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Improving infant food safety by avoiding hazards of chemical mixture effects using novel integrated methods based on bioassays and analytical chemistry

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ABSTRACT

Humans, including infants are exposed to complex mixtures of anthropogenic chemicals, and food is a major exposure route. Current risk assessment, however, typically does not evaluate mixture toxicity but rather focuses on single chemical exposure scenarios. Nevertheless, there is ample evidence that combined exposures to chemicals is involved in the etiology of major human diseases, and that infants are often more vulnerable than adults. Surprisingly hardly any efficient practical tools and guidelines have been defined to adequately assess mixture effects of food. Evaluation of levels of mixtures of dioxins and related compounds are a notable exception, although also in that area novel insights warrant reevaluation of the relevant compounds to be included in evaluation. Novel approaches are needed, since our knowledge on the toxicity of chemicals is lagging behind and even most of the industrial chemicals that are in common use have undergone no or limited safety testing, while the situation with natural compounds in food is even more challenging. Novel untargeted chemical analytical techniques and quantitative bioanalytical techniques that respond to toxic chemicals independent of prior knowledge on their structure or toxicity can be used to increase the knowledge on chemical mixtures. We discuss the complementarities between these bio- and chemical analytical methods that can be used in an integrated system to improve infant food safety by avoiding hazards of chemical mixture effects.

Introduction

Humans, including infants are exposed to complex mixtures of anthropogenic chemicals, never one at a time. Current risk assessment, however, typically focuses on single chemical exposure scenarios. Exposure to chemical mixtures and their combined effects require better risk assessment and management procedures to protect public health and the environment [1].

In the past, human adverse health issues have been reduced successfully through reduction of exposure to single highly toxic chemicals that posed significant health risk such as ubiquitously used persistent pesticides like DDT, and other persistent organic pollutants (POPs). Today, the focus is shifting to the less obvious effects of pollutants either alone or in mixtures and their influence on more chronic types of toxicities leading to e.g. cancer, disruption of the endocrine system, developmental toxicity, immune- and neurodevelopmental disorders.

Abbreviations: AhR, Aryl hydrocarbon receptor; AOP, Adverse outcome pathway; CALUX, Chemically activated luciferase expression; CP, Chlorinated paraffins; DDT, Dichloordifenyltrichloorethaan; ECHA, European Chemical Agency; ECVAM, European Centre for the Validation of Alternative Methods; EDA, Effect directed analyisis; EDC, Endocrine disrupting compound; EFSA, European Food Safety Authority; EPA, Environmental Protection Agency; FAO, Food and Agriculture Organization (WHO); HRMS, High resolution magnetic sector mass spectrometers; MIE, Molecular initiating event; OECD, Organisation for Economic Cooperation and Development; PBDD, Polybrominated dibenzodioxin; PBDF, Polybrominated dibenzofuran; PCDD, Polychlorinated dibenzodioxin; PCDF, Polychlorinated dibenzofuran; PCB, Polychlorinated biphenyl; PCN, Polychlorinated naphthalene; POP, Persistent organic pollutants; REACH, Registration Evaluation and Authorization of Chemicals; SOP, Standard operating procedure; SVHC, Substance of Very High Concern; TDS, Total diet study; TEF, Toxic equivalency factor; TEQ, Toxic equivalents; TRV, Toxicological reference value; TWI, Tolerable weekly intake; WHO, World Health Organization.

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These relationships are much more difficult to establish since there is no directly visible causal relationship between effect and exposure. Also, exposure in food typically is to mixtures of chemicals, making this analysis even more complicated. Nevertheless, there is ample evidence that combined exposures to chemicals is involved in the etiology of major human diseases.[1–4] Therefore, nowadays, consideration of mixture effects is mentioned in several relevant regulations but surprisingly hardly any efficient practical tools and guidelines have been defined in those regulations to adequately assess mixture effects.[1,5,6]

Food is a major exposure route to chemicals in humans. Food is a complex mixture of chemicals of both natural and synthetic origin.[7] The natural ingredients include nutrients, vitamins, but also natural contaminants and potential toxic natural compounds. For all these compounds toxicity becomes relevant when dosed at a sufficiently high level. Even if individual compounds do not pass a threshold of toxicity, combined doses of compounds that have similar toxicity can lead to adversities.[3,8] Therefore, these combined dose effects need to be assessed. Exposure of infants through food, starting at the earliest age through milk or infant formula is highly relevant because the children's metabolic defenses still need maturation and disturbance of developmental processes can lead to serious health effects.[9] However, our understanding of the effects of early life exposure is limited.[8, 10,11] Efficient methods to measure hazards of chemical mixtures in infant food are still in its infancy and not regularly used. Here we describe novel developments in this important area, and we give examples of advanced methods that have been developed for mixtures of specific compound groups, in particular dioxins and dioxin-like compounds, and the possibilities for their integrated use to comprehensively secure infant food safety.

