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Challenges in risk assessment of multiple mycotoxins in food

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REVIEW ARTICLE

Abstract

Most fungi are able to produce several mycotoxins simultaneously and, consequently, to contaminate a wide variety of foodstuffs. Therefore, the risk of human co-exposure to multiple mycotoxins is real, raising a growing concern about their potential impact on human health. Besides, government and industry regulations are usually based on individual toxicities, and do not take into account the complex dynamics associated with interactions between co-occurring groups of mycotoxins. The present work assembles, for the first time, the challenges posed by the likelihood of human co-exposure to these toxins and the possibility of interactive effects occurring after absorption, towards knowledge generation to support a more accurate human risk assessment. Regarding hazard assessment, a physiologically-based framework is proposed in order to infer the health effects from exposure to multiple mycotoxins in food, including knowledge on the bioaccessibility, toxicokinetics and toxicodynamics of single and combined toxins. The prioritisation of the most relevant mixtures to be tested under experimental conditions that attempt to mimic human exposure and the use of adequate mathematical approaches to evaluate interactions, particularly concerning the combined genotoxicity, were identified as the main challenges for hazard assessment. Regarding exposure assessment, the need of harmonised food consumption data, availability of multianalyte methods for mycotoxin quantification, management of left-censored data, use of probabilistic models and multibiomarker approaches are highlighted, in order to develop a more precise and realistic exposure assessment. To conclude, further studies on hazard and exposure assessment of multiple mycotoxins, using harmonised methodologies, are crucial towards an improvement of data quality and a more reliable and robust risk characterisation, which is central for risk management and, consequently, to prevent mycotoxins-associated adverse effects. A deep understanding of the nature of interactions between multiple mycotoxins will contribute to draw real conclusions on the health impact of human exposure to mycotoxin mixtures.

Keywords: mycotoxins mixtures, human risk assessment, combined toxicity, food safety, human health

1. Introduction

Food safety is threatened by numerous pathogens and toxins, including mycotoxins, which are associated to a variety of acute and chronic foodborne diseases. From an economic and public health standpoint, the foodborne mycotoxins that are considered as being relevant are aflatoxins (AFs), fumonisins (FBs), certain trichothecene mycotoxins (including deoxynivalenol (DON) and T-2 and HT-2 toxins), ochratoxin A (OTA), patulin (PAT) and zearalenone (ZEA). Briefly, AFs are strong hepatocarcinogens and have also been implicated in child growth impairment and acute toxicoses; FBs have been associated with oesophageal cancer and neural tube defects; DON and other trichothecenes, display immunotoxic effects and cause gastroenteritis; OTA has been associated to nephrotoxicity and renal cancer;

PAT induces gastrointestinal effects, as inflammation and ulcers; and ZEA affects the reproductive function (CAST, 2003; WHO, 2011; Wu *et al.*, 2014). These mycotoxins are able to naturally contaminate commodities at toxicologically relevant concentrations and are the focus of legislation and regulations in the European Union and beyond (Clarke *et al.*, 2014; Wu *et al.*, 2014). Maximum levels (MLs) are set in EU legislation to control these mycotoxin levels in food and feed (EC, 2002, 2006). Besides the parent forms, potential health effects of modified mycotoxins are also relevant due to their possibility to be hydrolysed to the more toxic parent mycotoxins and thus lead to an increased health risk (Berthiller *et al.*, 2013; Stoev, 2015).

All over the world, recent surveys highlight the fact that humans are more frequently exposed to multiple than to single mycotoxins (De Ruyck *et al.*, 2015; Grenier and Oswald, 2011; Solfrizzo *et al.*, 2014; Stoev, 2015) given the natural co-occurrence of mycotoxins in food and the globalisation of food markets (McKean *et al.*, 2006). Furthermore, climate changes towards an increase of temperature and humidity in certain European regions are expected to favour the growth of contaminating fungi, thereby increasing the likelihood of food commodities contamination with mycotoxins (Paterson and Lima, 2010). As a consequence, there has been an increasing concern about the health hazard from exposure to multiple mycotoxins (mycotoxin mixtures) in human and animals. Several studies have reported on the combined effects of mycotoxins (Alassane-Kpembi *et al.* 2016; Corcuera *et al.*, 2011; Klarić *et al.*, 2013; Speijers and Speijers, 2004; Tavares *et al.*, 2013, 2015) but the nature of the observed effect or the relative potencies of each mycotoxin in the mixture are not fully understood yet (Wan *et al.*, 2013), thus limiting the actual health risk assessment to their single effects.

Human risk assessment is based on a 4-step process, namely (1) hazard identification and (2) characterisation (both also considered as hazard assessment) examining if, and the conditions by which, a certain mycotoxin has the potential to cause a particular adverse health effect or disease and the numerical relationship between the level of dietary exposure (dose) and the associated adverse effect (response); (3) exposure assessment, estimating the frequency, intensity, and duration of ingestion of a mycotoxin; and (4) risk characterisation, integrating the results of the exposure assessment with those of hazard characterisation to estimate the degree of concern (FAO/WHO, 1997).

Historically, the health risk from human exposure to chemical contaminants has been evaluated on the basis of single-chemical and single-exposure pathway scenarios. In general, exposures to a chemical through the food were assessed independently, and no concerted effort had been made to evaluate potential multiple exposures simultaneously. However, in the last years a tiered approach has been proposed by the World Health Organization (WHO) and by the European Food Safety Authority (EFSA) in order to assess the risk of multiple chemicals, including contaminants (EFSA, 2013; Meek *et al.*, 2011). This hierarchical approach involves integrated and iterative considerations of exposure and hazard at all phases, with each tier being more refined (i.e. less conservative and uncertain) than the previous one, but more laborious and intensive. The framework comprises a tiered approach for exposure assessment, hazard assessment and risk characterisation and requires at the higher tiers increasing knowledge about the group of chemicals under assessment. Briefly, the tiers can range from tier 0 (default values, data poor situation) to tier 3 (full probabilistic models) (EFSA, 2013; Meek *et al.*, 2011).

Considering that risk assessment is intimately related to the establishment of regulatory guidelines, once the risk assessment is completed, an effort to reduce or manage the risk should be followed to protect public health (Renwick *et al.*, 2003). The food safety legislation through mycotoxins risk assessment is in place to control mycotoxins in food and includes single and sum of maximum levels for some mycotoxins in foodstuffs (EC, 2006). Some approaches are now being reported for the first time in the literature concerning the risk assessment of co-occurring mycotoxins in foodstuffs (Assunção *et al.*, 2015; De Boevre *et al.*, 2013; Han *et al.*, 2014) but far more research is required concerning the nature of interactions between multiple mycotoxins until real conclusions could be drawn concerning the health impact of mycotoxin mixtures.

