be formulated to be equivalent in their contents of major and minor nutrients considered to be of nutritional importance. This approach is recommended throughout a programme of animal testing and the nutrient standards employed should be appropriate for the age and physiological state of the species, e.g. growth vs. reproduction. In all cases where animal studies are deemed necessary, it is probable that a 90 day rodent study will be a common requirement and this may, in some instances, indicate the need for further targeted studies to be undertaken. *In vitro* systems may be suitable for certain nutrient availability assays, but *in vitro* toxicology assays, e.g. genotoxicity, present many difficulties when applied to complex foods, both in terms of execution and interpretation.

In all cases of chemically defined substances or simple mixtures thereof, it may be possible to follow the traditional toxicological approach of feeding sufficiently high quantities to identify the NOAEL and to apply a safety factor of 100 in order to establish an ADI. However, when considering chemically definable compounds with a nutritional effect, e.g. new sources of vitamins, minerals and similar types of compounds, the gap between the amount required for the intended nutritional effect and the level where adverse nutritional effects occur is often quite narrow and a different approach is needed.

For more complex novel food ingredients and whole foods, the ADI approach may not be possible. The proportion of the food that it is possible to include in a test diet is limited by the maintenance of nutritional balance, though it may still be physically quite high. In the absence of observed toxic effects a traditional ADI cannot be established and a different approach is required. Howlett et al. (2003) proposed that the highest safe intake identified in feeding studies should be compared with the expected human consumption, i.e. the estimated daily intake (EDI), to arrive at a margin of safety.

When the absence of adverse health effects has been sufficiently established, the option of studies in human volunteers becomes available to confirm the safety, including absence of adverse reactions; to demonstrate the nutritional suitability for purpose and for population sub-groups. In some cases, these studies may be supplemented by post launch monitoring to provide further reassurance within the totality of the accumulated information on the novel food in question. Data from human studies may also contribute to the establishment of safe levels of intake discussed above, providing additional reassurance to the safety assessment.

2. Scope of this paper

In the light of the new developments in toxicological and safety research in general, particularly with regard to the need for better understanding of the safe use of foods, this manuscript presents an overview of the possibilities for the application of new methodologies to food ingredients and the mixtures in which these are present in food matrices. The need for new strategies is also driven by the desire to develop more relevant models with respect to a better predictability for the human situation, as well as by the increasing number of compounds and mixtures thereof for which safety assessments are required. The challenge will be to apply these approaches to the safety assessment of foods and food ingredients, given their inherent complexity.

It is important to recognise the safety of foods and food ingredients, novel or already in use, is not solely based on toxicological considerations. The nutritional properties and other beneficial characteristics must also be taken into consideration.

This paper will present an overview of:

- the availability and applicability of methodologies for the safety assessment of (novel) foods, food ingredients and mixtures;
- the construction of a roadmap that can be applied to determine safe levels of exposure;
- the gaps in our knowledge and in the availability of the methodologies.

Furthermore, recommendations will be formulated for research needs in this area. In line with the recommendations in the NRC 2007 report (NRC, 2007), it is important to mention the possibility of creating strategies that are best suited to focus on the human risk assessment by implementing toxicity testing systems, e.g. derived from human tissues, and applying kinetic and dynamic modelling techniques specific for the human situation.

It is clear that there will not be a "one size fits all" strategy; the applicability for a number of roadmaps are described. The robustness and reliability of a number of chosen strategies will then be illustrated using a number of case studies. The roadmaps should be as small as possible and no larger than necessary, also taking account of the cost-effectiveness and practical applicability in food safety.

3. Food safety evaluation in the light of new developments in toxicological risk assessment paradigms

This paper reviews the possibilities and opportunities of implementing the recently developed concepts and methods for toxicity testing for the safety evaluation of food and food ingredients and thereby avoiding animal experimentation if possible. It extends the work in the ILSI-Europe FOSIE project (Smith, 2002; Eisenbrand et al., 2002). Since the publication of the NRC report "Toxicity testing in the 21st century, a vision and a strategy" (NRC, 2007), a range of activities has been started to interrogate the possibilities of integrating elements of the changes in the way toxicity (or safety) testing could be performed, as proposed in this seminal report. The views presented in that report can be considered a true paradigm shift for the science of toxicology and for safety assessments.

The main emphasis in this new vision is on the shift from using clinically and/or histopathologically observable apical endpoints for adverse effects of a substance in models consisting of intact animals, towards a more detailed description of the process of adversity that makes use of the mechanism of action at the molecular level. This runs in parallel with the opportunities provided by technological innovations (Leist et al., 2012) and the implementation of new scientific concepts with regard to new *in vitro* and *in silico* approaches, as well as concepts from the field of systems biology. The integration of the derived toxicity data into a systems biology-type description (Hartung et al., 2012), referred to as modes of action or adverse outcome pathways, together with computer-based kinetic modelling might then result in a risk or safety assessment and at the same time reducing the number of animal studies needed. When human-based cell or tissue cultures, or even human data, can be employed, a more direct relevance to the human situation can be obtained. This will allow a "fit-for-purpose" approach that can be flexibly adapted to the questions to be answered in safety assessments. Well-described problem formulations are the basis for testing strategies that should offer sufficient precision to solve the problem while, at the same time, avoid unnecessary testing.

The new paradigm opens up the opportunity for integral assessment and evaluation of risks related to mixtures of compounds to which individuals are exposed, i.e. description of exposome and the human toxome (Rappaport, 2011; Hartung and McBride, 2011). In this regard, scientific challenges lie in understanding the mechanisms underlying biological responses, in evaluation and extrapolation of data derived from pathway-based approaches, and in interpretation of complex toxicological endpoints for decision finding processes.

There is evidence from mechanistic toxicology studies aimed at Modes of Action (MoA) of compounds suggesting that the thousands of known harmful substances act by interfering with only a few cellular key molecular pathways (NRC, 2007). Information on involved pathways can be obtained by high-throughput and high-content screening systems, e.g. omics (Heijne et al., 2005) and modern imaging approaches, using human cells and organotypic tissue cultures. This information can be further analysed with modern methods of systems biology and bioinformatics. Implementation of this vision proposed by the NRC report (NRC, 2007) is needed and has been started via regulatory authorities such as OECD and US-FDA (Collins et al., 2008; Schiffelers MJ et al., 2012).

This concept also includes the concept that the knowledge of (quantitative) structure-activity relationships ((Q)SAR) of exposure and the knowledge of MoA of toxicants on these pathways allow predictions of toxicity at the level of the whole organism. The conceptual construct concerning the linkage between direct molecular initiating events and an adverse outcome at a biological level is also referred to as an adverse outcome pathway (AOP) (Ankley et al., 2010). Currently, several AOPs are under development under the management of OECD Extended Advisory Group on Molecular Screening and Toxicogenomics. With reference to the new concepts in toxicology, the safety evaluation of compounds is to begin with chemical properties and then proceed to the biological characterisation in multiple *in vitro* systems, i.e. test batteries. These functional assays address MoA of compounds result in information relevant to human physiology, which should be translated to hazard estimates (Blaauboer BJ et al., 2012).

Ideally, toxicological risk assessment should be based on the integration of the computational approach and the experimental profiling approach (Blaauboer, 2010). Data from experimental profiling refine the *in silico* approaches and results of computational approaches narrow the subset of test substances and provide matrices for interpretation and interpolation of the experimental profiling data. Examples of computational approaches and *in silico* predictive models are QSAR (Benfenati, 2013) and physiologically-based biokinetics models (PBBK) as well as (quantitative) *in vitro-in vivo* extrapolation ((Q)IVIVE) approaches (Polak, 2013). The QSAR approaches correlate descriptors of chemical characteristics of compounds with their biological activity. PBBK involves various mathematical models for description of adsorption, distribution, metabolism and excretion (ADME) of compounds within an organism on the basis of physiological (e.g. body fluid flows), physico-chemical (e.g. partition coefficients) and kinetic (e.g. metabolic rates) parameters. The PBBK provides a framework for conducting QIVIVE, as the prediction of biological activity of compounds implies the integration of data on the MoA with data on biokinetics (DeJongh et al., 1999; Blaauboer et al., 1999, 2000; Blaauboer, 2001, 2002; 2003; Verwei et al., 2006; Louisse et al., 2010). QIVIVE estimates the effect of compounds on tissues and on the whole organism, based on their effects in an *in vitro* toxicity test system at a certain exposure level (Yoon et al. 2012). The computational approaches depend on available existing data with regard to various endpoints and thus on the quality and extent of databases, which grow continuously due to the data from profiling approaches as well as due to epidemiological data.

This paper describes the more specific aspects of the safety assessment of food and food ingredients, also paying attention to the development of novel foods and the complexity of food composition and thus the complexity of safety testing in this area. This is followed by the description of a number of new developments and methodologies that have the potential to be applied in future food safety testing. A strategy is being proposed in which elements of the new developments could be implemented in a stepwise integrated roadmap for a practical use in food safety assessment in the near future. By describing some historical cases the usefulness of the suggested approach is demonstrated.

4. Considerations for the development of a roadmap for a future integrated strategy for safety assessment of foods and food ingredients

Today, the acceleration in knowledge generation, science progression and development of innovative technologies, open up the opportunity for combining new concepts in safety sciences and corresponding new technologies with good laboratory practices (van Thriel et al., 2012). New toxicological risk assessment strategies finally aim at *in silico* evaluation and literature/database search, to make use of existing and human/epidemiological data, and in parallel at *in vitro* based human-relevant hazard assessment and appropriate exposure assessment.

Also in the field of risk assessment of food and food ingredients it is very difficult to aim for one-to-one replacement of a traditional

animal-based method with a new approach. This strongly depends on the knowledge and understanding of the mechanisms underlying an adverse effect. For more complex endpoints several non-animal approaches may be required for characterisation of the impact of a compound on the relevant tissues. Examples are carcinogenicity or sensitisation.

For evaluation of the toxicological capacities and for nutritional assessment it is necessary to start with an exposure assessment. Exposure assessment is defined as the process for estimation and measuring the magnitude, frequency and duration of exposure to a compound, along with the number and characteristics of the population exposed, including the pathways, the routes, and also the uncertainties (Lioy, 2010). In the case of food and food ingredients, the exposure situation is to be considered as long-term repeated-dose exposure with the potential for systemic effects. This has consequences for selection criteria involved in choosing in vitro methods with regard to their significance and to the time frame within which they are able to deliver sensitive, robust and reproducible results. As the animal models do not always mirror human physiology adequately (Leist and Hartung, 2013) and as there are currently no in vitro approaches available to test compounds over a very long period, it stands to reason that in this field the efforts for risk assessment have to parallel the compilation of data from epidemiological and human studies. These data have the potential to improve the human risk assessment significantly, as they would provide information on long term effects, enable retrospective evaluation of results from in vitro methods, and lead to knowledge about effects on frequently affected populations and about differences in repeated response due to variations between populations.

Where there is a lack of relevant safety data on foods and food ingredients for the establishment of an adequate risk assessment in this field, innovative technologies and molecular techniques are investigated on how and if they can provide integral, human-relevant and reliable information. The roadmap for implementation of the new toxicological approaches in this field provides opportunity for elucidation of mechanisms/modes of action, inclusion of intraspecies and interspecies variabilities, correlation of animal data with in vitro data and epidemiological data (human studies and patients data), development of foods and food ingredients on the basis of new scientific findings, and the development of valid approaches (Hartung et al., 2013a) for integration of large datasets.

Current new approaches would allow addressing relevant organ-specific features, such as absorption and metabolism (Ramirez et al., 2013) by recruiting test systems mimicking human organs and involving new findings on the gene, protein and metabolite level. The intestinal system and the liver can be mimicked by 2D and 3D cell culture systems in bioreactors, and for these systems organotypic tissues and functional units, e.g. intestinal villus, are already available. Also ex vivo viable human tissue can be used for screening purposes. Communicating micro-reactors and organs-on-chip approaches would also allow investigating the influence of distinct organs on each other, i.e. intestine, liver, adipocytes. New bio-barrier systems for intestine, placenta and brain allow the investigation of transport phenomena, as well as the influence on the coherence of these barriers. The choice of the adequate system depends on significance, sensitivity, robustness and scientific validity of the system, but with the variety of promising approaches and technologies available this choice may also depend on demands of the experimenter and risk assessor.