Presence of chemical mixtures in infant food

Safety assessment of chemicals, such as industrial chemicals and pesticides traditionally focuses on single chemicals only and not to safety issues of chemical mixtures that may occur in relevant exposure scenario's.[6] Food, also infant food, typically is an exposure scenario to highly complex mixtures of chemicals, at concentrations that generally will cause no harm. The basis to make this assumption, however, contains weaknesses. In fact, our knowledge of the extremely complex chemical universe is very limited. Hundreds of thousands of anthropogenic chemicals exist, including their by-products, metabolites and abiotically formed transformation products[1]. Only a very small fraction of these chemicals has undergone safety testing.[6] Even most of the approximately 100,000 industrial chemicals that are in common use have undergone no or limited safety testing only. This situation is improving due to the REACH legislation, but still the vast majority of chemicals that we are exposed to will remain untested or even unknown. This will include industrial chemicals and food processing-derived contaminants, their metabolites and natural chemicals. These natural chemicals include some of the most toxic classes of chemicals that are known, like toxins produced by plants, fungi, and bacteria,[12, 13] but also ones identical to synthetic chemicals like a range of organohalogens.[14] Recently, it has been recognized that unexpected food contaminants, both with known and unknown toxicity and often related to use of contaminated starting products is an issue of concern.[15] Several of these contaminants may be picked up during routine screening in advanced quality control systems, but others may escape notice. Therefore, methods are being developed for non-targeted analysis to assess the presence of unsuspected and unknown contaminants and possible mixture effects.

The impact of early life exposure to toxic chemical mixtures

It has been estimated that approximately 3% of all developmental defects are attributable to exposure to toxic chemicals and physical agents, including environmental factors, and that 25% of all developmental defects may be due to a combination of genetic and environmental factors.[9] This percentage includes all structural or functional abnormalities at birth. This estimate lacks consideration of effects on disease outcomes that are manifest later in life, and also does not cover mixture effects of chemicals. In fact, knowledge in this area is mainly based on animal experiments and human exposure to relatively high dosages of single chemicals, including certain drugs. Little is known about mixture effects leading to either functional anomalies or impact on incidence of disease later in life. However, particularly endocrine systems may be deregulated through developmental exposure to chemical mixtures with consequences on the incidence of disease.[3] These so-called endocrine disrupting compounds (EDCs) almost exclusively are low molecular weight molecules that readily can enter the body and bind to nuclear receptors in cells, thereby disturbing their normal functioning. Main hormonal systems involved are those for the sex steroids and thyroid hormone, but a similar interaction also occurs with the receptor to which dioxins bind, the aryl hydrocarbon receptor (AhR). There still are many gaps in our understanding of this emerging area of research. Since food is a main route of exposure,[8] either via the mother or after birth directly to the developing child, this is an important area to explore using novel approaches to assure optimal infant food safety.

Mechanistic basis of adverse effects related to early life exposure to chemical mixtures

Normal cellular physiology is governed by a range of signaling pathways that secure proper cell growth and differentiation. Disruption of those pathways can lead to diseases, such as cancer and developmental disorders.[9,15] Certain chemicals can interfere with these pathways, often through binding to molecules that are the starting points of the pathway, the so-called molecular initiating events (MIEs). When this occurs sufficiently strong, the pathway can be activated. This not necessarily leads to a toxic, adverse effect, but when stimulation becomes too strong adversity can be a result. Therefore, these physiological pathways are also referred to as adverse outcome pathways (AOPs).[16] Similarly, when different chemicals affect the same pathway, the effect may add up to pass this threshold, leading to adversity. This has for instance been shown to occur with chemicals interacting with sex steroid receptors, leading to combined endocrine system disrupting effects,[3,5] and dioxins.[17,18] Typically, disruption of basic cellular and hormonal pathways can lead to a range of structural and functional defects at birth and disorders later in life. This is because the magnitude and nature of these disorders is dependent on the dosing, but also the timing of exposure, thereby affecting different processes in which the pathway is involved. For example, dioxin's toxic effects are mediated through a single receptor-mediated pathway. Nevertheless, a wide spectrum of structural and functional defects is related to developmental dioxin exposure including cleft palate, hydronephrosis, altered thyroid and immune status, altered neurobehavior at the level of hearing, psychomotor function, and gender-related behaviors, altered cognition, dentition, and development of reproductive organs, and delays in breast development, in addition to altered sex ratios among the exposed offspring.[17,18] The knowledge on these common mechanisms of toxicity has greatly expanded in recent decades, which forms the basis of novel methods to analyze toxicity of mixtures using mechanism-based bioassays.