Following the increasing interest of risk assessors, regulators and scientific community on the risk assessment of multiple mycotoxins in food, recent international meetings and research projects had pointed out the urgent need to address and discuss issues such as the co-occurrence of mycotoxins, their combined toxicity and cumulative risk assessment, namely: the 'International Conference on Food Contaminants: challenges in chemical mixtures', 2015 (ICFC2015) (http://hdl.handle.net/10400.18/3214); a course and a symposium within the 51st Congress of the European Societies of Toxicology, 2015 (http://www.eurotox2015.com); the Portuguese project entitled 'MycoMix, Exploring the toxic effects of mixtures of mycotoxins in infant food and potential health impact' (Alvito *et al.*, 2015); and the EU research project entitled 'EuroMix, A tiered strategy for risk assessment of mixtures of multiple chemicals' (https://www.euromixproject.eu/).

Risk assessment of combined human exposure to multiple mycotoxins poses several challenges to scientists, risk assessors and risk managers and open new avenues for research. This work aims to give a holistic overview of the main challenges and perspectives concerning the risk assessment of multiple mycotoxins in food, as a scientific

evaluation process. For this purpose, the following sections include a general overview and report recent advances in mycotoxin research and main challenges for each risk assessment step.

2. Hazard assessment of multiple mycotoxins

An overview of hazard assessment of single and multiple mycotoxins

Information on the hazard assessment of a chemical to which humans are exposed to is a determinant step within the human health risk assessment. Particular attention has been dedicated in last years to the occurrence of multiple chemicals in food leading to a change in the paradigm of the hazard assessment. The urgent need for a deep understanding of the potential effects of chemicals in mixture gave rise to the concept of combined toxicity which is defined as the 'response of a biological system to several chemicals, either after simultaneous or sequential exposure' (EFSA, 2013; Loewe and Muischnek, 1926). Combined toxicity can take three possible forms: concentration addition (CA), independent action (IA) or interaction (Loewe and Muischnek, 1926). According to the CA model, the joint action of multiple chemicals is the summation of individual toxicities, assuming the same Mode of Action (MoA) and/or at the same target cell, tissue or organ. In the IA model, the combined effects are estimated assuming that chemicals act independently by dissimilar MoA or at different target cells, tissues or organs and considers that the probability of toxicity from exposure to one chemical is independent from the probability of toxicity from exposure to another chemical in the mixture (Bliss, 1939; Jonker *et al.*, 2004; Meek *et al.*, 2011). These two reference models have found successful application to toxicological assessments of mixtures of similarly acting and dissimilarly acting compounds, both in ecotoxicology studies using a range of species (Backhaus *et al.*, 2004; Faust *et al.*, 2003; Loureiro *et al.*, 2010) and in human toxicity studies using cell lines or animal models (Mueller *et al.*, 2013; Tavares *et al.*, 2013). Deviation from these models include synergism (mixture effect greater than additive), antagonism (mixture effect less than additive) and more subtle interactions that depend on the actual doses of the mixture components (e.g. synergism at low doses and antagonism at higher doses) or on the ratio of doses between the compounds in the mixture (e.g. the extent of the synergism or the antagonism depends on the relative contribution of each compound in the mixture) (Jonker *et al.*, 2004, 2005). Because MoA of chemicals in mixtures is often unknown or incompletely understood, a frequent option has been the application of both models of CA and IA for actual effect prediction, rather than making a theoretically based choice. However, from a practical point of view the application of a single model for all situations is desirable and, being the CA the most conservative model, EFSA recommended its use within the risk assessment of food contaminants that includes mycotoxins (EFSA, 2013).

Particular attention must be dedicated to carcinogens in hazard assessment, including mycotoxins and other food contaminants (Jeffrey and Williams, 2005). Genotoxicity, i.e. the capacity of exerting a damaging effect on the cell's genetic material (DNA, RNA, chromosomes) affecting its integrity and/or function is a major mechanism that contributes to the carcinogenic process. Considering that genetic events, such as gene mutations, structural or numerical chromosomal aberrations and recombination are closely related with carcinogenesis, genotoxic effects can be characterised in a faster, easier, and inexpensive way using standard *in vitro* and *in vivo* genotoxicity assays, instead of performing long-term carcinogenesis assays in animals (Dearfield and Moore, 2005; Louro *et al.*, 2015). However, chemicals acting through a non-genotoxic mechanism, including induction of epigenetic events and mitogenesis, can be equally relevant to the carcinogenic process. Therefore, besides genotoxicity testing, mechanistic studies are also needed to clarify the MoA of carcinogenic agents. In this respect, it is generally assumed that a threshold of exposure may be determined for non-genotoxic carcinogens, below which no biologically significant effect will be induced (Dybing *et al.*, 2008). In contrast, from a conservative and health protection point of view, it has been assumed that genotoxic carcinogens act by a non-threshold mechanism, giving rise to linear dose-response curves. The decision on whether or not a chemical is genotoxic is thus of primary importance to select between a non-threshold or threshold risk assessment approach in the step of hazard identification.

A framework, similar to the one suggested by Spurgeon *et al.* (2010) to understand the effects of environmental chemical mixtures, may be proposed to investigate the combined toxicity of multiple mycotoxins in food and their potential impact on human health. The framework incorporates the concepts of external exposure or 'bioavailability' of the mixture in the environment together with its exposure in the target species associated with accumulation through toxicokinetics to the expression of toxicity as mediated via receptor interactions within toxicodynamics. A similar framework could be suggested to investigate the health effects of multiple mycotoxins in food reflecting physiological conditions occurring from ingestion of mycotoxins to their effects on target cells, tissues or organs and should include three sequential main concepts: (1) bioaccessibility, release of mycotoxins from its matrix into digestive juice in the gastrointestinal tract (Versantvoort *et al.*, 2005); (2) toxicokinetics; and (3) toxicodynamics, taking into account interactions between mycotoxins and/or any active metabolites and the target cells/tissues/organs.

A physiologically-based framework for hazard assessment of mycotoxins

In human risk assessment, ingestion of food is considered a major route of exposure to mycotoxins. Oral bioavailability, defined as the fraction of an orally ingested mycotoxin that reaches the systemic circulation and is distributed throughout the body to exert its toxic effects, can be seen as the resultant of three processes: (1) release of the mycotoxin from its matrix into digestive juice in the gastrointestinal tract (bioaccessibility); (2) transport of the mycotoxin across the intestinal epithelium into the vena Portae (intestinal transport); and (3) biotransformation of the mycotoxin in the liver (and intestine) (metabolism) (González-Arias *et al.*, 2013; Versantvoort *et al.*, 2005). The concept of bioaccessibility of mycotoxins has become important in the risk assessment domain considering that the amount of mycotoxin consumed via food (external dose) does not always reflect the amount available to the body (internal dose) to produce its toxic effects on target cells, tissues or organs (González-Arias *et al.*, 2013; Versantvoort *et al.*, 2005).