With regard to large datasets to be expected from high-content, high-throughput profiling approaches (-omics) and testing batteries, there is a need for development of tools for extraction and weighing of relevant information (Judson et al., 2013), especially in cases in which low probability events are in the centre of focus, which are in contrast to deterministic methods, which have the focus on worst-case estimates. As modern toxicological risk assessment approaches result in multiple pieces of evidence, the main conceptual requirements for a multi-test decision framework are a probabilistic strategy (addressing uncertainties and dependencies): a consistent feature in allowing reasoning in both causal and predictive directions and supportive with regard to hypothesis and data-driven approaches, where the hypotheses can be updated when new data are provided. The approach of probabilistic risk assessment with integrated testing strategies (Stefanini, 2013; Hartung et al., 2013b) is to be an essential part of the new toxicological risk assessment approach and is to be integrated in the roadmap for new risk assessment strategies of foods and food ingredients.

In conclusion, any strategy for evaluating the safety of food and food ingredients should be able to address at least the following items:

- Chemical identity/structural activity relationships
- Relevant exposure scenarios
- Digestibility/stability
- Absorption and distribution (internal exposure)
- Metabolism
- Genetic toxicity and Carcinogenicity
- Repeated dosing
- Tolerance/allergenicity
- Other systemic endpoints case by case (e.g. reproductive and developmental toxicity)
- Target population specific testing (e.g. term infants and pregnant women)

In the following chapter a few new technologies will be described that could fit into the above mentioned items.

5. Useful testing methods to be applied in food safety strategies

The number of newly developed methods to determine the adverse effects of substances in sophisticated in vitro and in silico systems is overwhelming. This manuscript focuses on a number of these methodologies that may be advantageous when incorporated in integrated strategies for toxicity testing in the area of food safety. This overview is therefore by no means meant to be exhaustive and can be supplemented with many other examples.

The choice of the adequate non-animal assays, e.g. in vitro cell culture, depends on the question the system is able to address. Ideally, the systems mirror the features and responsiveness of human tissue. A functional and physiological test or test battery mimics the regular amplitude of organ response and variations as well as the reactivity on gene, protein and metabolite level in a concentration-response manner. Furthermore, these systems need to be sensitive, robust and reliable, and they should deliver reproducible and quantified data. In addition, the biological non-animal methods should preferably be able to be downscaled and automated to enable higher throughput (Wang et al., 2013). Moreover, the biological material, e.g. cells, should be easily accessible also in high quantities and their implementation and use should be possible without rigorous efforts.

For the study of many toxicologically relevant endpoints specific cell culture systems are available (Adler et al., 2011; Basketter et al., 2012). Distinction needs to be made between the study of a-specific or basal cytotoxicity (i.e. the effect on any cell type, mainly due to membrane damage, disturbance of energy supply, cell compartmentalisation) and more specific functional cell function disturbances, that can be related to a particular target cell, tissue or organ. For studying the latter form of toxicity, the knowledge of an AOP for

the toxic action is desirable.

5.1. (Stem) cell cultures to study absorption and systemic toxicity

In many cases the use of primary cell cultures are considered the "golden standard", being a better representation of normal *in vivo* physiology than cell lines. However, in general, human primary cells are not readily available. Moreover, primary cultures have the tendency to lose their organ-specific differentiation characteristics over time. The majority of cell culture systems now in use are therefore cell lines, preferably of human origin, and the limitations of these cell lines should be taken into account.

There is an increasing number of *in vitro* systems based on stem cell technologies. Stem cells are capable of dividing indefinitely and have the potential to differentiate into any cell type. Induced Pluripotent Stem Cells (iPSC) derived from adult, differentiated cells, are to regain pluripotency and thus may provide even better *in vitro* models to include in toxicology assessments. Since stem cell-derived *in vitro* systems can be stably maintained over prolonged periods of time in culture, these systems can be used in repeated dose toxicity studies *in vitro* (Suter-Dick et al., 2015). The application of stem cells in the production of organoids, that better mimic *in vivo* structures is yet another interesting area that may be applicable in studying organ-specific (systemic) toxicity (Foster et al., 2014).

Stem cell lines are in use for a long time as models for embryotoxicity, avoiding the use of animal embryos, i.e. by recruiting mouse embryonic stem cell lines (mES) (Scholz et al., 1999; Rolletscheck et al., 2005). Human embryonic stem cells (hESC) are also more and more involved in toxicology and biomedical research (Thomson et al., 1998). The EC funded project "Vitrocellomics" established an *in vitro* hepatotoxicity assays based on hepatic cells derived from hESC, including screening assays combining the hepatic model system with micro-sensor platforms for measuring absorption, cytotoxicity and metabolism (Beckers et al., 2010).

iPSC driven from somatic cells show features of hESC (Takahashi et al., 2007; Yu et al., 2007) and are accepted as an innovative technology, and already recruited in toxicological experiments. As the iPSC generation requires genetic modification of the cell nuclei, this approach leads to establishment of genotype-specified cell lines with the characteristics of hESC.

5.2. 3D cell culture models and organotypic tissues for metabolism

In vitro human 3D organotypic models have significant advances compared to monolayer cell culture models (Alépéé et al., 2014). For risk assessment, these models provide important features as there are normal human cells (non-tumor cells), they have organotypic structures, they mimic the barrier functions, they open realistic exposure options, and as they show physiological metabolism features (Kandarova et al., 2009; Kaluzhny et al., 2011).

A challenge to the field of tissue engineering pose the gastrointestinal tract, as the intestinal epithelium is a complex, rapidly renewing tissue with a distinct functional architecture. The approaches for engineering the gastrointestinal tract relevant to toxicity testing are based on organoids. These organoids are obtained by section of intestinal tissue, which is then seeded to a scaffold made of biodegradable materials. Such approaches have already been published on tissues from oesophagus, small bowel, colon and stomach (Fuchs et al., 2001; Sato et al., 1997; Choi et al., 1998; Grikscheit et al., 2003; Hori et al., 2001).

A novel approach for establishment of crosstalk between different cell cultures -also in 3D-aiming at overcoming the difficulty of translation of milliscale to microscale (e.g. with organ-on-chip approaches), is the modular multi-compartmental bioreactor array, the *quasi vivo* approach. The *quasi vivo* approach enables the transfer of microwell protocols directly to the bioreactor modules, offering mechanical stimuli from flow and biochemical stimuli from cells placed in connected modules (Mazzei et al., 2010).

Another major challenges is engineering an organ, e.g. the liver, as the *in vitro* models do not show the regenerative features, they show loss of functionality beginning after 24 h and as there are rapid changes in gene expression *in vitro*. As liver is a complex tissue, the different cell types have to be combined for establishment of an *in vitro* model, and also a rigorous perfusion system has to be integrated. Here new bioreactor technologies may open up to new possibilities, as these can include better oxygenation, e.g. by introducing gas-permeable fibers into the 3D tissue. This innovative approach resulted in higher metabolic activity, promotion of different cell types to liver-organotypic organisation, to significant elongation of functionality (20 days) and also spontaneous development of bile canaliculi and sinusoid-like structures (Gerlach et al., 1990; Zeilinger et al., 2002, 2004). Microarray bioreactors involving spheroids with integrated fluid flow could already show to be able to mimic the liver blood flow dynamics (Powers et al., 2002). In addition, in co-culture of hepatocytes and non-parenchymal cells (in a flat-bed bioreactor with controlled oxygen gradients) hepatic zonal patterns (region specific zonation) could be established, which were comparable with the zonal patterns *in vivo* (Allen et al., 2005).

5.3. Organs-on-chips

For mimicking the human physiology with *in vitro* methods it is inevitable to involve more cell types. With the complexity the likelihood of unpredictability and variations increases. The organ-on-chip approaches are micro-engineered microfluidic models including physiological micro-environment parameters, e.g. geometrical, mechanical and biochemical factors, for *in vitro* measurement, and are prone to be accessible as high throughput approaches.

As the organ-on-chip technology is based on modulation of fluid flow (microfluidic), it stand to reason that the vascular system and the endothelial response (e.g. to shear stress) can be addressed easily by this technology (van der Meer et al., 2009). Besides the readily accessible shear stress studies on endothelia in blood vessel-on-chip approaches also other features, e.g. cholesterol uptake and cytokine-induced direction on leukocytes could already be studied with this approach (Song et al., 2005; Shin et al., 2004; van der Meer et al., 2010; Srigunapalan et al., 2011).

The gut-on-chip approach is construct that is to mimic the geometry of intestinal villi as a more physiological and functional *in vitro* model of the intestine (Sung et al., 2011). The Nutrichip poses a microfluidic system for *in vitro* investigation of the immunomodulatory function of food ingredients. This approach involves Caco-2 cells (Ferruzza et al., 2013) in co-culture with THP1 cells aiming to address immune cell biomarkers (Ramadan et al., 2013).

There are numerous records of liver-on-chip approaches for screening purposes and for addressing the liver function (Baudoin et al. 2007; van Midwoud, 2011). It could also be shown, that the culturing of primary hepatocytes (clusters) on micro-patterned spots surrounded by fibroblasts will lead to preservation of hepatic functions for a significant longer period (Khetani and Bhatia, 2008).

The kidney-on-chip approaches could show, that cultured renal epithelial cells exposed to physiological levels of fluid flow and to a two-compartment microenvironment form differentiated and functional monolayers with apical-basolateral polarity (Ferrell

et al., 2010; Jang et al., 2010).

There are various successful attempts to establish a pancreas-on-chip system, involving pancreatic islets of Langerhans. This approaches aim at examination of hormone kinetics (Wang et al., 2010).

The brain-on-chip approaches have also been shown to be relevant in constructing neuronal models as the outgrowth can be directed in two or three dimensions by generating surface-bound or soluble biochemical gradients, and as this directed outgrowth can be combined with electrical activity, physiologically relevant measurements can be made (Wang et al., 2009).

The heart-on-chip approach aims at examination of mechanical (shear stress, stretch and relaxation) and electrical stimulation of matured and differentiating cardiomyocytes, revealing clear advantages of the microengineered system (Ghafar-Zadeh et al., 2011).

5.4. Models to investigate digestion and bioaccessibility

In contrast, macro systems mimicking the gastrointestinal tract are also successfully established and validated for assessment of nutritional and functional properties of foods and ingredients under simulated physiological digestion conditions, i.e. TIM-1 and TIM-2. These systems are multi-compartmental, dynamic computer-controlled models, whereas TIM-1 simulates the digestive processes of stomach and small intestines and TIM-2 represents the colon and includes a rich microbial gut-derived flora (Havenaar, 2011).

Another promising approach for analysis of gastrointestinal tract is the approach of immobilised digestive enzymes in microfluidic process reactors, which can give insight in which metabolites and intermediates are present during the digestion also by subsequent proteome analysis (Asanomi et al., 2011).

5.5. Biokinetics (ADME)

For the evaluation of a compound's toxicity it is essential to obtain knowledge on its biokinetic behaviour. An analysis of the absorption, distribution and metabolic or renal clearance provides insights in the exposure of possible toxicological targets *in vivo*. For estimating absorption via the oral route a number of well-established *in silico* and *in vitro* methods are available. However, although these methods are in use in the area of pharmaceutics, only limited information is available for other areas, including food ingredients and matrices. For estimates of ADME behaviour the use of physiologically-based biokinetic (PBBK) models are very helpful and in use in many areas, and since these methods can be to a great extend based on non-animal methods (Bouvier D'Ivoire et al. 2007; Bessems et al., 2014), this will be an important element of novel test strategies.