Mechanism-based bioassays to assess mixture effects of food-derived chemicals

Based on the knowledge on the mode of action of toxicants, modern mechanism (or effect)-based bioassays have been generated. One early well-known example of a mechanism-based bioassay that is used and accepted very frequently is the Ames mutagenesis assay that assesses chemically-induced mutations in bacterial DNA.[19] Using the knowledge

of the mechanisms of toxicity of chemicals modern mechanism-based bioassays have been developed covering a wide range of key mechanisms using human cells.[20] This includes assays with a higher predictive value for human genotoxicity than the Ames test.[21] These and other mechanism-based bioassays can be used as alternative methods to assess safety of chemicals and chemical mixtures. More recently, the throughput of analysis has been greatly enhanced using robotics.[22,23] The assays are highly specific and measure interference with distinct toxicity pathways through the CALUX reporter gene technology (Fig. 1).[20] In CALUX assays this interaction with key cellular pathways is made easily measurable through incorporation in a recipient cell line of a so-called reporter gene construct which measures activation of the relevant transcriptional pathway. Activation of that pathway is coupled to expression of the firefly luciferase gene, which leads to an easily measurable product in the mammalian cells (Fig. 1). Many of the assays measure interference with a specific type of nuclear hormone receptors that are frequently targets of pollutants,[20,24] while others focus on assessing influences of chemicals on pathways involved in basic cellular signaling which are for instance relevant for acute toxicity and carcinogenesis.[21]

This panel of mechanism-based assays can be linked to adversities that are important for risk assessment, as established in experimental animals and humans via adverse outcome pathways.[16] The assays have been extensively validated and shown to be predictive of effects in animal studies, as used in current chemical safety legislation. The specificity and sensitivity of the assays is particularly meant to facilitate measurements and interpretation of the results in complex mixtures present in food, feed, water, and a wide range of different environmental- and clinical samples.[20,25,26] Results are quantitative and expressed in toxic equivalents (TEQ) relative to a reference standard of a relevant pathway-activating chemical. Various of the CALUX assays have been used intensively and successfully, initially often for non-regulatory purposes, followed by incorporation in relevant national- and international guidelines.[27-32] This includes the DR CALUX assay that measures activation of the relevant target molecule, the dioxin receptor (AhR). By coupling to a specific workup method, a selection for the most relevant stable ligands is made.

Chemical analytical-based mixture effect assessment as used for dioxin mixtures

The advantage of targeted approaches is that through establishment of the chemical identities, source identification and risk reduction measures are facilitated. In bioanalysis, the contribution of the different chemicals to the TEQ value of chemical mixtures is integrated, while the relative contribution of different chemicals cannot readily be assessed. In chemical analytics the reverse is true since chemical analytics targets exact quantification of single chemicals. However, there are possibilities to estimate mixture effects using chemical analysis, of which the system to analyze dioxins is among the most advanced. Because of their toxicity at extremely low dosages, dioxins are of great concern. Since dioxins are present at significant levels in food, including breast milk and infant formula, measures have been put in place in Europe to reduce intake through this major route of exposure. The approach taken is unique in that the chemical analysis of a range of major congeners is used to estimate their combined biological effect. To do this, the concentration of individual congeners is multiplied by a corresponding toxic equivalency factor (TEF) which expresses its toxicity relative to the most toxic form of dioxins, 2,3,7,8-TCDD. In this way a TEQ value is derived for the respective congener, and by adding the values of the congeners used in this system, the expected sumTEQ value of the mixture is estimated, which, if all relevant congeners are included, would be equivalent to the TEQ measured with a relevant bioassay, like DR CALUX (Fig. 2). Nowadays a range of stable chlorinated dioxins, furans and PCBs are included in this chemical analytical estimating of total dioxin toxicity of a mixture. For mixtures, this approach can be taken if all relevant toxic compounds are known affecting one biological pathway, in this case activation of the dioxin receptor. Since dioxins are among the most studied toxic chemicals with many data on toxicity of individual congeners, this gives confidence that this estimation was correct. However,