To determine the bioaccessibility, several models of different complexities have been proposed to simulate food digestion. Simulated digestion methods typically include the oral, gastric and small intestinal phases, and, occasionally, large intestinal fermentation. The majority of models reported in literature are the static ones (Gil-Izquierdo *et al.*, 2002; Hur *et al.*, 2011; Versantvoort *et al.*, 2005). However, more sophisticated dynamic *in vitro* models are also available and although more realistic, they are complex and expensive (Avantaggiato *et al.*, 2004; González-Arias *et al.*, 2013). A recent review (González-Arias *et al.*, 2013) on the bioaccessibility of single mycotoxins reported, in general, high bioaccessibility values for AFs and FBs, (70-100%), intermediate values for PAT and DON (30-70%) and lower values for ZEA. The bioaccessibility of OTA has proven to be very variable, including values near 100%, but also below 30%. Until now, few data on the bioaccessibility of co-occurring mycotoxins are available, namely for AFs (Kabak and Ozbey, 2012), AFB_1 and OTA (Kabak *et al.*, 2009; Raiola *et al.*, 2012; Versantvoort *et al.*, 2005), enniatin (Meca *et al.*, 2012; Prosperini *et al.*, 2013), trichothecenes T-2 and HT-2 toxin (Monaci *et al.*, 2015). A very recent preliminary study evaluated the possible interactions that could happen when PAT and OTA co-occur in cereal-based baby foods (Assunção *et al.*, 2016).

The *in vitro* digestion models could also be used in combination with intestinal models (e.g. Caco-2 cells) to address further mechanistic questions, such as intestinal transport, contributing to an accurate mycotoxin risk assessment, offering a more complete picture of what happens during digestion in the intestinal tract (De Nijs *et al.*, 2012; Meca *et al.*, 2012; Prosperini *et al.*, 2012; Versantvoort *et al.*, 2005).

Regarding toxicokinetics, absorption occurs mainly through ingestion for chemicals in food and a central issue relates to the passage across the gut wall and entering into the blood circulation, although for some chemicals, uptake is restricted to the epithelium of the gastrointestinal tract (FAO/WHO, 2009). The sites of passive or active uptake across body barriers, depending on the chemical nature of the toxins, are potential points of interactions because one toxin might affect the efficiency of the uptake or elimination of other components of the mixture. In cases where toxins enter or are eliminated through selective transporters, competition between mycotoxins at relevant surface transporters can take place and can result in changes in uptake, bioaccessibility and toxicity, depending on the relative affinity of each toxin for the transporter.

Once (multiple) chemicals have entered the systemic circulation they can interact, inhibit or induce a range of metabolic pathways including phase I enzymes (e.g. cytochromes P450, (CYPs)), phase II enzymes (e.g. glutathione-S transferases) and antioxidant defence enzymes (e.g. superoxide dismutase or catalase) (Streetman *et al.*, 2000). These systems provide a network of responses directed mainly to the detoxification of chemicals but in some cases lead the production of toxic metabolites. This is the case of AFB_1 biotransformation by CYP3A4 and 1A2 that results in the formation of an exo-epoxide and AFQ_1, whilst CYP1A2 can lead to the formation of some exo-epoxide but also a high proportion of endo-epoxide and AFM_1 (Dohnal *et al.*, 2014). The exo-epoxide binds to DNA and forms a pre-mutagenic lesion that mediates AFB_1 mutagenicity and carcinogenicity (Bedard and Massey, 2006; Wild and Turner, 2002). It is plausible that interactive effects may occur when two or more mycotoxins are metabolised through the same pathway, so that one mycotoxin might substantially impact on the detoxification of the other ones. This may be mediated either by a competitive inhibition or by an over-induction of the metabolic system and a faster biotransformation of the mycotoxins. In the work by Corcuera *et al.* (2011) antagonistic genotoxic effects of OTA and AFB_1 combinations were observed in liver-derived HepG2 cells, concomitantly with an increase of intracellular reactive oxygen species (ROS). The authors hypothesised that competition between both toxins to the same CYP enzymes could have resulted in a lower amount of the mutagenic AFB_1 exo-epoxide molecules and thus in a lower level of DNA damage. Concerning interactions at the level of phase II metabolism, Tavares *et al.* (2013) proposed that the co-existence of OTA and AFM_1 in Caco-2 cells might have resulted in a competition for the glutathione molecules, decreasing the level of ROS produced by OTA and hence leading to an antagonistic cytotoxic effect.

A further source of mixture interactions relates to its toxicodynamics, i.e. at the level of the dynamic contact of a toxicant with its biological target, possibly impacting

on its biological effects at target cells/tissues/organs. For mycotoxins with a similar MoA, the assumption within the CA model is that both toxins are present at the target site, and each one is able to bind freely with no stimulatory or competitive influences. In mammalian systems, the toxicodynamic consequences of receptor-binding have been associated with a variety of effects, ranging from neurotoxicity, renal toxicity and cardiovascular toxicity (Dorne *et al.*, 2007). Carefully designed experiments and informatics approaches can be used to investigate the mechanistic basis of mixture effects.

Evaluation of combined effects of multiple mycotoxins

The number of studies addressing the combined effects of mycotoxins using *in vitro* and *in vivo* models and several endpoints (e.g. cytotoxicity, immunotoxicity and genotoxicity) has been steadily increasing in the last decade giving rise to a set of data that might greatly contribute to hazard assessment of multiple mycotoxins. Šegvić Klarić (2012) and, more recently, Alassane-Kpembi *et al.* (2016) have comprehensively reviewed combined toxicity studies involving regulated groups of mycotoxins, particularly, OTA, AFs, *Fusarium* toxins, trichothecenes and emerging mycotoxins, e.g. beauvericin and enniatins. Cytotoxicity assays, using a diversity of cell lines have been widely used because they are fast and economic assays that may help predicting the *in vivo* toxicity of combinations of mycotoxins co-occurring in food (Creppy *et al.*, 2004; Tiemann and Dänicke, 2007), with the advantage of allowing the reduction of the number of animals under experimentation, in compliance with the European Union recommendations (EC, 2010). Combinations involving AFB_1 or OTA are among the most frequently assessed, due to concerns related to their recurrent occurrence in several mixtures and their severe chronic adverse effects that can be even amplified if a synergistic effect is identified.