Apart from being relevant for the toxicodynamic characterisation of a compound, the use of *in vitro* systems also need to be relevant with regard to the cellular exposure to the compound. First of all, this implies a good characterisation of the biokinetics in the *in vitro* systems themselves (Kramer et al., 2015). This includes also the information on the intracellular concentrations, on the concentrations in different cellular compartments as well as on the extracellular concentrations To perform an *in vitro* to *in vivo* extrapolation (IVIVE), the real concentration of a compound has to be determined (Coecke et al., 2012: Groothuis et al., 2015), as the free concentration of a compound is not necessarily identical to the nominal concentration; compounds may bind to the device material or to biomolecules or they may evaporate or be metabolised quickly (Kramer et al., 2012).

The other element of the biokinetic considerations in a safety assessment based on *in vitro* methods is the in *vitro-in vivo* extrapolation of the data. This implies the translation of the effective concentrations in the *in vitro* systems to an exposure scenario *in vivo*, making use of reverse dosimetry (Yoon et al., 2012; Blaauboer et al., 2012). The tool to apply is physiologically-based biokinetic modelling. PBBK models describing the kinetics are being made for individual compounds, which hampers the application in risk assessment. However, generic PBBK models are under development which will largely decrease resources (time and costs) and accelerate implementation (Bessems et al., 2014). PBBK should be extended for evaluation of biodynamic processes and also extended for involvement of a higher number of compounds. This methodology should be also modified to allow combination of *in vitro* toxicity and ADME data with *in vivo* dose-response curves. QSAR, PBBK, IVIVE are often used as stand-alone approaches, but the integration of these approaches may lead to testing strategies that result in replacement or reduction of animal tests.

5.6. Biotransformation

Since a compound's toxicity is highly determined by its metabolism, either by minimising the toxicity through detoxifying reactions or by enhancing toxicity (bioactivation), the relevance of using *in vitro* systems to predict toxicity is depending on the presence (qualitatively as well as quantitatively) of biotransformation reactions in these systems. In many existing *in vitro* systems biotransformation is not an appropriate mirror of the *in vivo* situation (Coecke et al., 2006). Therefore, results of *in vitro* experiments need to be interpreted with care, especially when a compound's metabolism is located in one organ (e.g. the liver) while the toxicity (of the metabolite) is expressed in another tissue or organ.

The use of appropriate *in vitro* systems (Rossini and Hartung, 2012) allows the quantification of the relevant biotransformation reactions and systems have been described to scale the outcomes up to the formation of metabolites for the *in vivo* situation (Yoon et al., 2012, 2014).

5.7. QSARs

In silico modelling is already a prerequisite in many areas of the risk assessment field (Hartung and Hoffmann, 2009; Raunio, 2011) and is to be also an integral part of toxicological assessment of foods and food ingredients. Non-testing data can be generated by several approaches, including grouping approaches, which consist of read-across and chemical category formation, structure—activity relationship (SAR) and quantitative SAR (QSAR). A structural physico-chemical reactivity characterisation of compounds is currently done routinely (Valerio, 2009, 2011). Further, provided that the subset of nutritional compounds shares structural similarity with chemicals or drugs this approach may be able to use the larger amount of SAR and QSAR approaches available from pharmaceuticals and industrial chemicals.

For the QSAR approach there is a need for development of methods to assess mixture effects, and also for development of intuitive software or artificial intelligence matrices for more comprehensive use of databases. QSAR also needs possibilities for software matrices for more sensitive and selective prediction models.

5.8. The usefulness of the above-mentioned technologies for the safety evaluation of food and food ingredients

In chapter 4 the essential elements for these safety assessments are listed. For some of these elements the necessary data can be acquired by methods that are outside the realm of testing, e.g. for

the determination of relevant exposure scenarios, the chemical identity of compounds and the composition of more complex food and food matrices. Stability and digestibility are elements that may require chemical and biochemical test methods.

For a determination of absorption, which parameter is determining the internal exposure to compounds, progress has been made to determine this in *in silico* (QSAR) and *in vitro* systems. The same applies for estimating biotransformation; the formation of metabolites can be determined in *in vitro* systems and parameters such as intrinsic clearance can be scaled up, e.g. to serve as input parameters for PBBK modelling. A drawback of these methods is that analytical tools are needed to quantify metabolite formation, thus hampering the incorporation in high-throughput systems.

The use of *in vitro* evaluation of genotoxicity has been performed over a long period. The increasing knowledge of the mechanisms involved in the effects on the genome and its relation to carcinogenic endpoints also opens possibilities to include this in an evaluation scheme less relying on animal studies.

The ever increasing number of test systems focussing on the mechanism of toxicity with specific target tissues and organs is now even faster progressing. Depending on the issue at stake, cell culture systems consisting of 2D, single cell cultures might answer the more basal questions, while the application of 3D cultures (spheroids), stem cell-derived cultures etc. would be more applicable for specific toxicity. For the future, organ-on-chip models or bioreactor-type approaches are promising tools. The more systematic way in which modes of action, e.g. by describing adverse outcome pathways, is also opening possibilities to better include *in vitro* toxicity data in risk evaluations. And although in the past the focus has been on short-term effects that were most of the time related to acute toxicity, a number of studies have been done in which prolonged or repeated exposure were evaluated with *in vitro* methods (e.g. the Predict-iv project, Wilmes et al., 2013).

The more specific endpoints such as tolerance, allergenicity, reproductive and developmental toxicity have been studied in *in vitro* systems, however, work needs to be done to evaluate these methods for the area of food and food ingredients.

Many of the results of these mechanistic studies based on *in vitro* methods will have to be evaluated for their relevance in an intact (human) organism. These *in vitro-in vivo* evaluations will have to be quantified (QIVIVE) to be useful in a risk or safety evaluation.

If specific target populations are the issue, the parametrisation of the QIVIVE process should be tailor-made for such situations, including a probabilistic rather than a point-estimate approach.

For all the above-mentioned methods and technologies it should be kept in mind that these will need to cover the range of less or more complex situations often observed in the food area, ranging from individual compounds and enzymes to increasingly complex mixtures.

6. An evaluation roadmap

Based on the above considerations a roadmap for safety assessment of food and ingredients (Fig. 1) was developed. The roadmap consists of a number of blocks that describe activities or decision steps to be taken. Each block activity aims at answering specific questions that then may lead to activities in the following block. It is also possible that iterations to earlier blocks in the roadmap are necessary. The specific aspects of each block are further described in the explanatory text.

Explanations of steps, routing and considerations for Fig. 1.

1) Define the type of food: is it a chemically defined ingredient? a complex food ingredient? a non-nutritional compound? what is intended target population and use? This information helps to define what kind of data should be collected.
2) Provide information on: physico-chemical properties (incl. solubility) and characterisation of the material (material specification): identification of (non) nutritional compounds: changes in composition due to production process (impurities).
3) Apply computational approaches to predict e.g.:
   - QSAR/QSPR (impurities);
   - Bioaccessibility and bioavailability (behaviour in GI tract);
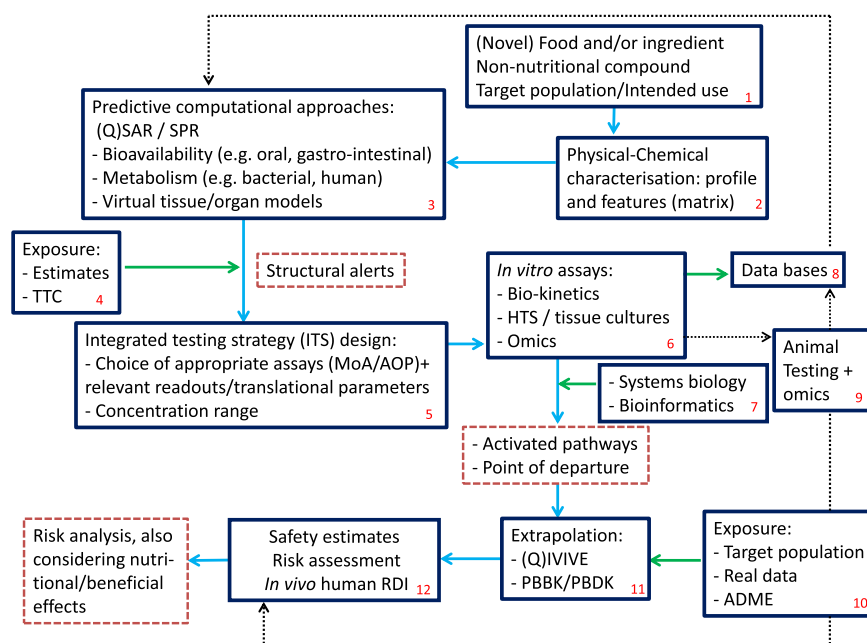
Fig. 1. Evaluation roadmap for safety assessment of food and ingredients. Numbers represent the flow and the solid blocks with blue arrows the main stream (methods explained in text). The information provided by the blocks with green arrows is related to exposure. The dotted blocks are 'outcomes' of the previous blocks and the dotted lines are feedback routes and may provide additional information to (re)consider the next steps.

- Metabolism (e.g. bacterial, liver);
- Virtual tissue/organ models for safety/efficacy assessment.

4) Exposure assessment: estimation of daily exposure and apply TTC concept in case of non-nutritive compounds or impurities.

Consideration: if *in silico* predictions lead to one or more alerts (thus not only 'approved' alerts such as genotoxicity) and/or the exposure is estimated to exceed the threshold of toxicological concern, which is often the case for foods and food ingredients, further testing is needed.

5) Design an integrated testing strategy with appropriate assays (choices should be based on the alerts, but also including considerations regarding a specific target population (pregnant women, infants)) that can identify mode of action(s), determine dose response relations and measure parameters/read-outs that are translational to human population.

6) Perform *in vitro* assays (consider both nominal and measured dose concentrations), preferably medium/high throughput and based on human cells or tissues. Make use of new technologies, such as omics, imaging, etc. and include biokinetic data.

7) Apply bioinformatics tools and systems biology to integrate data and identify signatures (finger prints) and mode of actions.

Consideration: can activated pathways be identified? When will they become adverse? If so, what will be the point of departure for the *in vitro-in vivo* extrapolation and the final safety assessment?

8) Data obtained from the *in vitro* assays should be collected and stored in databases, in such a way that the current *in silico* tools can be improved and/or new QSAR models can be build.

9) In cases where *in vitro* assays do not lead to conclusive results or do not address the relevant endpoint, or approval is needed *in vivo*, animal studies might be more considered. These tests should be designed using information from the other approaches, such as mechanistic data (e.g. from omics analyses) and can be directly used for quantitative risk assessment. These data should also be included in the databases to 'validate' the *in vitro* assays.

10) Measure the real exposure and ADME (human data), specifically for target groups such as children, elderly and obese population.

11) Combine exposure data and *in vitro* data to extrapolate from *in vitro* to *in vivo*, by using PBPK modelling, assessing how well the in vitro system mimics in vivo and considering any uncertainties.

12) Perform risk assessment, determine safety levels and human ADI or reference daily intake (RDI) for general public and target groups

13) Rational and mode of action supporting the beneficial or technological effects of the food or food ingredient should be considered as part of the effect spectrum, to be able to interpret potential adverse effects related to the same mode of action. This information also serves to make a risk benefit assessment

Consideration: What is the margin of safety? Is information on nutritional (beneficial) effect present? If so, is it achievable to weigh risk and benefit, taking into account target populations, severity of the effect (deficiency versus toxicity)?

7. Case studies

Cases are selected on foods and food ingredients for which safety assessment are available by EFSA, JECFA, making use of animal and human data. Taking these cases through the decision tree, while making use of new available methods may demonstrate in what way new methodologies could improve food safety assessment.

Cases should have at least the following elements:

1) A well-defined question regarding the safety of a food ingredient or product.
2) The collection of information, where possible, on the structural physico-chemical properties, matrix, mixture etc.
3) Collection of information on possible exposure scenarios for consumers (e.g. possibility of application of TTC).
4) Based on this information: leads for selection of possible MoA for toxic effects, target tissues and endpoints (qualitative info).
5) Selection of appropriate *in vitro* and *in silico* methods to evaluate these MoA/endpoints: are these available, are these evaluated?
6) Integrate knowledge on possible toxicity with knowledge on kinetics (e.g. bioavailability after oral (human) consumption, distribution, metabolism, clearance), data preferably derived from *in vitro* or *in silico* data.
7) Extrapolate data to a human exposure scenario: reverse dosimetry, quantitative *in vitro-in vivo* extrapolation.