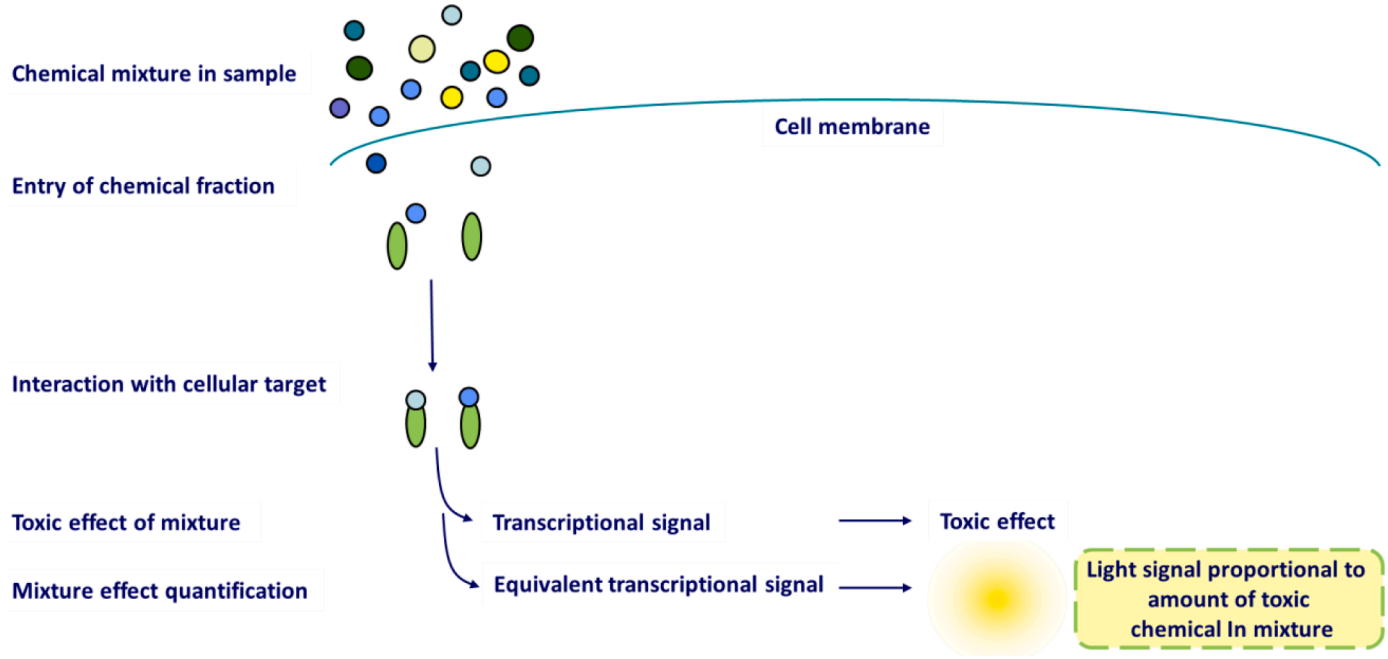

Fig. 1. General principle of a CALUX assay. Exposure of cells to chemicals will lead to a change in gene expression and a consequential change in cellular behavior that is instrumental in the toxic effect of the chemical(s). This response is mediated through a transcriptional response that drives expression of endogenous genes, and as a result the toxic effect. In a CALUX® reporter gene assay this response is modulated in such a way that activation of a signaling pathway is linked to transcription of a stably introduced luciferase gene. Upon addition of a substrate a light signal is generated which is proportional to the amount of bioactive chemical in a sample.

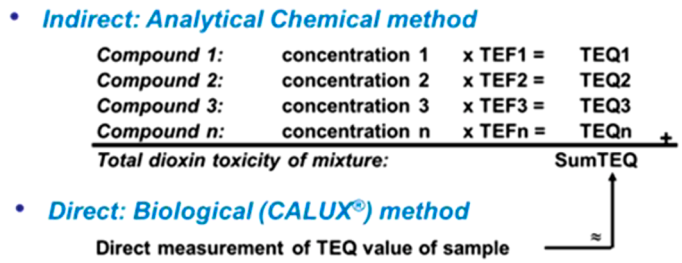

Fig. 2. Determination of the toxicity of mixtures of dioxin-like chemicals. Analytical chemical methods determine compounds of known concern and add up the product of individual concentrations and a relevant toxicity factor, the TEF value. In this way an estimate of the expected biological activity, expressed as sumTEQ, is made. Biological methods, like CALUX measure the SumTEQ through an interaction with the dioxin receptor, and do not rely on prior knowledge on toxicity of individual chemicals.

new data suggest that the current coverage of relevant compounds may not be sufficient (see next section).