The potential of mycotoxins to elicit a cytotoxic response is transversal to many recent works, but some controversy still remains about the pattern that better describes mycotoxins combined effect. Table 1 compiles data from the joint effects of AFB_1 or OTA as components of several mixtures assessed *in vitro* and, more rarely, *in vivo*. For instance, combinations of OTA, AFB_1 and FB_1 in three different mammalian cell lines showed synergistic cytotoxic effects with regard to mitochondrial integrity although binary mixtures of the same mycotoxins followed the additivity pattern (Clarke *et al.*, 2014). Noteworthy, no interactive effect was observed for mycotoxin mixtures tested at the EU regulatory limits, which highlights the relevance of using an appropriate dose-range in the *in vitro* studies. For binary mixtures involving OTA, additive toxic effects were observed for combinations with AFB_1 in kidney cells (Golli-Bennour *et al.*, 2010) and in hepatoma-derived cells (Corcuera *et al.*, 2011), whereas synergistic effects were reported in rat brain glioma, Caco-2 and Vero cells (Creppy *et al.*, 2004); antagonism was described for the combined toxicity of OTA and AFM_1 in Caco-2 cells (Tavares *et al.*, 2013). In addition, synergistic effects between OTA and CIT were identified in renal cells *in vitro* (Bouslimi *et al.*, 2008; Heussner *et al.*, 2006) and *in vivo* (Pfohl-Leszkowicz *et al.*, 2008). Recently, the effect of the ternary mixture of OTA, citrinin and sterigmatocystin was explored in a human hepatocellular cancer cell line (Hep3B), showing a synergistic effect at low toxin doses that shifted to antagonism at higher concentrations (Anninou *et al.*, 2014). A similar dose-dependent interactive effect was found for the joint effects of OTA and FB_1 in human hepatoma and human renal cells (Tavares *et al.*, 2015). Apart from mixtures involving OTA or AFB_1, the combined effects of a number of other mycotoxins combinations have been also addressed in the last years, including *Fusarium* and *Alternaria* toxins (Vejdovszky *et al.*, 2016), DON, NIV and their acetylated derivatives (Alassane-Kpembi *et al.*, 2015) or BEA, DON and T2-toxin (Ruiz *et al.*, 2011), to name only some studies. Factors related to the experimental design, including the concentration range of the single toxins and relative concentration of each toxin in the mixture or the metabolic capacity of the target cell are central to the combined final effect and have to be carefully controlled.

Another important aspect relates to the approaches that have been applied to quantitatively measure the dose-effect relationships of single mycotoxins and its combinations and to ascertain putative interactive effects. Among them, the usefulness of the combination index (CI)-isobologram equation by Chou (2006) and Chou and Talalay (1984), which is based on the median-effect principle (mass-action law) that demonstrates that there is an univocal relationship between dose and effect, independently of the number of substrates or products and of the mechanism of action or inhibition (Alassane-Kpembi *et al.*, 2013; Bernhoft *et al.*, 2004; Ruiz *et al.*, 2011) (Table 1). This method involved plotting the dose-effect curves for each compound and their combinations in multiple diluted concentrations and is based on the assumption that when two compounds are combined and subjected to several dilutions, the combined mixture of the two compounds behaves as the third compound for the dose-effect relationship. The CI indicates not only the type of interaction (additivity, synergism or antagonism) but also the magnitude of the interaction found. In addition, the conceptual models of CA and IA incorporating also a set of deviation functions within a nested framework (Jonker *et al.*, 2004, 2005) have been effectively applied to the analysis of cytotoxic effects of mycotoxins mixtures (Tavares *et al.*, 2013; Table 1).

Several prerequisites are required to allow the application of these models, implicating a careful experimental design that will depend on the number of chemicals in the mixture and the degree of detail needed concerning the dose-effect relationship for single and combined toxicity; the single

Table 1. Combined toxicity and interactive effects concerning multiple mycotoxins studies.

Mixture[1]	Biological effects: methodologies[2]	Experimental system	Combined effect	Data analysis/modelling[3]	Reference
AFB_1-OTA-FB_1, OTA-FB_1	Cytotoxicity: high content analysis endpoints	MDBK cell line	Synergism (binary and tertiary mixtures)	comparison between observed and expected additive effects	Clarke *et al.*, 2015
AFB_1-OTA	Genotoxicity: Comet assay, micronucleus assay	rat kidney and liver, bone marrow	Antagonism (liver and bone marrow)	statistical comparison of data from single and combined effects	Corcuera *et al.*, 2015
AFB_1-ZEA-DON, AFB_1-ZEA, AFB_1-DON	Cytotoxicity: MTT	BRL 3A rat liver cells	Synergism (binary mixtures)	central composite design	Sun *et al.*, 2015
AFB_1-OTA-FB_1, AFB_1-OTA, OTA-FB_1	Cytotoxicity: MTT, NR	Caco-2 cell line, MDBK, Raw 264.7	Synergism (tertiary mixture) Additivity (binary mixtures)	comparison between observed and expected additive effects	Clarke *et al.*, 2014
AFB_1-ZEA, AFB_1-DON, AFB_1-ZEA-DON	Cytotoxicity: MTT, LDH Apoptosis: PI/Annexin Oxidative stress: DFD	PK15 cell line	Synergism (AFB_1-ZEA or -DON) Antagonism (apoptosis) (AFB_1-ZEA) Antagonism (AFB_1-ZEA low doses) Synergism (high doses AFB_1-ZEA or -DON)	central composite design; comparison between observed and expected dose-response curves	Lei *et al.*, 2013
AFB_1-AFB_2, AFM_1-AFM_2	Cytotoxicity: MTT Immunotoxicity: flow cytometry, NO_2 assay	J774A.1 cell line	Synergism	statistical comparison of data from single and combined effects	Bianco *et al.*, 2012
AFB_1-OTA	Cytotoxicity: MTT Genotoxicity: Comet assay	HepG2 cell line	Additivity Antagonism	statistical comparison of data from single and combined effects	Corcuera *et al.*, 2011
AFB_1-OTA	Cytotoxicity: MTT Genotoxicity: Comet assay	Vero cell line	Additivity Additivity	interactive index calculation	Golli-Bennour *et al.*, 2010
AFB_1-T2	Cytotoxicity: WST-1 cell proliferation assay system	HepG2 cell line, BEAS-2B	Additivity Synergism	interactive index calculation	McKean *et al.*, 2006
OTA-DON	Cytotoxicity: MTT Oxidative stress: DFD	Caco-2 cell line	Additivity/synergism	comparison between single and combined effects	Cano-Sancho *et al.*, 2015
OTA-STE, CIT-STE	Cytotoxicity: MTT Genotoxicity: SCE	Hep3B cell line	Additivity Additivity/antagonism	calculation of the coefficient of drug interaction	Anninou *et al.*, 2014
OTA-CIT	Cytotoxicity: NR Genotoxicity: micronucleus assay	V79 cell line	Additivity Synergism (low doses) Antagonism (high doses)	comparison between observed and expected data	Föllmann *et al.*, 2014
OTA-AFM_1	Cytotoxicity: NR	Caco-2 cell line	Antagonism	full or partial factorial design; mathematical functions for CA/IA and dependent deviations	Tavares *et al.*, 2013
OTA-BEA	Genotoxicity: Comet assay	PK15 cell line, human lymphocytes	Additivity/synergism Additivity (lymphocytes)	comparison between observed and expected additive effects	Klarić *et al.*, 2010
OTA-FB_1-BEA	Genotoxicity: micronucleus assay	PK15 cell line	Additivity	comparison between observed and expected additive effects	Klarić *et al.*, 2008