8. Selected cases

In the next paragraphs a number of foods and food ingredients following the roadmap in Fig. 1 were evaluated. The product specification or manufacturing processes involved as well as the history of use or the intended use of the products in which the food was or would be present was taken into account, assuming there was no existing safety data knowledge based on animal studies.

8.1. Case 1: steviol glycosides

Box 1. Intended Use; Food Application; Use levels; Target population.

Stevia rebaudiana is a plant native to South America and has been used to sweeten beverages and food for several centuries. The plant has also been distributed to Southeast Asia. Steviol glycosides, the principle sweetening components are low-calorie, high-intensity sweeteners (~200—300 times sweeter than sucrose) of similar taste quality as sucrose, which provide an alternative to the already approved high-intensity sweeteners and are stable to heat. The uses and use levels for steviol glycosides intended to be used in food categories, targeting the general population, reflect those currently permitted for aspartame in the EU with some major exceptions.

Box 2. Physical-chemical characterisation.

The manufacturing process comprises two main phases: the first involving water extraction of the leaves of the *S. rebaudiana Bertoni* plant and preliminary purification of the extract by employing ion exchange chromatography to yield a steviol glycoside primary extract, and the second involving recrystallisation of the steviol glycosides from methanol or aqueous ethanol resulting in a final product.

The steviol glycosides produced are chemically defined

mixtures that comprise not less than 95% stevioside and/or rebaudioside A. Stevioside and/or rebaudioside A are more than 95% of the mixture in some of the available products. In another product, rebaudioside A is the major component of the mixture (≥95%) together with other glycosides. In addition, smaller amounts of rebaudiosides B, C, D, E and F, steviolbioside, rubusoside and dulcoside A are present in the compositions of final mixtures.

The mixtures are described as white to light yellow powders, odourless or having a slight characteristic odour, about 200–300 times sweeter than sucrose, freely soluble in water and a pH between 4.5 and 7.0 (1 in 100 solution).

The JECFA specifications outlines that the purity of steviol glycosides should not be less than 95% of the total amount of the seven named glycosides (stevioside, rebaudioside A, rebaudioside C, dulcoside A, ubusoside, steviolbioside and rebaudioside B) on the dried basis with the major glycosides in the product being stevioside and rebaudioside A. In the EU specifications it was proposed to also include rebaudioside D and rebaudioside F, two minor steviol glycosides, which may also be present in the final mixture.

According to JECFA (2007), impurities occurring in extracts of Stevia leaves are typical plant materials, such as pigments and saccharides. One literature study reports identification of the following substances in the non-glycosidic fractions of extracts of Stevia leaves, obtained using Supercritical Carbon-dioxide Fluide Extraction (SCFE): spathulenol; decanoic acid; 8,11,14-ecosatrienoic acid; 2-methyloctadecane; pentacosane; octacosane; stigmasterol; β-sitosterol; α- and β-amyrine; lupeol; β-amyrin acetate; and pentacyclic triterpene. These substances (corresponding to approximately 5% of the steviol glycosides preparation) represent 56% of the total non-glycosidic extracts, while 44% remain unidentified. The specified additive (>95% total steviol glycosides) will contain, in addition to saccharides other than those associated with the individual steviol glycosides, residual extraction/recrystallisation solvent and possibly also residues of ion-exchange resins used in the manufacturing process.

According to JECFA, the level of the non-glycosidic fraction, because of its highly non-polar character, can be considered insignificant in the additive.

Several other related steviol glycosides that may be generated as a result of the production process but do not occur naturally in the leaves of S. rebaudiana plant, have been identified in small amounts (0.10–0.37%, w/w) by High Performance Liquid Chromatography (HPLC) in the steviol glycoside bulk material. Some of them share the same steviol aglycone backbone structure as rebaudioside A and differ only with respect to the number of glucose units, while the remaining compounds have slight structural differences in the aglycone backbone like an endocyclic double bond, an additional hydroxyl group or isosteviol instead of steviol aglycone.

Box 3. Information on GIT metabolism/bioavailability.

In vitro assays with human/animal enzymes: *These experiments have been conducted and could well be used following the roadmap:*
Stevioside metabolism was studied by adding various digestive enzymes or fluids like salivary α-amylase, pancreatic α-amylase, saliva, pepsin, gastric secretion, pancreatin and intestinal brush border membrane enzymes of rodents as well as by the intestinal microflora of various species including humans (Hutapea et al., 1997). None of these enzymes digested stevioside. However, the caecal microflora of all species tested was able to metabolise stevioside to steviol the aglycone. A transient formation of steviol-16, 17 α-epoxide was observed in mouse caecal contents and human feces. It was suggested that steviol is the major metabolite produced by caecal microflora from various animal species and humans. The *Stevia* mixture, stevioside and rebaudioside A appeared also to be hydrolysed to the aglycone steviol by human intestinal microflora after incubation with human intestinal microflora (faeces).

Intestinal transport of steviol was studied in Caco-2 cells: steviol permeability was 200–300 times higher compared to stevioside or rebaudioside, indicating poor oral bioavailability of the glycosides.

The hepatic metabolism was studied of steviol, by using human liver microsomes versus rat to include also species difference: steviol undergoes glucuronidation and low conversion into oxidative metabolites, no major difference in metabolic profile between species was observed.

Human studies: *These experiments have been conducted and could also be used following the roadmap: because of the history of human use, it would not be unethical to conduct these single dose studies without in vivo animal studies, except with regard to potential allergenicity:*

Regarding possible allergenicity of stevioside: available data concerning anaphylaxis-like reactions by stevioside in children with atopic eczema do not raise concern regarding the potential for oral exposure to steviol glycosides to trigger anaphylactic reactions. Sparse *in vitro* and *in vivo* data indicate that stevioside may have immunostimulating effects and modulatory activities on inflammation. Immunostimulating and immunomodulating effects of steviol glycosides in cell lines and rodent models have not been demonstrated in a robust and reproducible way, which could enable them to be used as pivotal studies for risk assessment. These observations deserve more in-depth examination as, if they are confirmed, they may raise concern regarding the use of steviosides in some sub-groups of the population, particularly for individuals suffering from auto-immune diseases or inflammation of the gastrointestinal tract.

In human volunteers exposed orally (dose ranging from 375 to 750 mg/day) to stevioside or rebaudioside A, no free steviol was detected in the blood but steviol glucuronide was found to be the main metabolite in plasma. No steviol epoxide, which may be mutagenic, was detected in human plasma. Steviol glucuronide appeared in the plasma after administration of rebaudioside A or stevioside, with median plasma peak time values of 12 and 8 h post-dose, respectively. In both cases, two plasma peaks occurred at 6–12 and 24 h post-dose. Steviol glucuronide was eliminated from the plasma, with similar half-life values of approximately 14 h for both compounds. The presence of multiple peaks in time of plasma concentrations of steviol glucuronide indicates enterohepatic circulation of steviol in humans. Steviol glucuronide was also reported to be the main metabolite found in the urine of subjects receiving stevioside or rebaudioside A; this elimination pathway accounted for about 60% of the dose. Steviol was reported to be the main metabolite found in the faeces of humans receiving oral stevioside or rebaudioside A. Steviol glucuronide is excreted primarily via the urine in humans.

Box 4. Exposure estimates.

Structural alerts should be addressed first, since the exposure is estimated to be considerable. TTC approach is therefore not applicable. Small children and adults are both part of the target population.

The dietary exposure to steviol glycosides (E 960) ranges for toddlers from 2.0 to 4.3 mg/kg bw/day at the 95th percentile. The exposure levels for children are estimated to be between 1.3 and 3.9 mg/kg bw/day at the 95th percentile. Exposure estimates calculated for adolescents of 0.6–1.8 mg/kg bw/day for high level exposure. In adults, the exposure levels are up to 2.2 mg/kg bw/day at the high exposure levels. In the elderly, the high exposure level is estimated up to 1.3 mg/kg/bw.

Test metabolic ceacal fractions in in vitro genotox assays (Ames, chromosomal abb.), although from human ADME studies it is demonstrated that possible genotoxic metabolites are absent from the systemic circulation, site of contact tissue (GIT) should be looked at.

Steviol and some of its oxidative derivates showed clear evidence of genotoxicity *in vitro*, particularly in the presence of a metabolic activation system.

Box 10, 11, 12. Extrapolation in vitro dose to in vivo human dose and risk assessment.

Randomised, placebo controlled clinical trials were conducted to study the effect of steviol glycosides on glycemic response. Single doses of 1000 mg steviol glycosides/person/day (97% rebaudioside A) corresponding to approximately 330 mg steviol equivalents/day) did not affect glucose homeostasis and did not affect blood pressure in individuals with normal glucose tolerance or type-2 *diabetes mellitus*. Also repeated use for 16 weeks of 1000 mg rebaudioside A/ person/day did not alter glucose homeostasis in individuals with type-2 *diabetes mellitus*. Blood pressure parameters were not significantly affected by oral intake of 1000 mg rebaudioside A/ person/day for 4 weeks in individuals with normal and low systolic blood pressure. This daily dose corresponds to 16.6 mg of rebaudioside A/kg bw for a person weighing 60 kg and to approximately 5.5 mg steviol equivalents/kg bw/day.

In conclusion, while following the roadmap and disregarding all existing *in vivo* information on steviosides: the human kinetics of steviosides are well addressed using the *in vitro* systems making use of human enzymes and enzymes of different animal species. No species difference was observed, and in humans the glucuronidation of steviol appears the main metabolic pathway, also supported by *in vivo* human data.

Further *in vitro* investigations also pointed towards clear evidence of genotoxicity of steviol and some of its oxidative derivates, particularly in the presence of a metabolic activation system and towards potential immunomodulating properties. With regard to the possible genotoxic potential especially site of contact tissue (GIT) should be further investigated, since systemically the glucuronidation does not appear to be saturated at relevant levels of exposure. It should be investigated if relevant *in vitro* models are available and how do these translate to the human situation.

In addition regarding the target population including pregnant and lactating women and small children, specific endpoints on reproduction toxicity and teratogenicity should be addressed. Currently no *in vitro* models are available replacing the established *in vivo* animal models.

8.2. Case 2: synthetic lycopene

This case study is based on a proposal for the use of synthetic (crystalline) lycopene as a food ingredient/food supplement which was reviewed by EFSA (EFSA Journal, 2008). The question that was addressed was 'Is the synthetic (crystalline) lycopene safe to use by the general population at levels of 8 or 15 mg/dosing in supplements, at levels up to 2.5 mg/100 g in beverages and dairy products, up to 4 mg/100 g in breakfast cereals, up to 8 mg/100 g in cereal bars?'.

For the safety assessment of synthetic lycopene the approach taken was to compare the synthetic lycopene with naturally occurring lycopene (from tomatoes and tomato products) to determine if a read across approach could be taken based on

chemical similarity or, if there were any differences, were there any components of concern. This was followed by a comparison of the human exposures that could result when using synthetic lycopene and whether this is qualitatively and quantitatively similar to what is already occurring with the uses of natural lycopene.

Following Fig. 1 the relevant questions are addressed:

Box 1. Intended Use; Food Application; Use levels; Target population.

The novel food ingredient consists of synthetic (crystalline) lycopene to be marketed in three different formulations. These are lycopene 10%, lycopene 10 cold water dispersion (CWD) and lycopene dispersion 20%. Synthetic lycopene will be used in food supplements at levels of 8 or 15 mg/dosing, in beverages and dairy products at levels of up to 2.5 mg/100 g, in breakfast cereals up to 4 mg/100 g, in cereal bars up to 8 mg/100 g, in fats and dressings up to 4 mg/100 g and also in dietary foods for special medical purposes at levels suited to those that the products are relevant to. The target population will include both adults and children.

Box 2. Provide information on physical chemical properties (incl. solubility) and characterisation of the material: identification of (non)nutritional compounds. Include changes in composition due to production process (impurities).