Uncertainties in current dioxin-related chemical mixture effect assessment

To reduce uncertainty in the food chain monitoring using chemical analytics one of the major challenges is the choice of chemicals to be monitored while toxicity data on most chemicals are not available.[6] This even applies to one of the most advanced systems of estimating dioxin-related chemical toxicity as present in food. The current system focuses on chemically very stable chlorinated dioxins, furans and PCBs that all can activate the dioxin receptor and are known to cause adverse effects in experimental animals and humans, when dosed at sufficiently high levels. In the light of the missing knowledge on the toxicity of most chemicals it would be surprising that all relevant dioxin receptor-interacting molecules would be known already. Indeed, recent research suggests that there are important omissions in the routinely measured panel of dioxin receptor interacting compounds. As such, various halogenated compounds groups have been identified that pose possible risks that are mediated through dioxin receptor activation, including chlorinated paraffins, polychlorinated naphthalenes, and brominated dioxins and furans.

Chlorinated paraffins (CPs), are complex mixtures of hundreds of isomeric groups with varying linear carbon chain length and chlorine number, themselves comprising hundreds of isomers[28,33]. A fraction of the synthesized volumes of CPs is unintentionally released into the environment during the production, use or destruction of products containing them. Due to their lipophilic properties and stability, CPs enter the human food chain through processes like those described for similar halogenated substances like dioxins, furans and PCBs. They can also be accumulated and redistributed from reservoirs related to industrial processing and food preparation, such as in kitchen ovens. In this context, assessing the risk associated with these contaminants in relation to human exposure is a pressing need. Recently, the relevant European Food Safety Agency (EFSA) panel of experts established lowest adverse effect levels for several of these CPs,[33] while some have been classified as persistent organic pollutants (POPs), under the Stockholm Convention (Persistent Organic Pollutants Review Committee 2017) and have been placed on the Candidate List of Substances of Very High Concern (SVHC) under the REACH Regulation. However, the clear lack of toxicological and exposure data previously highlighted limits in the risk assessment associated with dietary exposure to CPs. A key factor explaining the lack of data relates to the challenge of analyzing relevant CP classes that remain despite the recent advances.[34]

Polychlorinated naphthalenes (PCNs) are legacy contaminants gathering 75 congeners. They have been listed by the Stockholm convention, initially for reduction of inadvertent production and ultimately, for elimination. They originate through releases from older electrical equipment, inadvertent contamination in industrial chemicals and from combustion processes such as incineration. Recent advances in measurement techniques have allowed a greater characterization of PCN occurrence, yielding more specific data including individual PCN congener concentrations. Emerging data on food shows widespread occurrence in most commonly consumed foods from different parts of the world. Concurrently, toxicological studies have also allowed a greater insight into the potencies of some congeners, a number of which are known to elicit potent, aryl hydrocarbon receptor (AhR) mediated responses, referred to as dioxin-like toxicity. The dietary pathway is widely recognized as the most likely route to non-occupational human exposure. Overall, the data that are currently available on PCN occurrence in foods suggest a widespread current distribution of these contaminants in foods and food webs. This is remarkable given the time that has elapsed since the unrestricted use of these compounds, and those other commercial chemicals such as PCBs which are known sources of PCNs, ceased (particularly in Western Europe and North America), and underlines the persistence and ubiquity of PCNs. Although the reported contribution is smaller than PCDD/Fs and PCBs, PCN toxicity is likely to add to the cumulative toxicity of other dioxin-like compounds.[35]

Polybrominated dioxins and furans (PBDD/F) are brominated counterparts of the traditionally measured chlorinated compounds. It, however, has been found that polybrominated dioxins and furans have comparable affinity to the human dioxin receptor.[36,37] When using the DR-CALUX bioassay both chlorinated and brominated congeners will contribute to biological activity in a way relevant for human toxicity. Chlorinated dioxins are going down in the diet, but brominated ones are increasing, often as breakdown products of flame retardants. They are found at high levels in children's toys that are made of recycled plastics,[38] and are also entering the food chain.[37] Reported PBDD/F dietary intakes suggest that some population groups, particularly young children, may exceed the revised tolerable weekly intake for dioxin-like contaminants, even for mean consumption estimated with lower bound data. It is evident that the omission of PBDD/Fs from the TEQ scheme results is a significant underestimation of the cumulative toxicity and associated risk arising from this mode of action.[37,38]

Although for several of these novel relevant AhR ligands mass spectrometer (MS)-based analytical methods have been developed already, they have not been incorporated in the international TEF/TEQ-based methodology to assess total dioxin receptor-mediated toxicological burden.[39] Also, very likely novel relevant AhR interacting compounds will be identified in the future, and therefore this methodology will need to be updated regularly. Because of the advances in biological and chemical analytical methods novel, more comprehensive, integrated methods become feasible.