Table 1. Continued.

Mixture[1]	Biological effects: methodologies[2]	Experimental system	Combined effect	Data analysis/modelling[3]	Reference
OTA-CIT	Cytotoxicity: MTT Genotoxicity: Comet assay Oxidative stress: DFD Apoptosis	HepG2 cell line	Synergism	comparison of IC_{50} values between individual and combined effects	Gayathri *et al.*, 2015
OTA-CIT	Cytotoxicity/genotoxicity: DNA fragmentation, chromosome aberrations *in vivo*	Vero cell line, mouse bone marrow cells	Synergism	comparison of IC_{50} or LD_{50} between individual and combined effects	Bouslimi *et al.*, 2008
OTA-PAT-CIT-GLI	Cytotoxicity: MTT Immunotoxicity: T cell function, ELISA	human lymphocytes	Antagonism	comparison between observed and expected dose-response curves using the response addition and CA models	Tammer *et al.*, 2007
OTA-PAT, OTA-CIT	Cytotoxicity: MTT	LLC-PK1 cell line	Synergism	central composite design; comparison between observed and expected dose-response curves	Heussner *et al.*, 2006
OTA-CIT, OTA-PAT, CIT-PAT	Cytotoxicity: lymphocyte proliferation test	porcine lymphocytes	Synergism (OTA-CIT) Antagonism (OTA-PAT) Additivity (CIT-PAT)	isobologram analysis	Bernhoft *et al.*, 2004
OTA-FB_1	Cytotoxicity: NR	C6-glioma cell line, Vero, Caco-2	Synergism	Statistical comparison of data from single and combined effects	Creppy *et al.*, 2004

[1] AFB_1 = aflatoxin B_1; AFM_1 = aflatoxin M_1; BEA = beauvericin; CIT = citrinin; DON = deoxynivelanol; FB_1 = fumonisin B_1; GLI = gliotoxin; OTA = ochratoxin A; PAT = patulin; STE = sterigmatocystin; T2 = T-2 toxin; ZEA = zearalenone.
[2] DFD = dichloro-fluorescein diacetate; ELISA = enzyme-linked immunosorbent assay; MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; NR = Neutral Red uptake assay; PI = propidium iodide; SCE = sister chromatid exchanges;
[3] IA = independent action model; CA = concentration addition model.

compounds in the mixture should be tested at a constant dose ratio, using a full factorial design. This approach was employed by Tavares *et al.* (2013) to assess the combined effect of OTA and AFM_1 in an intestine cell line, following determination of the inhibitory concentration at 50% (IC_{50}) of the single toxins. Both CA and IA models were applied to derive potential interactions at concentrations below the individual IC_{50}. Whereas the well-known CA model assumes that both toxins have a similar MoA, the IA model assesses the probability of toxicity from exposure to one mycotoxin being independent from the toxicity of the other toxin in the mixture (Jonker *et al.*, 2004, 2005). A fairly good agreement was obtained for both models, in that antagonism was found after the CA model fit, while a dose level deviation was observed after IA modelling, where antagonism was observed at low dose levels and synergism at high dose levels. However, when dealing with a mixture of several chemicals, where the number of experimental groups increases exponentially, fractionated factorial designs can be used to identify interactions in a manageable way (Groten *et al.*, 2004). Another possibility is to apply a tiered approach as suggested by Tajima *et al.* (2002) for *Fusarium* mycotoxins, starting from the study of the whole mixture effect, followed by a screening of interactions using a fractionated factorial design and, finally the confirmation of interaction through a full factorial design.

In spite of the number of studies addressing the cytotoxicity of multiple toxins, genotoxicity is of a greater concern for most of the mycotoxins and their mixtures due to its association to carcinogenesis. However, the specific genotoxic properties of multiple mycotoxins are much more difficult to address comprehensively in complex combinatory experiments and thereby studies reporting combined genotoxic effects of mycotoxins are more limited. Interestingly, among the *in vitro* studies available, additivity or even antagonism are the predominant joint effects of mixtures containing AFB_1 or OTA (Table 1). A common drawback of *in vitro* studies is the use of dose-ranges that are much higher than those that have been found in biological fluids of exposed humans. Thus, further studies using realistic concentrations that follow a carefully planned experimental design are still needed. Moreover, for those mixtures showing interactive effects in *in vitro* assays, confirmatory *in vivo* assays should be used to better predict the effects on humans. For instance, the combined genotoxic effect of OTA and CIT was explored in a Chinese hamster lung cell line showing a synergism at low doses that changed to antagonism at higher doses (Föllmann *et al.*, 2014). A synergistic effect was also observed through the analysis of chromosome aberrations in bone marrow cells from exposed mice (Bouslimi *et al.*, 2008), confirming that low doses are more realistic and thus more relevant to predict *in vivo* effects. On the other hand, Corcuera *et al.* (2011) showed that the mixture of OTA and AFB_1 produced an antagonistic DNA damaging effect, comparatively to each single toxin. The observed antagonism was further confirmed in liver and bone marrow cells of exposed rats using the comet and the micronucleus assays, respectively (Corcuera *et al.*, 2015). These two examples suggest that carefully designed *in vitro* studies on combined genotoxic effects of mycotoxins may have a good predictive value for their *in vivo* joint effect, as advocated by Creppy *et al.* (2004). Nevertheless, more *in vivo* genotoxicity studies are urgently needed, using the oral route of exposure, realistic concentrations derived from human exposure data and allowing the quantification of several endpoints (e.g. DNA breaks, chromosome numerical and structural anomalies, gene mutations) to confirm the value of *in vitro* approaches. Furthermore, the application of mathematical models to ascertain genotoxic interactive effects, similarly to what has been done for combined cytotoxicity assessment, is highly relevant to have firm and reliable conclusions about genotoxic interactive effects. In this sense, Ermler *et al.* (2014) have already shown that the CA, IA and hybrid CA/IA models are applicable to data obtained for several model compounds with similar and dissimilar MoA, using the micronucleus assay.