Lycopene is a carotenoid with the formula $C_{40}H_{56}$ (molecular weight of 536.85 and CAS 502-65-8).

Lycopene occurs in food predominantly in an all-*trans* form (Cronin, 2000; Boileau et al., 2002). Tomatoes and tomato products contain the all-E (*trans-*)isomers of lycopene (between 35 and 96% of total lycopene content), but also some Z (*cis-*)isomers, mainly as 5Z, 9Z, 13Z and 15Z in percentages varying between 1 and 22% (Schierle et al., 1997).

Synthetic lycopene consist mainly of the all *trans*-lycopene (>70%) with 5-*cis*-lycopene (max. 20%) and up to 3.5% other *cis* isomers. It contains the same *cis* isomers found in tomatoes and tomato products.

The synthetic lycopene considered in this case study included other components in the specification but these were all considered to be standard food grade materials and therefore not of any safety concern. However, for one of the proposed formulations (20% dispersion) it was noted that no additional stabilisers were included which might result in oxidation of the material. Specifications were set for purity of >96% and for the presence of contaminants such as heavy metals.

There were no aspects of the production process that were considered to add any safety concerns to the material.

Based on the comparison of the chemistry and any components of concern the safety of synthetic lycopene can be based on similarity to naturally occurring lycopene using a History of Safe Use approach (Constable et al., 2007). Therefore next question to consider in the roadmap would be Box 4.

Box 4. Exposure estimation.

It was estimated that intakes of lycopene from natural dietary sources in different populations are, according to dietary surveys, estimated to average between 0.5 and 5 mg/day, with high exposures up to about 8 mg/day. High consumption of fruits and vegetables, especially tomato products, may result in occasional intakes of 20 mg lycopene/day or more.

Intake of lycopene from supplements is not expected to be more than 21 mg/day among supplement users, based on the combined use of one lycopene supplement (providing maximal 20 mg of

lycopene) and one multi-vitamin supplement (providing max 1 mg of lycopene).

Lycopene intake via lycopene fortified products is estimated to be 28—30 mg/day for children up to 9y, 37 mg/day for males and 33 mg/day for females aged 10—18y, and 25 mg/day for males and 23 mg/day for females over 19y. Expressed per kg bw the estimated 95th percentile intakes will be highest for children (1—3y), being 2.2 mg/kg bw/day, intermediate for children (4—9y), 1.3 and 1.4 mg/kg bw/day, for girls and boys respectively, and lowest for men and women (19y) 0.32 and 0.35 mg/kg bw/day, respectively.

Overall intakes from the proposed uses of synthetic lycopene would lead to intakes substantially higher than dietary intake of lycopene, and could lead to daily intakes from 10.5 to 30 mg/day at the mean and to 52—95 mg/day as high intakes. These values amount from 0.175 to 0.5 mg/kg bw/day and 0.87—1.58 mg/kg bw/day for a 60 kg person and are for the high intake estimates substantially higher than the ADI recently established by the AFC Panel as a group ADI of 0.5 mg/kg bw/day for lycopene from all sources (EFSA, 2008). This ADI is in line with the ADI of 0—0.5 mg/kg bw/day established by JECFA (JECFA, 2006).

It was concluded that intakes at the average level are in line with the ADI but there may be some excursions above this in high level consumers.

Box 10. Measure the real exposure and ADME, specifically for target groups such as children, elderly and obese population.

Lycopene absorption from purified or synthetic lycopene has been demonstrated to be comparable to tomato-based lycopene (Hoppe et al., 2003). In a study in healthy adults synthetic and tomato-lycopene resulted in significant increases above baseline of serum total lycopene by 0.58 and 0.57 micro mol/L, trans-lycopene by 0.34 and 0.41 micro mol/L, and total-cis-lycopene by 0.24 and 0.16 micro mol/L, whereas no significant changes were found in the placebo treatment. The mean serum total lycopene response to synthetic and natural lycopene was not significantly different.

Interactions, both competitive and synergistic, between carotenoids have been shown to occur during the various stages of absorption (e.g., incorporation into mixed micelles, intracellular transport within enterocytes, and chylomicron assemblage), as well as during post-absorptive distribution (Furr and Clark, 1997; Van den Berg, 1999). However, the mechanisms via which this occurs are not clear, and definite relationships between specific carotenoids have not been established. The specific carotenoids in the synthetic lycopene are comparable with the natural lycopene and therefore interactions are not expected. However, it may be prudent to consider a study to investigate uptake of individual components from the synthetic lycopene to ensure they are within those of natural lycopene.

Little is known about metabolism and degradation of lycopene in mammals but it would not be expected that this would differ between the synthetic and natural lycopene based on their similar structure. Analysis of lycopene metabolism and degradation could be made as part of a human study.

Box 12. Perform risk assessment, determine safety levels and human ADI or reference daily intake (RDI) for general public and target groups.

An ADI of 0—0.5 mg/kg bw/day was established by JECFA (JECFA, 2006) for lycopene from all sources.

There are numerous published short-term and sub-chronic toxicity studies on natural and other synthetic lycopene materials. These have been summarised as part of a review by EFSA on lycopene as a colour (EFSA, 2008). These confirmed the basis of the ADI set by JECFA using a NOAEL in a 1 year rodent study of 50 mg/kg/day. It was confirmed that there might be higher intakes on occasions which would exceed the ADI. However, there would be no difference expected in the safety profile of the novel synthetic lycopene with natural or synthetic lycopene already in use and therefore no further work would be required.

8.3. Case 3: botanical extracts as beverages

The majority of our foods are complex materials, and not single molecular entities. The first two cases considered are examples of well-defined single molecules, or fairly well characterised simple mixtures, used as additives, or ingredients into a wide variety of foods. More challenging in the context of this present discussion is how to assess the safety of complex mixtures, such as actual foods. Botanicals such as traditional herbal medicines, is an area which receives a lot of attention. The applicability and availability of *in silico* methodologies and phytochemical data sources were reviewed by Barlow et al. (2012). Pelkonen et al. (2012) discussed the potential impact of omics techniques on R&D and regulation of complex herbal products, and propose using omics techniques and a systems biology approach to integrate data from *in vitro* and *in vivo* tests, preclinical and clinical toxicity, pharmacokinetics, pharmacodynamics and efficacy tests. Current and new (alternative) methods for assessing genotoxicity, teratogenicity and nephrotoxicity of herbal medicines and mushrooms have been reviewed (Ouedraogo et al. 2012). For example, useful *in vitro* methodologies for teratogenicity, covering all aspects of prenatal development are available, however a major drawback is that the lack of placenta means that real foetal exposure cannot be determined.

An appropriate example for the food industry to take is aqueous botanical extracts which are consumed as beverages. Many common foods can be included in this class of products, such as coffee, tea, herbal teas. The raw materials used to prepare the different beverages can be different parts of plants (roots, leaves, flowers, fruit), and subjected to a variety of processing before being used to prepare the water infusions which are consumed. Powdered extracts can also be prepared, which are added to water to prepare 'instant' beverages. As well as simple hot water infusions, organic solvent extracts may be used if the aim is to enrich in certain 'bioactives'. Such liquids which are to be consumed as such are also more amenable to testing in *in vitro* assays, with potentially less technical difficulties and bias than solid foods.

Booth et al. (2012) evaluated the safety of a cranberry leaf extract. This is an example of a new food product, for which a history of use is known for the fruit of the species, and is a common food, but no knowledge exists on the safety of the leaves as consumed. Based on literature search of suspected compounds, on analytical profiling, on expected exposures and comparison to health based reference values (TTC, extrapolation from animal studies, ADIs etc.), no safety concern was identified in the study, and no further requirements for further safety testing would likely be needed before human consumption. This conclusion could be further substantiated using the steps in our roadmap.

Problem formulation: 'Are there indications for health concerns from a beverage prepared from water extraction of Cranberry leaves?

Box 1. Intended Use; Food Application; Use levels; Target population.

There is no history of use of the leaf of this plant species (*Vaccinium macrocarpon*), although there is a common use of the fruit (cranberry). A powdered water extract is to be developed as an ingredient for a new family of commercial beverages. The intended

application will be an extract equivalent to 2.4 g leaves in a 250 ml beverage, with a possible ingestion of 2 servings/day.

Box 2. Physical-chemical characterisation.

Literature search was performed on *V. macrocarpon*, extended to other members of *Vaccinium* genus and *Ericaceae* family for potential chemical components. These were grouped into classes of compounds, with a reference indicator compound (as available) for each class. It was acknowledged that appropriate standard operating procedures have to be developed to prevent contamination with undesired species (weeds, and any associated toxins), and authenticity purposes (HACCP, supply chain control).

Chemical analyses were performed on representative samples (from different harvest seasons and locations) of plant leaf material. Analyses included general parameters such as heavy metals, microbes, pesticides, ash content, % foreign material, % solids, moisture content, water activity. Additionally, a screen was made for organic compounds identified from literature to be of greatest toxicological concern.

Compounds known to be of high toxicological concern such as Ephidrene alkaloids, aristolocholic acid, gossypol, pyrrolidizine alkaloids, cardiac glycosides were not considered in this particular case as these are found in unrelated plants.

Box 4. Exposure estimation.

Commercial (or batch scale) batches was prepared, standardised on anthocyanin content of 33.4 mg/8 oz. serving (approx. 250 ml). It was assumed that all compounds are 100% transferred into water extract, and so 8 oz. serving would be equivalent of consuming 2.4 g dried leaf, 2 servings.

Box 12. Perform risk assessment.

It was evaluated whether consumer exposure to key phytochemicals (e.g. arbutin, indole alkaloids, quinolizidine alkaloids, grayanotoxins) is acceptable by using established Acceptable Daily Intakes (ADIs), or establishing such reference values based on available toxicity data, and comparing against Estimated Daily intakes (EDIs).

Based on the absence of compounds of high toxicological concern, no alert for concern was identified, and product development could continue.

To further strengthen the argument of absence of concern, some *in vitro* assays could be performed for bioavailability, genotoxicity, and liver toxicity. There would be a need to consider bacterial metabolism in the gut as well as liver metabolites. If still no alerts are identified, then there would be no requirement for an integrated testing strategy. However, additional screening and an ITS could be done if an incomplete characterisation was performed, uncertainties remained, and if there were alerts for the presence of compounds structurally related to known toxins. If from literature searches case reports are found of health concern, then targeted studies would be required to investigate such reports. For example, as in the case of concentrated green tea extract, liver toxicities have been reported. This could then indicate ITS concentrating on liver toxicity.

8.4. Case 4: Cetyl Myristoleate Complex (CMC)

Cetyl Myristoleate Complex (CMC) is a powder consisting of cetylated fatty acids (CFAs), and an application made in Europe through the Novel foods regulation. EFSA have evaluated this complex several times (EFSA, 2010, 2013; 2014), each time concluding that the safety of the CMC had not been established. The major concerns of the EFSA Dietetic Products, Nutrition and Allergies (NDA) Panel were that in the absence of data on the absorption, distribution, metabolism and excretion of unhydrolysed CFAs, that the existing toxicological data was not adequate.

The applicant provided data from literature on the ADME of CFAs. They proposed that CFAs in the CMC complex are expected to be nearly completely absorbed. Most of the CFA will be hydrolysed into cetyl alcohol and fatty acids. Cetyl alcohol is then oxidised to palmitic acid and esterified to phospholipids, triglycerides and cholesterol esters. However, the material tested in the studies were not considered to be representative of the CMC for which the application had been made, and it appears that a small amount of intact unhydrolysed CFAs (approx. 10%) remained in various organs of the body. The panel considered that the existing information was not sufficient to determine the ADME of the remaining intact CFAs. An acute LD50 toxicity study with cetylated myristoleate was submitted, but such studies are of limited value for assessing food ingredients. Otherwise, only limited human trial data were available. Further data were supplied by the applicant, namely a dedicated 90 day toxicological study. Although a NOAEL of 1000 mg/kg bw/day was identified, the highest dose tested, the NDA panel saw too many shortcomings in the experimental design and execution of the study. A study report on the *in-vitro* hydrolysis of cetyl myristoleate and cetyl myristate demonstrated a low rate of hydrolysis in simulated digestive fluids. This only re-iterated the request from the panel for more safety information on unhydrolysed CFAs.