Novel approaches in infant food safety assessment

Food safety assessment is challenging not only due to the lack of knowledge on toxicity of many chemicals, but also because of changes in raw materials, processing, packaging and storage methods and consumer practices, and thus the need to efficiently monitor at critical control points. To do this, methods need to be reviewed and updated using the latest scientific insights and technological developments. The complexity of food and feed samples, together with the low concentrations at which contaminants occur (ppb (ng.g-1) to ppt (pg.g-1)), requires highly sensitive, selective and robust analytical techniques. These requirements need to be reviewed and upgraded, when needed. At the end of 2018, the EFSA CONTAM expert group carried out a re-evaluation of the Toxicological Reference Value (TRV) for dioxins and dioxin-like PCBs (DL-PCBs) in food. A new Tolerable Weekly Intake (TWI) was proposed, amounting to 2 picograms per kilogram of body weight (pg. kg-1 bw). This TWI is seven times lower than the previous TWI set by the former European Commission's Scientific Committee on Food in 2001. The main reasons for this decrease in level are the availability of new epidemiological and experimental data on the toxicity of these

substances in animals, as well as the emergence of more sophisticated modelling techniques to predict the levels of these substances in the human body over time. This provides novel analytical challenges.

For decades, the analysis of dioxins and furans has been performed by GC coupled to high resolution magnetic sector mass spectrometers (HRMS).[40] Recently, tandem mass spectrometry coupled to gas chromatography (GC-MS/MS) has been added in the European Union (EU) legislation as an alternative to HRMS for the confirmatory analysis of dioxins and DL-PCB in food and feed.[41] In this context, innovative ionization techniques have demonstrated increased sensitivity to perform analyses with the required sensitivity and selectivity for this field.[42]

To review the results of current monitoring programs contaminant occurrence data need to be collected and evaluated. Collecting occurrence data for risk assessment purposes relies on the implementation of two distinct strategies. The first one allows gathering occurrence data from routine monitoring programs conducted at the level of a specific country to check compliance of contaminants.[43] This approach has recently been further encouraged through a novel European regulation (Reg 2017/625/EC). An alternative to relying on data from food control systems is the use of the Total Diet Study (TDS) approach. These studies are based on a standardized method as recommended by WHO, FAO and EFSA: steps characterizing a TDS include the selection of foods based on food consumption data to represent as best as possible a typical diet, their preparation to food as consumed and the subsequent pooling of related foods before analysis.[44] Regarding dioxins and furans, the main contributors to the average dietary exposure for most age groups in European countries are fish (in particular oily fish), cheese and cattle meat[13]. In its latest Total Diet Study (TDS) dedicated to children's food, the French risk assessment agency Anses concluded that dietary exposure to dioxins and furans was a cause for concern, recommending to reduce exposures, in particular via everyday food products that contribute strongly to exposure to these molecules in the most exposed children (milk, ultra-fresh dairy products and fish).[45] At the European level, EFSA has recently confirmed the conclusion of previous assessments that dietary exposure to dioxins and dioxin-like PCBs is a health concern. The data collected in Europe indicate that the tolerable intake recently updated by EFSA is exceeded for all age groups. Average and high exposures are respectively 5 to 15 times higher than the new Tolerable Upper Intake Level for adolescents, adults and the elderly. Young children and children under 10 years of age also show a similar exceedance of the TW.[46]

As mentioned above, the analysis of known chemical hazards in complex biological matrices such as food requires sensitive, selective, and robust methods. To achieve the performance levels, the methods are usually targeted, in the sense that they only observe what is being looked for. Targeted methods are by definition selective, they thus do not detect substances that are not considered to be priorities, not suspected to be present in the matrix under consideration or not yet described, e.g. degradation products of known or unknown substances. New strategies which are known as global or non-targeted, have been reported over the last years to seek unknown/emerging exposure substances or unknown degradation products that may be considered as many potential emerging hazards. The recent period has indeed witnessed spectacular advances in chromatography and high-resolution mass spectrometry (HRMS), opening the way to non-targeted full scan fingerprints as a new methodological approach. It combines classical analytical chemistry tools, with sophisticated data analysis.[47] When certain molecular characteristics are targeted, such as the presence of halogens, specific signal processing algorithms can then be implemented to identify emerging POPs-type contaminants.[48] In the future, the development of such global approaches can be increased with the introduction of new analytical techniques which offer a new dimension in addition to chromatography and mass spectrometry for improved analysis of complex mixtures such as food.[49] These advanced novel non-targeted analytical approaches nevertheless remain targeted towards chemical groups with distinct characteristics. By combining with novel bioanalytical tools, additional opportunities for comprehensive, non-targeted chemical safety monitoring possibilities can be obtained.