Main challenges in hazard assessment of multiple mycotoxins

Knowledge of the real percentage of mycotoxins that can be absorbed in the small intestine would enable a more accurate risk assessment. Several factors may affect the bioaccessibility of single and multiple mycotoxins. Variability within mycotoxin bioaccessibility values depends on the compound, food product, contamination level and way of contamination (spiked or naturally contaminated) (Kabak *et al.*, 2009). Additionally, the diversity of *in vitro* digestion models used to access the bioaccessibility of mycotoxins constitutes another important challenge. The individual static *in vitro* digestion models described in the literature exhibit significant variations in the *in vitro* digestion parameters as pH, mineral type, ionic strength and digestion time, which alter enzyme activity (González-Arias *et al.*, 2013; Minekus *et al.*, 2014). Consequently, this fact hampers the possibility to compare results across research-groups and to deduce general findings. To overcome this difficulty, recently a standardised static *in vitro* digestion model suitable for food was developed within the COST action INFOGEST (https://www.cost-infogest.eu/). This standardised digestion method (Egger *et al.*, 2015; Minekus *et al.*, 2014) is based on the current state of knowledge on *in vivo* digestion conditions and describes a detailed line-by-line protocol (https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg) with recommendations and justifications on the experimental procedures applied.

Mycotoxin absorption constitutes another challenge within risk assessment considering that toxins could reach intestine as the parent compound or as metabolites formed

during the digestion, and methods for their detection are not yet developed or currently available (González-Arias *et al.*, 2013).

The level of effort required for hazard assessment of the combined effect of multiple chemicals to humans or to the ecosystem is quite high and should be initially weighted by the magnitude of potential risks, the objective (e.g. priority setting or screening for additional focus or risk management) and scope (e.g. local and national interest) (Meek *et al.*, 2011). Since testing the combined effects of all possible combinations of mycotoxins is not feasible in a reasonable timeframe, priorities for hazard assessment need to be set in order to put more effort into the most relevant mixtures. The rational for priority setting may be based on the frequency of its co-occurrence in food, the hazardous potency of the single toxins, the structure-activity relationship pointing to a strong probability of interactions or on preliminary data suggesting synergistic effects. Even though the available data concerning combined toxic effects of mycotoxins have been growing, a considerable degree of inconsistency is noted when comparing the outcomes of studies focused on similar mixtures and therefore, more studies are needed to allow firm conclusions. Given that these studies are laborious and time consuming, the future utilisation of simpler and faster electrochemical biosensors (exemplified in Gu *et al.*, 2015) and high content analysis that allows the simultaneous examination of a large set of endpoints with high sensitivity (Clarke *et al.*, 2015) are promising advances in combinatory toxicology. Studies on combined genotoxic effects of multiple mycotoxins are still scarce and more studies should be developed in order to go further on the risk assessment and provide information for risk assessors and, subsequently, for risk managers and regulators. In addition, exploring interactions at the mechanistic level remains a challenging issue and more studies are needed in order to clarify the biochemical, cellular or molecular mechanisms underlying the observed interactive effects. For this purpose, a systems toxicology approach, i.e. a toxicogenomic approach can provide useful information about genes expression, proteins or biochemical pathways within a reasonable timeframe from which mechanisms of toxicity can be established (Altenburger *et al.*, 2012).

3. Exposure assessment of multiple mycotoxins

Overview of methods for estimating dietary exposure

Exposure assessment is a key element for quantifying risk and constitutes one of the four steps included in the risk assessment process and is usually defined as the qualitative and/or quantitative evaluation of the likely intake of agents via food as well as exposures from other sources (FAO/WHO, 2009). Dietary exposure assessment consists of combining deterministically or probabilistically food consumption figures with occurrence of a given chemical substance in a number of food categories (EFSA, 2013).

Food consumption data reflect what individuals or groups consume in terms of solid foods, beverages, and dietary supplements. National food consumption surveys are the principal sources of information for determining real food consumption habits in a population of consumers. In addition to the general population, the risk assessments generally also consider the exposure of specific consumer groups, such as infants, children, and people following specific diets (e.g. vegetarians) (FAO/WHO, 2009). Food consumption can be estimated through food consumption surveys, including records/diaries, food frequency questionnaires and dietary recall (EFSA, 2011). The food records or food diaries require the report of all foods consumed during a specific period (usually ranging between 24 hours to 7 days). Food frequency questionnaires consist of a structured listing of individual foods or food groups where the respondent is asked to estimate the number of times the food is usually consumed per day, week, month or year (FAO/WHO, 2009).

Considering the increasing evidence that co-contamination of food matrices is the rule, not the exception (Stoev, 2015), the proposal to quantify the simultaneous occurrence of multiple contaminants goes on the direction of developing multi-analyte methods combining a generic sample preparation protocol with a highly selective method exhibiting sufficient detection capacity, such as LC-MS (Malachová *et al.*, 2014; Turner *et al.*, 2015). EFSA suggested that Total Diet Studies (TDSs) provide the most accurate estimates of mean contamination by the chemicals in the food consumed by the population or collective group of individuals (FAO/WHO/EFSA, 2011). As TDSs consider total exposure from whole diets and are based on food contamination 'as consumed' rather than contamination from raw commodities, they are considered to ensure a more realistic exposure measure than exposure studies based on monitoring programs and surveillance data (Papadopoulos *et al.*, 2015). Within the general framework of chemical risk assessment, a difficult step in dietary exposure assessment is the handling of concentration data reported to be below the limit of detection (LOD) of the analytical method. These data are known as non-detects and the resulting distribution of occurrence values is left-censored. EFSA has so far mainly used substitution methods (EFSA, 2010). The most common approaches are the substitution methods that replace non-detects by LOD divided by 2 or producing an upper and lower bound by substitution of non-detects by LOD or 0, respectively (EFSA, 2010).

Food consumption and occurrence data are then combined to perform the dietary exposure assessment. A deterministic (point estimate) or probabilistic (stochastic) approach is

generally applied to perform dietary exposure assessment studies. The structure of the probabilistic and deterministic approaches is similar and is based on the same basic equations whereby food consumption data are combined with concentration data to estimate dietary exposure. The fundamental difference is that at least one variable is represented by a distribution function instead of a single value and the model sample from each distribution is a distribution of potential dietary exposures generated using several thousand iterations. Monte Carlo simulation is the technique that has been applied to a wide variety of modelling scenarios in probabilistic dietary exposure assessment (FAO/WHO, 2009; Han *et al.*, 2014).