Question Formulation: How can the safety of CMC, with specific focus on the ADME of unhydrolysed CFAs, be best assessed? Are there adequate *in silico/in vitro* assays that could substitute for better performed *in vivo* data?

Specifications indicate that CMC contains ≥48% oil which is composed of ≥38% cetyl myristoleate (C14:0, Hexadecyl ester of tetradecanoic acid), 40% cetyl myristate (C14:1, hexadecyl ester of tetradecenoic acid), with 10% CFAs with Carbon chain of >C14:0, and 10% with >C14:0. The rest of the CMC is cornstarch (48%) and 2% silicon dioxide. Analyses for heavy metals, dioxins, PCBs, PAHs and pesticides did not indicate any concern. Microbial analyses were compliant to the respective EC regulations.

A daily dose of 3.3 g CMC, corresponding to 1.65 g cetylated fatty acids, with 660 mg cetyl myristoleate and cetyl myristate.

9. Conclusions and recommendations

This paper described the developments in the fields of toxicology and risk assessment, which areas are undergoing an extensive shift in the paradigms governing the experimental and evaluation work that is the basis of evaluating the safe use of chemicals and products in general. This shift was well-covered in the 2007 NRC report (NRC, 2007) and can be summarised as a departure from the emphasis on animal model-based evaluations of apical endpoints of toxicity towards an approach that is more focused on mechanisms of toxicity, kinetic knowledge of internal exposure and modelling methods. In line with these developments, the possibilities to implement these new approaches in the field of foods and food ingredients were evaluated, also in the light of the need to evaluate novel foods. A roadmap is described consisting of a stepwise evaluation of the different aspects needed for a safety evaluation. These steps consider e.g. the possible exposure scenarios, kinetics to evaluate the internal exposure, methods to evaluate (target-specific) toxicities, mechanisms of toxicity, *in vitro/in vivo* evaluations, as well as considerations of the benefits vs. the risk of adversity.

On the basis of the – limited number of – cases described above,

it is concluded that the use of the roadmap can be very helpful in concluding on safety issues with regard to the substances described in the cases, while avoiding the classical animal-based methods for such safety evaluations as much as possible. However, in some instances the use of animal models may still be required for addressing particular questions, for example in developmental toxicity.

Similar activities have been carried out with other categories of chemicals, e.g. in the ILSI-HESI Risk-21 project (Pastoor et al., 2014; Simon et al., 2014). A focus on food safety was recently described by Schilter et al. (2014).

However, more work needs to be done to expand the experience with the roadmap and the knowledge about the suitability and applicability of available *in vitro* and *in silico* test systems in the context of food safety assessment. The selected cases appeared to be rather data rich, it will be clear that for many substances further experimental work making use of e.g. *in vitro* systems, kinetic models, etc. will be necessary. The tool described in the roadmap can then be of help in selecting the (battery of) experimental systems needed to come to a conclusion. It is highly recommended to invest in work testing this approach.

On the basis of this, the following recommendations for the stakeholder parties in this area of safety evaluation can be made.

1. Gaps in our knowledge should be addressed. One gap is the applicability of a number of the *in vitro* methods for the field of foods and food ingredients. This applies for example on the use of *in silico* and *in vitro* data for oral absorption, for biotransformation and elimination as well as the applicability of many of the methods described for more complicated mixtures and food matrices.
2. Cooperation with other sectors such as pharma, chemical use, pesticides. In these areas also evaluation schemes based on newer paradigms are under development or highly needed (e.g. Toxcast, Risk21, EUToxRisk).
3. There is a need to extend the practical applicability of the roadmap presented here by taking up more case studies. The formation of a task force of stakeholders and supported by experts in these applications can be a tool.
4. The further development of the Threshold of Toxicological Concern (TTC), with emphasis on internal rather than external exposure should be made applicable to the area of foods and food ingredients.

Acknowledgements

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2016.02.019.

References

Adler, S., Basketter, D., Creton, S., Pelkonen, O., van Benthem, J., Zuang, V., Andersen, K.E., Angers-Loustau, A., Aptula, A., Bal-Price, A., Benfenati, E., Bernauer, U., Bessems, J., Bois, F.Y., Boobis, A., Brandon, E., Bremer, S., Broschard, T., Casati, S., Coecke, S., Corvi, R., Cronin, M., Daston, G., Dekant, W., Felter, S., Grignard, E., Gundert-Remy, U., Heinonen, T., Kimber, I., Kleinjans, J., Komulainen, H., Kreiling, R., Kreysa, J., Leite, S.B., Loizou, G., Maxwell, G., Mazzatorta, P., Munn, S., Pfuhler, S., Phrakonkham, P., Piersma, A., Poth, A., Prieto, P., Repetto, G., Rogiers, V., Schoeters, G., Schwarz, M., Serafimova, R., Tähti, H., Testai, E., van Delft, J., van Loveren, H., Vinken, M., Worth, A., Zaldivar, J.M., 2011. Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Arch. Toxicol. 85 (5), 367–485.

Alépée, N., Bahinski, A., Daneshian, M., De Wever, B., Fritsche, E., Goldberg, A., Hansmann, J., Hartung, T., Haycock, J., Hogberg, H., Hoelting, L., Kelm, J.M., Kadereit, S., McVey, E., Landsiedel, R., Leist, M., Lübberstedt, M., Noor, F., Pellevoisin, C., Petersohn, D., Pfannenbecker, U., Reisinger, K., Ramirez, T., Rothen-Rutishauser, B., Schäfer-Korting, M., Zeilinger, K., Zurich, M.G., 2014. State-of-the-art of 3D cultures (organs-on-a-chip) in safety testing and pathophysiology. ALTEX 31 (4), 441–477.

Allen, J.W., Khetani, S.R., Bhatia, S.N., 2005. *In vitro* zonation and toxicity in a hepatocyte bioreactor. Toxicol. Sci. 84 (1), 110–119.

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29 (3), 730–741.

Asanomi, Y., Yamaguchi, H., Miyazaki, M., Maeda, H., 2011. Enzyme-immobilized microfluidic process reactors. Molecules 16 (7), 6041–6059.

Barlow, D.J., Buriani, A., Ehrman, T., Bosisio, E., Eberini, I., Hylands, P.J., 2012. In silico studies in Chinese herbal medicines research: evaluation of in silico methodologies and phytochemical data sources, and a review of research up to date. J. Ethnopharmacol. 140, 526–534.

Basketter, D.A., Clewell, H., Kimber, I., Rossi, A., Blaauboer, B., Burrier, R., Daneshian, M., Eskes, C., Goldberg, A., Hasiwa, N., Hoffmann, S., Jaworska, J., Knudsen, T.B., Landsiedel, R., Leist, M., Locke, P., Maxwell, G., McKim, J., McVey, E.A., Ouédraogo, G., Patlewicz, G., Pelkonen, O., Roggen, E., Rovida, C., Ruhdel, I., Schwarz, M., Schepky, A., Schoeters, G., Skinner, N., Trentz, K., Turner, M., Vanparys, P., Yager, J., Zurlo, J., Hartung, T., 2012. A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing – t4 report*. ALTEX 29 (1), 3–91.

Baudoin, R., Corlu, A., Griscom, L., Legallais, C., Leclerc, E., 2007. Trends in the development of microfluidic cell biochips for *in vitro* hepatotoxicity. Toxicol. In Vitro 4, 535–544.

Beckers, S., Noor, F., Müller-Vieira, U., Mayer, M., Strigun, A., Heinzle, E., 2010. High throughput, non-invasive and dynamic toxicity screening on adherent cells using respiratory measurements. Toxicol. In Vitro 24 (2), 686–694.

Benfenati, E., Pardoe, S., Martin, T., 2013. Using toxicological evidence from QSAR models in practice. ALTEX 30 (1), 19–40.

Bessems, J.G., Loizou, G., Krishnan, K., Clewell, H.J., Bernasconi, C., Bois, F., Coecke, S., Collnot, E.M., Diembeck, W., Farcal, L.R., Geraets, L., Gundert-Remy, U., Kramer, N., Küsters, G., Leite, S.B., Pelkonen, O.R., Schröder, H., Testai, E., Wilk-Zasadna, I., Zaldívar-Comenges, J.M., 2014. PBTK modelling platforms and parameter estimation tools to enable animal-free risk assessment. Recommendations from a joint EPAA — EURL ECVAM ADME workshop. Regul. Toxicol. Pharmacol. 68 (1), 119–139.

Blaauboer, B.J., Barratt, M.D., Houston, J.B., 1999. The integrated use of alternative methods in toxicological risk evaluation. ECVAM integrated testing strategies task force report 1. Altern. Lab. Anim. 27, 229–237.

Blaauboer, B.J., Forsby, A., Houston, J.B., Beckman, M., Combes, R.D., Dejongh, J., 2000. In: Balls, M., van Zeller, A.-M., Halder, M.E. (Eds.), An Integrated Approach to the Prediction of Systemic Toxicity Using Biokinetic Models and Biological *in vitro* Test Methods. Progress in the Reduction Refinement and Replacement of Animal Experimentation. Elsevier, Amsterdam, pp. 525–536.

Blaauboer, B.J., 2001. Toxicodynamic modelling and the interpretation of *in vitro* toxicity data. Toxicol. Lett. 120, 111–123.

Blaauboer, B.J., 2002. The necessity of biokinetic information in the interpretation of *in vitro* toxicity data. Altern. Lab. Anim. 30 (Suppl. 2), 85–91.

Blaauboer, B.J., 2003. The integration of data on physico-chemical properties, *in vitro*-derived toxicity data and physiologically based kinetic and dynamic as modelling a tool in hazard and risk assessment. A commentary. Toxicol. Lett. 138, 161–171.

Blaauboer, B.J., 2010. Biokinetic modeling and *in vitro-in vivo* extrapolations. J. Toxicol. Environ. Health B Crit. Rev. 13, 242–252.

Blaauboer, B.J., Boekelheide, K., Clewell, H.J., Daneshian, M., Dingemans, M.M., Goldberg, A.M., Heneweer, M., Jaworska, J., Kramer, N.I., Leist, M., Seibert, H., Testai, E., Vandebriel, R.J., Yager, J.D., Zurlo, J., 2012. The use of biomarkers of toxicity for integrating *in vitro* hazard estimates into risk assessment for humans. ALTEX 12 29 (4), 411–425.

Boileau, T.W., Boileau, A.C., Erdman Jr., J.W., 2002. Bioavailability of all-trans and cis-isomers of lycopene. Exp. Biol. Med. (Maywood) 227 (10), 914–919.

Booth, N.L., Kruger, C.L., Hayes, A.W., Clemens, R., 2012. An innovative approach to the safety evaluation of natural products: cranberry (vaccinium macrocarpon Aiton) leaf aqueous extract as case study. Food Chem. Toxicol. 50, 3150–3165.

Bouvier D'Yvoire, M., Prieto, P., Blaauboer, B.J., Bois, F.Y., Boobis, A., Brochot, C., Coecke, S., Freidig, A., Gundert-Remy, U., Hartung, T., Jacobs, M.N., Lavé, T., Leahy, D.E., Lennernäs, H., Loizou, G.D., Meek, B., Pease, C., Rowland, M., Spendiff, M., Yang, J., Zeilmaker, M., 2007. Physiologically-based kinetic

modelling (PBK modelling): meeting the 3Rs agenda. The report and recommendations of ECVAM workshop 63. ATLA Altern. Lab. Anim. 35 (6), 661—671.

Choi, R.S., Riegler, M., Pothoulakis, C., Kim, B.S., Mooney, D., Vacanti, M., Vacanti, J.P., 1998. Studies of brush border enzymes, basement membrane components and electrophysiology of tissue-engineered neointestine. J. Pediatr. Surg. 33 (7), 991—996.