Integrated analytical approaches to assure infant food safety

Targeted chemical analytics measures the presence of known toxic chemicals specifically, and therefore will not detect relevant unknown ones and their mixture effects. Non-targeted methods can greatly improve the number of chemicals addressed, but still cannot be directly linked to a measure of toxicity. Thus, a link to toxicity assessment is always needed, which can be based on prior knowledge, that is available for a subset of chemicals only. This knowledge is largely based on animal experimentation, but increasingly also on the use of *in vitro* assays. Mixture safety assessment can also make use of these *in vitro* assays, requiring no prior knowledge on safety of chemicals assessed. The net toxic effect of all contaminants in a sample can be measured regardless of their chemical structure and prior knowledge on their toxicity. Although the latter could be regarded as an alternative to current chemical analysis, there are several reasons why a combined system with targeted- and non-targeted chemical analytics will likely be more effective to assure safety of complex mixtures such as infant food. The targeted approaches will allow exact quantification of individual toxicants. which is important in source identification and risk management once a sample is identified with unexpected high bioactivity (Fig. 3). Generation on knowledge on the toxicity of yet uncharacterized "emerging" toxicants can be generated through untargeted chemical analytics or bioanalysis. For the latter, to identify the chemical or chemicals responsible for unexpected bioactivities in sample so-called effect-directed analysis (EDA)[50,51] can be used. In this procedure which involves step-wise fractionation of the chemical mixture, coupled to identification of the fraction with bioactivity leads to purification of the chemical responsible for the bioactivity of interest. When sufficiently pure, the unknown chemical can then be identified using advanced analytics. Another emerging possibility to estimate contribution of unexpected chemicals to mixture effects is to use nontargeted chemical analysis to get a view on additional compounds present in the mixture and link these to existing knowledge on their toxicological properties. Although this approach will be limited due to the limited knowledge on the toxicological properties of chemicals, the introduction of rapid bioanalytical methods and storage of analytical results in databases will increase the possibilities of this approach in the future. If a novel toxicologically relevant compound is identified, it can be added to the range of targeted compounds to be measured (Fig. 3; feedback loop no 2).

It should also be noted that, although the knowledge on toxicity pathways has greatly expanded, some chemical toxicities still are difficult to measure with modern bioanalytics, since no relevant *in vitro* assay has been developed, and thus targeted chemical analytics of those compound groups of special concern is required. If a positive result in a targeted analysis is not matched by a response in a bioassay, this will give a starting point to further improve the bioassay panel (Fig. 3; feedback loop no 1). In this way, an integrated system with chemical and biological analytics can be generated which is much stronger than the individual components.

The property of bioassays not to select chemicals to which they respond can give background issues. Non-specific toxicity to the cells when samples contain compounds that disturb their normal physiological environment, e.g. through strong effects on pH or osmolarity. For this, preventive measures can be installed. Generally, a method is needed that extracts toxicologically relevant molecules, leaving behind large molecules like proteins and other irrelevant ones like salts.

In the evaluation of the test results, it should be kept in mind that for all chemicals, also very toxic ones, being alone or in mixtures, a threshold can be defined below which there is no concern. The absence of establishing a threshold for chemical carcinogen has resulted in far too many chemicals being assigned as carcinogens.[52] Also, food contains