Worldwide exposure assessment of co-occurring mycotoxins in food

Human exposure is a crucial element in the risk assessment of mycotoxins. Table 2 summarises most recent reports on exposure assessment of worldwide populations to multiple mycotoxins in food, published in the last six years. Children and adults are the main population groups considered in these studies. Cereal based products are important commodities prompt to mycotoxins contamination and as such were an obvious target in the reported exposure assessment studies (wheat and maize foods, breakfast cereals). Nuts and dried fruits were also assessed (Cano-Sancho *et al.*, 2013; Cressey and Reeve, 2013; Van de Perre *et al.*, 2015). AFs are the mycotoxin group most assessed (Assunção *et al.*, 2015; Cano-Sancho *et al.*, 2013; Cressey and Reeve, 2013; García-Moraleja *et al.*, 2015; Signorini *et al.*, 2012; Sirot *et al.*, 2013; Van de Perre *et al.*, 2015). Other evaluated mycotoxin groups include FBs, ochratoxins, PAT, trichothecenes and ZEA and their respective metabolites, enniatins, beauvericin, sterigmatocystin and *Alternaria* toxins, with a maximum of 48 mycotoxins assessed simultaneously (Sirot *et al.*, 2013; Sprong *et al.*, 2016b). For the exposure assessment studies, food consumption data were mainly obtained from national food consumption surveys, including different data collection methodologies, namely 1-day (Cressey and Reeve, 2013; Han *et al.*, 2014), 2-day (De Boevre *et al.*, 2013; Sprong *et al.*, 2016a; Van de Perre *et al.*, 2015) and 3-day 24-hour recalls (Zhao *et al.*, 2015), 3-day food diary (Assunção *et al.*, 2015) and 7-day food diary (Sirot *et al.*, 2013), all reported in Table 2.

LC/MS-MS was the most used technique for the quantification of multiple mycotoxins in foodstuffs although LC-FD is also used in several studies for AFs quantification (Assunção *et al.*, 2015; Cano-Sancho *et al.*, 2013; Sirot *et al.*, 2013). Recently, an LC-MS/MS 'dilute and shoot' method for the determination of 295 fungal and bacterial metabolites was optimised and validated according to the guidelines established in the Directorate General for Health and Consumer Affairs of the European Commission (SANCO) document No. 12495/2011 (Malachová *et al.*, 2014; SANCO, 2011). Based on this study, Malachová *et al.* (2014) considered that a quantitative determination of mycotoxins by LC-MS/MS based on a 'dilute and shoot' approach is also feasible in case of complex matrices. TDSs were developed to estimate the exposure of populations to food contaminants, including mycotoxins. From the studies presented in Table 2, Sirot *et al.* (2013) and Sprong *et al.* (2016a) evaluated the exposure of French and Dutch populations, respectively, to mycotoxins.. According to Lee *et al.* (2015), countries as Australia, France, Korea and China had developed their country-specific TDSs including mycotoxins but all countries are encouraged to conduct total diet studies to assess the safety and nutritional quality of diet of their population since TDS is not only a cost-effective tool but also a realistic tool for risk assessment of chemicals in foods. Actually, the TDS EXPOSURE project aims to provide guidance for future TDSs, as well as for new TDSs in countries that have no TDS experience (http://tds-exposure.eu/). Despite the mentioned advantages, TDS also present some limitations (e.g. lack of harmonisation on how to build up the list of foods or food categories) and other type of sampling procedures for food could be applied (e.g. duplicate portion). The substitution methods most used for the handling of non-detects are the replacement of non-detects by LOD values, half LOD values or 0 (EFSA, 2010). Although, some authors decided also to include the LOQ in the management of non-detects (García-Moraleja *et al.*, 2015; Sirot *et al.*, 2013). Until now, the assessment of mycotoxin exposure is mainly based on deterministic approaches, however, there are an increasing number of studies applying probabilistic models in last years, as shown in Table 2 (Assunção *et al.*, 2015; Cano-Sancho *et al.*, 2013; De Boevre *et al.*, 2013; Han *et al.*, 2014; Signorini *et al.*, 2012; Van de Perre *et al.*, 2015).

Main challenges in exposure assessment to multiple mycotoxins

It is quite challenging to estimate the usual food consumption considering the limited amount of information from national surveys, since not all age groups (infant, toddlers, young children or older children, adolescents or adults) are included in each national dietary survey. Usually, they are conducted on a limited number of days (up to seven) and use different methodologies (24h dietary recall, food diary, food frequency questionnaire), as summarised in Table 2. This lack of harmonisation compromises an accurate mycotoxin exposure assessment and does not allow the generation of European estimates of dietary exposure. To overcome this limitation a recent study was executed to assess how existing consumption data could be improved by developing a 'Compiled European Food Consumption Database' (Vilone *et al.*, 2014). According to this study, this database provides a fundamental tool to perform exposure assessments at the European level.

Table 2. Recent reports on worldwide exposure assessment to multiple mycotoxins in food products, published between 2010 and 2016.