Coecke, S., Ahr, H., Blaauboer, B.J., Bremer, S., Casati, S., Castell, J., Combes, R., Corvi, R., Crespi, C.L., Cunningham, M.L., Elaut, G., Eletti, B., Freidig, A., Gennari, A., Ghersi-Egea, J.F., Guillouzo, A., Hartung, T., Hoet, P., Ingelman-Sundberg, M., Munn, S., Janssens, W., Ladstetter, B., Leahy, D., Long, A., Meneguz, A., Monshouwer, M., Morath, S., Nagelkerke, F., Pelkonen, O., Ponti, J., Prieto, P., Richert, L., Sabbioni, E., Schaack, B., Steiling, W., Testai, E., Vericat, J.A., Worth, A., 2006. Metabolism: a bottleneck in In Vitro toxicological test development. The report and recommendations of ECVAM workshop 54. ATLA Altern. Lab. Anim. 34 (1), 49—84.

Coecke, S., Pelkonen, O., Leite, S.B., Bernauer, U., Bessems, J.G., Bois, F.Y., Gundert-Remy, U., Loizou, G., Testai, E., Zaldívar, J.M., 2012. Toxicokinetics as a key to the integrated toxicity risk assessment based primarily on non-animal approaches. Toxicol. In vitro S0887—S2333.

Constable, A., Jonas, D., Cockburn, A., Davi, A., Edwards, G., Hepburn, P., Herouet-Guicheney, C., Knowles, M., Moseley, B., Oberdörfer, R., Samuels, F., 2007. History of safe use as applied to the safety assessment of novel foods and foods derived from genetically modified organisms. Food Chem. Toxicol. 45 (12), 2513—2525.

Collins, F.S., Gray, G.M., Bucher, J.R., 2008. Toxicology. Transforming environmental health protection. Science 319, 906—907.

Cronin, J.R., 2000. Lycopene: the powerful antioxidant that makes tomatoes red. Altern. Complement. Ther. 6 (2), 92—94.

Daneshian, M., Botana, L.M., Dechraoui Bottein, M.Y., Buckland, G., Campàs, M., Dennison, N., Dickey, R.W., Diogène, J., Fessard, V., Hartung, T., Humpage, A., Leist, M., Molgó, J., Quilliam, M.A., Rovida, C., Suarez-Isla, B.A., Tubaro, A., Wagner, K., Zoller, O., Dietrich, D., 2013. A roadmap for hazard monitoring and risk assessment of marine biotoxins on the basis of chemical and biological test systems. ALTEX 30 (4), 487—545.

DeJongh, J., Nordin-Andersson, M., Ploeger, B.A., Forsby, A., 1999. Estimation of systemic toxicity of acrylamide by integration of in vitro toxicity data with kinetic simulations. Toxicol. Appl. Pharmacol. 158 (3), 261—268.

Dreisig, K., Taxvig, C., Birkhøj Kjærstad, M., Nellemann, C., Hass, U., Vinggaard, A.M., 2013. Predictive value of cell assays for developmental toxicity and embryotoxicity of conazole fungicides. ALTEX 30 (3), 319—330.

EFSA, 2008. Safety of synthetic lycopene. Scientific opinion of the panel on scientific panel on dietetic products, nutrition and Allergies. EFSA J. 676, 1—25.

EFSA, 2010. Scientific opinion on the safety of 'cetyl myristoleate complex' as a food ingredient. EFSA panel on dietetic products, nutrition and Allergies (NDA). EFSA J. 8 (7), 1686.

EFSA, 2013. Statement on the safety of 'cetyl myristoleate complex' as an ingredient in food supplements. EFSA panel on dietetic products, nutrition and Allergies (NDA). EFSA J. 11 (6), 3261.

EFSA, 2014. Statement on the safety of 'cetyl myristoleate complex' as an ingredient in food supplements. EFSA panel on dietetic products, nutrition and Allergies (NDA). EFSA J. 12 (7), 3758.

Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B.J., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J.–C., Pieters, R., Kleiner, J., 2002. Methods of In Vitro toxicology. Food Chem. Toxicol. 40, 193—236.

European Commission, 1997. Recommendation of 29 July 1997 Concerning the Scientific Aspects and the Presentation of Information Necessary to Support Applications for the Placing on the Market of Novel Foods and Novel Food Ingredients, pp. 1—36. L253.

FAO/WHO, 1996. Biotechnology and Food Safety. FAO Food and Nutrition Paper 61.

Ferrell, N., Desai, R.R., Fleischman, A.J., Roy, S., Humes, H.D., Fissell, W.H., 2010. A microfluidic bioreactor with integrated transepithelial electrical resistance (TEER) measurement electrodes for evaluation of renal epithelial cells. Biotechnol. Bioeng. 107 (4), 707—716.

Ferruzza, S., Rossi, C., Sambuy, Y., Scarino, M.L., 2013. Serum-reduced and serum-free media for differentiation of Caco-2 cells. ALTEX 30 (2), 159—168.

Forster, R., Chiba, K., Schaeffer, L., Regalado, S.G., Lai, C.S., Gao, Q., Kiani, S., Farin, H.F., Clevers, H., Cost, G.J., Chan, A., Rebar, E.J., Urnov, F.D., Gregory, P.D., Pachter, L., Jaenisch, R., Hockemeyer, D., 2014. Human intestinal tissue with adult stem cell properties derived from pluripotent stem cells. Stem Cell Rep. 2 (6), 838—852.

Fuchs, J.R., Nasseri, B.A., Vacanti, J.P., 2001. Tissue engineering: a 21st century solution to surgical reconstruction. Ann. Thorac. Surg. 72 (2), 577—591.

Furr, H.C., Clark, R.M., 1997. Intestinal absorption and tissue distribution of carotenoids. J. Nutr. Biochem. 8, 364—377.

Gerlach, J., Kloppel, K., Stoll, P., Vienken, J., Müller, C., 1990. Gas-supply across membranes in bioreactors for hepatocyte culture. Artif. Organs 14 (5), 328—333.

Ghafar-Zadeh, E., Waldeisen, J.R., Lee, L.P., 2011. Engineered approaches to the stem cell microenvironment for cardiac tissue regeneration. Lab. Chip 11 (18), 3031—3048.

Grikscheit, T.C., 2003. Tissue engineering of the gastrointestinal tract for surgical replacement: a nutrition tool of the future? Proc. Nutr. Soc. 62 (3), 739—743.

Groothuis, F.A., Heringa, M.B., Nicol, B., Hermens, J.L.M., Blaauboer, B.J., Kramer, N.I., 2015. Dose metric considerations in in vitro assays to improve quantitative in vitro-in vivo dose extrapolations. Toxicology 332, 30—40.

Hartung, T., Hoffmann, S., 2009. Food for thought... on in silico methods in toxicology. ALTEX 26, 155—166.

Hartung, T., McBride, M., 2011. Food for Thought... on mapping the human toxome. ALTEX 28 (2), 83—93.

Hartung, T., van Vliet, E., Jaworska, J., Bonilla, L., Skinner, N., Thomas, R., 2012. Systems toxicology. ALTEX 29 (2), 119—128.

Hartung, T., Hoffmann, S., Stephens, M., 2013a. Mechanistic validation. ALTEX 30 (2), 119—130.

Hartung, T., Luechtefeld, T., Maertens, A., Kleensang, A., 2013b. Integrated testing strategies for safety assessments. ALTEX 30 (1), 3—18.

Havenaar, R., 2011. Intestinal health functions of colonic microbial metabolites: a review. Benef. Microbes 2 (2), 103—114.

Health Canada, 1994. Guidelines for the Safety Assessment of Novel Foods.

Heijne, W.H., Kienhuis, A.S., van Ommen, B., Stierum, R.H., Groten, J.P., 2005. Systems toxicology: applications of toxicogenomics, transcriptomics, proteomics and metabolomics in toxicology. Expert Rev. Proteom. 2 (5), 767—780.

Hoppe, P.P., Krämer, K., van den Berg, H., Steenge, G., van Vliet, T., 2003. Synthetic and tomato-based lycopene have identical bioavailability in humans. Eur. J. Nutr. 42 (5), 272—278.

Hori, Y., Nakamura, T., Matsumoto, K., Kurokawa, Y., Satomi, S., Shimizu, Y., 2001. Experimental study on in situ tissue engineering of the stomach by an acellular collagen sponge scaffold graft. ASAIO J. 47 (3), 206—210.

Howlett, J., Edwards, D.G., Cockburn, A., Hepburn, P., Kleiner, J., Knorr, D., Kozianowski, G., Müller, D., Peijenburg, A., Perrin, I., Poulsen, M., Walker, R., 2003. The safety assessment of novel foods and concepts to determine their safety in use. Int. J. Food Sci. Nutr. 54, S1—S32.

Hutapea, A.M., Toskulkao, C., Buddhasukh, D., Wilairat, P., Glinsukon, T., 1997. Digestion of stevioside, a natural sweetener, by various digestive enzymes. J. Clin. Biochem. Nutr. 23 (3), 177—186.

Jang, K.J., Suh, K.Y., 2010. A multi-layer microfluidic device for efficient culture and analysis of renal tubular cells. Lab. Chip 10 (1), 36—42.

JECFA, 2006. Evaluation of Certain Food Additives and Contaminants: Sixty-seventh Report of the Joint FAO/WHO Expert Committee on Food Additives. WHO technical report series; no. 940.

Judson, R., Kavlock, R., Martin, M., Reif, D., Houck, K., Knudsen, T., Richard, A., Tice, R.R., Whelan, M., Xia, M., Huang, R., Austin, C., Daston, G., Hartung, T., Fowle 3rd, J.R., Wooge, W., Tong, W., Dix, D., 2013. Perspectives on validation of high-throughput assays supporting 21st century toxicity testing. ALTEX 30 (1), 51—56.

Kaluzhny, Y., Kandarova, H., Hayden, P., Kubilus, J., d'Argembeau-Thornton, L., Klausner, M., 2011. Development of the EpiOcular(TM) eye irritation test for hazard identification and labelling of eye irritating chemicals in response to the requirements of the EU cosmetics directive and REACH legislation. Altern. Lab. Anim. 39, 339—364.

Kandarova, H., Hayden, P., Klausner, M., Kubilus, J., Sheasgreen, J., 2009. An in vitro skin irritation test (SIT) using the EpiDerm reconstructed human epidermis (RHE) model. J. Vis. Exp. 29, 1366.

Khetani, S.R., Bhatia, S.N., 2008. Microscale culture of human liver cells for drug development. Nat. Biotechnol. 26 (1), 120—126.

Kramer, N.I., Krismartina, M., Rico-Rico, A., Blaauboer, B.J., Hermens, J.L.M., 2012. Quantifying processes determining the free concentration of phenanthrene in basal cytotoxicity assays. Chem. Res. Toxicol. 25 (2), 436—445.

Kramer, N.I., De Consiglio, E., Blaauboer, B.J., Testai, E., 2015. Biokinetics in repeated-dosing in vitro drug toxicity studies. Toxicol. In Vitro 30, 217—224.

Leist, M., Hartung, T., 2013. Inflammatory findings on species extrapolations: humans are definitely no 70-kg mice. Arch. Toxicol. 87 (4), 563—567.

Leist, M., Lidbury, B.A., Yang, C., Hayden, P.J., Kelm, J.M., Ringeissen, S., Detroyer, A., Meunier, J.R., Rathman, J.F., Jackson Jr., G.R., Stolper, G., Hasiwa, N., 2012. Novel technologies and an overall strategy to allow hazard assessment and risk prediction of chemicals, cosmetics, and drugs with animal-free methods. ALTEX 29 (4), 373—388.

Lioy, P.J., 2010. Exposure science: a view of the past and milestones for the future. Environ. Health Perspect. 118, 1081—1090.

Louisse, J., De Jong, E., van de Sandt, H., Blaauboer, B.J., Woutersen, R.A., Piersma, A.H., Rietjens, I.M.C.M., Verwei, M., 2010. The use of in vitro toxicity data and physiologically based kinetic modeling to predict dose-response curves for in vivo developmental toxicity of glycol ethers in rat and man. Toxicol. Sci. 118, 470—484.

Mazzei, D., Guzzardi, M.A., Giusti, S., Ahluwalia, A., 2010. A low shear stress modular bioreactor for connected cell culture under high flow rates. Biotechnol. Bioeng. 106 (1), 127—137.