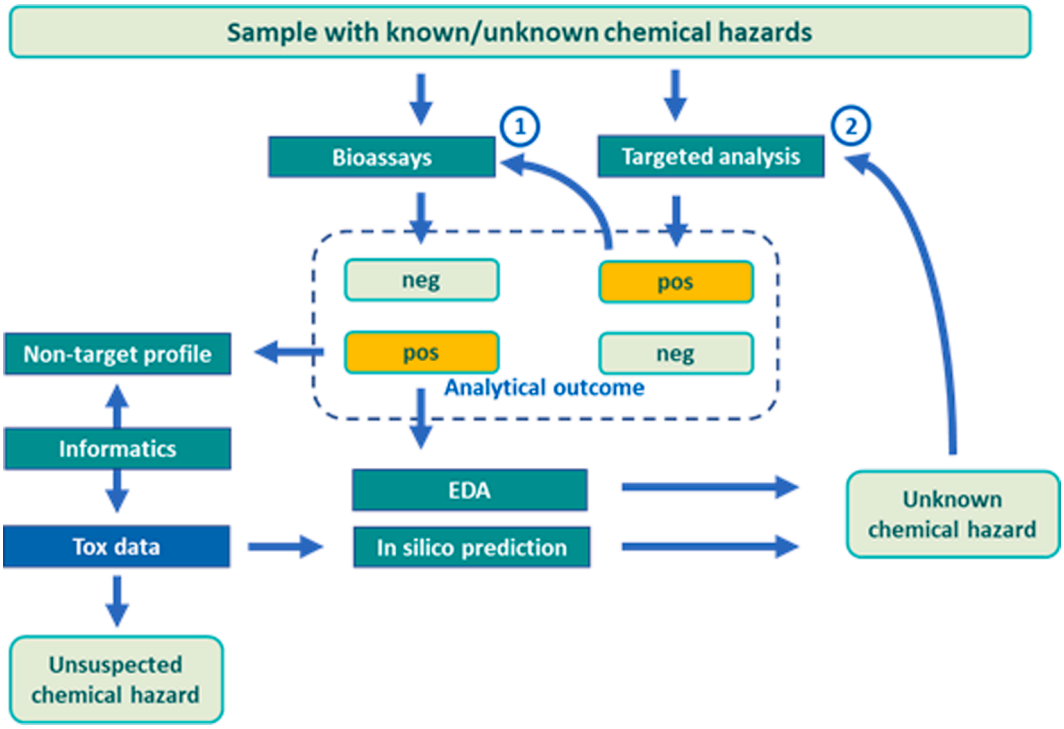

Fig. 3. Integrated analysis of food safety using chemical and biological methods. By using complementary possibilities of bio analytics and chemical analytics an integrated system can be built that can better assure protection against unknown and unexpected contaminants. Through feedback loops 1 and 2, a learing system is generated that will improve bio analytics and chemical analytics, respectively (see text for details).

considerable background levels of natural compounds having toxicological properties, but only when consumed at high levels. This is something to which the human body is adapted, and to which elaborate defense mechanisms are in place that often are much more elaborated than that of short living organisms (Ames and Gold, 2000). Therefore, for any bioassay that is used to assess toxicity it is important to establish a threshold of activity below which there is no concern for adversity in humans. For several CALUX assays this has been defined already for various applications.[25,26,53,54]

The CALUX reporter gene assays have been designed for robustness, sensitivity and specificity and therefore are particularly suitable for analysis of complex mixtures. With the proper conditions in place a wide range of studies have been executed successfully in a wide range of complex and polluted mixtures. In the area of infant safety CALUX bioanalysis for instance has been applied to assess chemical exposure *in utero* (in cord blood samples) or after birth, e.g. through mother's milk, indoor house dust samples and plastic toys.[38,55–59]

In the area of infant food safety, the importance of exposure to dioxins has been studied. Some of the earlier studies have focused on the relationship between dioxin exposure and health outcomes in children. Based on animal studies and human exposure to known chlorinated dioxins developmental dioxin exposure has been linked to a range of disorders, including cancer in all tissues, and endocrine and reproductive effects among the most sensitive ones.[60] The impact of more comprehensive biologically active dioxin mixtures has been studied using the DR CALUX assay. It has been used successfully to assess correlations between developmental exposure to total biologically active stable AhR ligands and some health outcomes and relevant clinical markers. As a result of the limited studies performed to date it was shown that there indeed is a relationship between total dioxin load, hormone action and the ano-genital distance, particularly in boys.[55,61] Further studies are needed to explore the relationship with the suspected wide array of health effects linked to developmental exposure to biological active dioxin-like compounds. This is of particular importance in the light of the newly discovered relevant AhR ligands, including brominated dioxins and furans.[36-38] Since several effects are linked to modulating the effects of the sex steroids, more comprehensive studies should also consider direct interactions of chemical mixtures as present in food with sex steroid receptors. Tools for such studies, including suitable extraction methods have become available recently, and particularly the androgen receptor was found to be suppressed in its activity by chemical mixtures present in mother's milk.[59,62] A relationship of this suppression with possible health outcomes remains to be established.

Conclusions

- Chemical food safety assessment is hampered by the limited knowledge on toxicity of chemicals.
- Both bioanalytical and chemical analytical methods have witnessed huge progress in past decades.
- There are great opportunities to reduce uncertainties in monitoring programs through integrated assessment using recent developments in biological- and chemical analytics.
- Chemist and biologist should increasingly work together to improve the coverage of relevant toxic chemicals stepwise further to be monitored in infant food in an efficient integrated manner, taking advantage of the complementary opportunities of novel developments in chemical- and biological analytics.

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