NRC, 2007. Committee on Toxicity Testing and Assessment of Environmental Agents, National Research Council. Toxicity Testing in the 21st Century: a Vision and a Strategy. The national academies press. http://www.nap.edu/catalog.php?record_id=11970.

OECD, 1993. Safety Evaluation of Foods Derived by Modern Biotechnology: Concepts and Principles.

Ouedraogo, M., Baudoux, T., Stévigny, C., Nortier, J., Colet, J.M., Efferth, T., Qu, F., Zhou, J., Chan, K., Shaw, D., Pelkonen, O., Duez, P., 2012. Review of current and 'omics' methods for assessing the toxicity (genotoxicity, teratogenicity and nephrotoxicity) of herbal medicines and mushrooms. J. Etnopharmacol. 140, 492—512.

Palafox-Carlos, H., Ayala-Zavala, J.F., González-Aguilar, G.A., 2011. The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. J. Food Sci. 76 (1), R6—R15.

Paparella, M., Daneshian, M., Hornek-Gausterer, R., Kinzl, M., Mauritz, I., Mühlegger, S., 2013. Uncertainty of testing methods—what do we (want to)

know? ALTEX 30 (2), 131–144.

Pastoor, T.P., Bachman, A.N., Bell, D.R., Cohen, S.M., Dellarco, M., Dewhurst, I.C., Boobis, A.R., 2014. A 21st century roadmap for human health risk assessment. Crit. Rev. Toxicol. 44 (Suppl. 3), 1–5.

Pelkonen, O., Pasanen, M., Lindon, J.C., Chan, K., Zhao, L., Deal, G., Xu, Q., Fan, T.P., 2012. Omics and its potential impact on R&D and regulation of complex herbal products. J. Ethnopharmacol. 140, 587–593.

Polak, S., 2013. In vitro to human in vivo translation - pharmacokinetics and pharmacodynamics of quinidine. ALTEX 30 (3), 309–318.

Powers, M.J., Janigian, D.M., Wack, K.E., Baker, C.S., Beer Stolz, D., Griffith, L.G., 2002. Functional behavior of primary rat liver cells in a three-dimensional perfused microarray bioreactor. Tissue Eng. 8, 499–513.

Ramadan, Q., Jafarpoorchekab, H., Huang, C., Silacci, P., Carrara, S., Koklü, G., Ghaye, J., Ramsden, J., Ruffert, C., Vergeres, G., Gijs, M.A., 2013. NutriChip: nutrition analysis meets microfluidics. Lab. Chip 13 (2), 196–203.

Ramirez, T., Daneshian, M., Kamp, H., Bois, F.Y., Clench, M.R., Coen, M., Donley, B., Fischer, S.M., Ekman, D.R., Fabian, E., Guillou, C., Heuer, J., Hogberg, H.T., Jungnickel, H., Keun, H.C., Krennrich, G., Krupp, E., Luch, A., Noor, F., Peter, E., Riefke, B., Seymour, M., Skinner, N., Smirnova, L., Verheij, E., Wagner, S., Hartung, T., van Ravenzwaay, B., Leist, M., 2013. Metabolomics in toxicology and preclinical research. ALTEX 30 (2), 209–225.

Rappaport, S.M., 2011. Implications of the exposome for exposure science. J. Expo. Sci. Environ. Epidemiol. 21 (1), 5–9.

Raunio, H., 2011. In silico toxicology – non-testing methods. Front. Pharmacol. 2, 33.

Rolletscheck, A., Blyszczuk, P., Wobus, A.M., 2005. Embryonic stem cell derived cardiac neuronal and pancreatic cells as model systems to study toxicological effects. Physiol. Rev. 85, 635–667.

Rossini, G.P., Hartung, T., 2012. Towards tailored assays for cell-based approaches to toxicity testing. ALTEX 29 (4), 359–372.

Sato, M., Ando, N., Ozawa, S., Miki, H., Hayashi, K., Kitajima, M., 1997. Artificial esophagus. Mater Sci. Forum 250, 105–114.

Schierle, J., Bretzel, W., Bühler, I., Faccin, N., Hess, D., Steiner, K., Schüep, W., 1997. Content and isomeric ratio of lycopene in food and human blood plasma. Food Chem. 59 (3), 459–465.

Schiffelers, M.J., Blaauboer, B.J., Hendriksen, C.F., Bakker, W.E., 2012. Regulatory acceptance and use of 3R models: a multilevel perspective. ALTEX 29 (3), 287–300.

Schilter, B., Benigni, R., Boobis, A., Chiodini, A., Cockburn, A., Cronin, M.T., Lo Piparo, E., Modi, S., Thiel, A., Worth, A., 2014. Establishing the level of safety concern for chemicals in food without the need for toxicity testing. Regul. Toxicol. Pharmacol. 68 (2), 275–296.

Scholtz, G., Pohl, I., Genschow, E., Klemm, M., Spielmann, H., 1999. Embryotoxicity screening using embryonic stem cells in vitro: correlations with in vivo teratogenicity. Cells Tissues Org. 165, 203–211.

Shin, M., Matsuda, K., Ishii, O., Terai, H., Kaazempur-Mofrad, M., Borenstein, J., Detmar, M., Vacanti, J.P., 2004. Endothelialized networks with a vascular geometry in microfabricated poly(dimethyl siloxane). Biomed. Microdevices 6 (4), 269–278.

Simon, T.W., Simons Jr., S.S., Preston, R.J., Boobis, A.R., Cohen, S.M., Doerrer, N.G., Fenner-Crisp, P.A., McMullin, T.S., McQueen, C.A., Rowlands, J.C., 2014. The use of mode of action information in risk assessment: quantitative key events/dose-response framework for modeling the dose-response for key events. Crit. Rev. Toxicol. 44 (Suppl. 3), 17–43.

Smith, M., 2002. Food safety in Europe (FOSIE): risk assessment of chemicals in food and diet: overall introduction. Food Chem. Toxicol. 40 (2–3), 141–144.

Song, J.W., Gu, W., Futai, N., Warner, K.A., Nor, J.E., Takayama, S., 2005. Computer-controlled microcirculatory support system for endothelial cell culture and shearing. Anal. Chem. 77 (13), 3993–3999.

Srigunapalan, S., Lam, C., Wheeler, A.R., Simmons, C.A., 2011. A microfluidic membrane device to mimic critical vascular components of the vascular microenvironment. Biomicrofluidics 5 (1), 13409.

Stefanini, F.M., 2013. Bayesian network integrated testing strategy and beyond. ALTEX 30 (3), 386–390.

Sung, J.H., Yu, J., Luo, D., Shuler, M.L., March, J.C., 2011. Microscale 3-D hydrogel scaffold for biomimetic gastrointestinal (GI) tract model. Lab. Chip 11 (3), 389–392.

Suter-Dick, L., Alves, P.M., Blaauboer, B.J., Bremm, K., Brito, C., Coecke, S., Flick, B., Fowler, P., Hescheler, J., Ingelman-Sundberg, M., Jennings, P., Kelm, J.M., Manou, I., Mistry, P., Moretto, A., Roth, A., Stedman, D., Van De Water, B., Beilmann, M., 2015. Stem cell-derived systems in toxicology assessment. Stem Cells Dev. 24 (11), 1284–1296.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872.

Thomson, J., Itskovitz-Eldor, J., Shapiro, S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147.

US Food and Drug Administration (US FDA), 1992. Foods derived from new plant selections. Fed. Regist. 57 (104), 22984.

Valerio Jr., L.G., 2009. In silico toxicology for the pharmaceutical sciences. Toxicol. Appl. Pharmacol. 241, 356–370.

Valerio Jr., L.G., 2011. In silico toxicology models and databases as FDA Critical Path Initiative toolkits. Hum. Genom. 5, 200–207.

van den Berg, H., 1999. Carotenoid interactions. Nutr. Rev. 57 (1), 1–10.

van der Meer, A.D., Poot, A.A., Duits, M.H., Feijen, J., Vermes, I., 2009. Microfluidic technology in vascular research. J. Biomed. Biotechnol. 823148.

van der Meer, A.D., Vermeul, K., Poot, A.A., Feijen, J., Vermes, I., 2010. Flow cytometric analysis of the uptake of low-density lipoprotein by endothelial cells in microfluidic channels. Cytom. A 77 (10), 971–975.

van Midwoud, P.M., Verpoorte, E., Groothuis, G.M., 2011. Microfluidic devices for in vitro studies on liver drug metabolism and toxicity. Integr. Biol. (Camb) 3 (5), 509–521.

van Thriel, C., Westerink, R.H., Beste, C., Bale, A.S., Lein, P.J., Leist, M., 2012. Translating neurobehavioural endpoints of developmental neurotoxicity tests into in vitro assays and readouts. Neurotoxicology 33, 911–924.

Verwei, M., van Burgsteden, J.A., Krul, C.A., van de Sandt, J.J., Freidig, A.P., 2006. Prediction of in vivo embryotoxic effect levels with a combination of in vitro studies and PBPK modelling. Toxicol. Lett. 165 (1), 79–87.

Wang, J., Ren, L., Li, L., Liu, W., Zhou, J., Yu, W., Tong, D., Chen, S., 2009. Microfluidics: a new cosset for neurobiology. Lab. Chip 9 (5), 644–652.

Wang, S., Houtman, R., Melchers, D., Aarts, J., Peijnenburg, A., van Beuningen, R., Rietjens, I., Bovee, T.F., 2013. A 155-plex high-throughput in vitro coregulator binding assay for (anti-) estrogenicity testing evaluated with 23 reference compounds. ALTEX 30 (2), 145–157.

Wang, Y., Lo, J.F., Mendoza-Elias, J.E., Adewola, A.F., Harvat, T.A., Kinzer, K.P., Lee, D., Qi, M., Eddington, D.T., Oberholzer, J., 2010. Application of microfluidic technology to pancreatic islet research: first decade of endeavor. Bioanalysis 2 (10), 1729–1744.

Wilmes, A., Limonciel, A., Aschauer, L., Moenks, K., Bielow, C., Leonard, M.O., Hamon, J., Carpi, D., Ruzek, S., Handler, A., Schmal, O., Herrgen, K., Bellwon, P., Burek, C., Truisi, G.L., Hewitt, P., Di Consiglio, E., Testai, E., Blaauboer, B.J., Guillou, C., Huber, C.G., Lukas, A., Pfaller, W., Mueller, S.O., Bois, F.Y., Dekant, W., Jennings, P., 2013. Application of integrated transcriptomic, proteomic and metabolomic profiling for the delineation of mechanisms of drug induced cell stress. J. Proteom. 79, 180–194.

Yoon, M., Campbell, J.L., Andersen, M.E., Clewell, H.J., 2012. Quantitative in vitro to in vivo extrapolation of cell-based toxicity assay results. Crit. Rev. Toxicol. 42 (8), 633–652.

Yoon, M., Efremenko, A., Blaauboer, B.J., Clewell, H.J., 2014. Evaluation of simple in vitro to in vivo extrapolation approaches for environmental compounds. Toxicol. In Vitro 28 (2), 164–170.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., Slukvin II, Thomson, J.A., 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917–1920.

Zeilinger, K., Sauer, I.M., Pless, G., Strobel, C., Rudzitis, J., Wang, A., Nüssler, A.K., Grebe, A., Mao, L., Auth, S.H., Unger, J., Neuhaus, P., Gerlach, J.C., 2002. Three-dimensional coculture of primary human liver cells in bioreactors for in vitro drug studies: effects of the initial cell quality on the long-term maintenance of hepatocyte-specific functions. Altern. Lab. Anim. ATLA 30 (5), 525–538.

Zeilinger, K., Holland, G., Sauer, I.M., Efimova, E., Kardassis, D., Obermayer, N., Liu, M., Neuhaus, P., Gerlach, J.C., 2004. Time course of primary liver cell reorganization in three-dimensional high-density bioreactors for extracorporeal liver support: an immunohistochemical and ultrastructural study. Tissue Eng. 10 (7–8), 1113–1124.