Country	Population group (age, years old)[1]	Samples	Number of analysed mycotoxins (toxin group)[2]	Food consumption (data collection)	Analytical method: occurrence[1,3]	Handling non-detects: substitution method[1]	Exposure assessment	References
Argentina	adults (n.r.)	cow's milk	3 (AFs, TTCs, ZEA)	Argentinean Ministry of Agriculture	ELISA	n.r.	probabilistic	Signorini *et al.*, 2012
Belgium	adults (>15)	cereal-based foods	13 (TTCs+; ZEA+)	Belgian National Food Consumption Survey 2004 (2-day 24h recall)	LC-MS/MS	0, LOD/2, LOD	deterministic & probabilistic	De Boevre *et al.*, 2013
Belgium	adults (>15)	nuts, dried fruits	2 (AFs, OTs)	Belgian National Consumption Survey 2004 (2×24 h recall)	n.r.	0	probabilistic	Van de Perre *et al.*, 2015
China P.R.	children & adults (>7)	wheat & maize foods	3 (TTCs+)	Shangai Food Consumption Survey 2012-13 (24-h recall)	LC-MS/MS	LOD/2	deterministic & probabilistic	Han *et al.*, 2014
China P.R.	children & adults (2-100)	wheat based foods	4 (*Alternaria* toxins)	China National Nutrient and Health Survey 2002 (3×24 h recall)	UPLC-MS/MS	0, LOD/2,LOD	deterministic	Zhao *et al.*, 2015
France	children (3-17); adults (18-79)	212 core foods (*as consumed*)	25 (AFs, TTC+, FBs, OTs, PAT, ZEA+)	Second National Individual Dietary Consumption Survey 2006-7 (7-day food record diary)	LC-FD; LC-MS/MS	0, LOD, LOQ	deterministic	Sirot *et al.*, 2013
New Zealand	children (5-14); adults (>15)	maize-based foods, nuts & nuts foods, dried fruits, spice	4 (AFs)	National Nutrition Survey 1997 (24h recall)	n.r.	LOD/2, 0	deterministic	Cressey and Reeve, 2013
Portugal	infants (1-3)	breakfast cereals	12 (AFs, TTCs, OTs)	Pilot survey 2014 (3-day food diary)	LC-FD; GC-MS, UPLC-MS/MS	0, LOD/2, LOD	deterministic & probabilistic	Assunção *et al.*, 2015
Spain	infants (0-3); children (4-9); adolescents (10-19); adults (20-65); elders (>65); immigrants (17-51) celiac sufferers (16-75)	cereal food, baby food, dried fruits	4 (AFs)	Catalonia survey (food frequency questionnaire)	LC-FD	LOD/2	probabilistic	Cano-Sancho *et al.*, 2013
Spain	adolescents & adults (n.r.)	coffee	21 (AFs, TTCs+, FBs, OTs, ENNs, BEA, STE)	Spanish Agency for Food Safety Survey 2009	LC-MS/MS	0	deterministic	García-Moraleja *et al.*, 2015
The Netherlands	children (2-6) & adults (7-69)	88 composite samples (as consumed)	48 (PAT, AFs, OTA, FBs, ZEA, TTCs, ergot alkaloids, *Alternaria* toxins, BEA, ENNs)	Dutch National Food Consumption Surveys: 2005/2006 and 2007/2010 (questionnaire, 2-day food diary, 2×24 h recall)	LC-MS/MS, LC-FD	LOD, LOQ	probabilistic	Sprong *et al.*, 2016a,b

[1] n.r. = not reported; LOD = limit of detection; LOQ = limit of quantification.

[2] AFs = aflatoxins; BEA = beauvericin; ENNs = enniatins; OTA = ochratoxin A; OTs = ochratoxins; FBs = fumonisins; PAT = patulin; STE = sterigmatocystin; TTCs = trichothecenes; ZEA = zearalenone; + includes metabolites of that mycotoxin group.

[3] ELISA = enzyme-linked immunosorbent assay; FD = fluorescence detection; GC = gas chromatography; LC = liquid chromatography; MS = mass spectrometry; UPLC = ultrahigh performance liquid chromatography.

Recent surveys highlight the fact that humans are more frequently exposed to multiple than to single mycotoxins. LC-MS/MS has been the method mostly used for analysis and quantification of multiple mycotoxins and their metabolites in food (Berthiller *et al.*, 2016; Malachová *et al.*, 2014; Turner *et al.*, 2015). As mycotoxins comprise a wide range of chemical properties, the extraction and chromatographic conditions have to be compromised (Capriotti *et al.*, 2012). The influence of matrix effects is the major challenge in developing reliable quantitative multi-analyte methods therefore, considerable efforts to control matrix effects should be carried out to obtain accurate results (Turner *et al.*, 2015), namely, the inclusion of a sample clean-up step (e.g. using QuEChERS) and the compensation of the signal suppression/enhancement through the usage of matrix matched standards (Berthiller *et al.*, 2016; Malachová *et al.*, 2014; Turner *et al.*, 2015).

Mycotoxin contamination datasets are characterised by the presence of non-detects or none quantified values which constitutes an important issue for the exposure assessment studies (Assunção *et al.*, 2015; Cano-Sancho *et al.*, 2013; Sirot *et al.*, 2013). Therefore, a representative food sampling design (selecting the most susceptible foods, considering a large set of individual and/or composite samples), an accurate chemical analysis method (with low detection limits) and a suitable method to manage left-censored data will be decisive to obtain realistic exposure estimations with low level of uncertainty. This could be particularly important for the exposure assessment of vulnerable population groups such as small children that are generally exposed to higher levels of mycotoxins than adults (Alvito *et al.*, 2010; Assunção *et al.*, 2015; Cano-Sancho *et al.*, 2013; Sirot *et al.*, 2013).

In order to draw more sophisticated exposure scenarios an increasing number of authors applied information technology on probabilistic models instead of deterministic ones. One of the main drawbacks of the deterministic approach is that it does not allow calculating complicated statistics such as high quartiles. Defining high-level consumers is crucial for the outcome of risk assessment. In practice, it determines the proportion of the population that would exceed a health-based limit. Therefore, when refinements are required, simulation methods are proposed as the best approach, particularly for high quartiles (Marin *et al.*, 2013).

The indirect approach obtained with the combination of data of mycotoxin occurrence in food and food consumption patterns is associated with some limitations for the mycotoxins exposure assessment, including the heterogeneous distribution of mycotoxins in food, the possible exposure through other exposure routes than ingestion, the presence of masked mycotoxins, the influence of food processing, inter-individual variation in absorption, distribution, metabolism and excretion (ADME), and the under- and overestimation in food consumption data (Arcella and Leclercq, 2004; Heyndrickx *et al.*, 2014). These limitations could lead to an under- and/or overestimation of the exposure, and biomarkers have been proposed as a suitable alternative. Human biomonitoring is considered a quite new frontier for establishing the real human exposure to mycotoxins. Recent results on this domain (Gerding *et al.*, 2014, 2015; Heyndrickx *et al.*, 2015; Warth *et al.*, 2012a,b) surprisingly revealed a level of exposure to mycotoxins above the widely accepted tolerable daily intake values, especially to DON, highlighting the importance to perform mycotoxin biomonitoring studies. Typical biomarkers of exposure are the parent toxins themselves, protein or DNA adducts, and/or major phase I or phase II metabolites (e.g. glucuronide conjugates), which are measured in biological fluids such as urine or plasma/serum, and are related to the actual intake of the toxin through contaminated food (Warth *et al.*, 2013). Table 3 summarises studies developed in different countries and performed using a multibiomarker approach to determine the human mycotoxin exposure. LC/MS-MS methods were the mainly used for the quantification of biomarkers contents in urine, the biological fluid mostly used to determined mycotoxin biomarkers contents (Table 3). The number of analytes studied simultaneously varied between four (Warth *et al.*, 2012a) and 33 (Heyndrickx *et al.*, 2015) compounds. The use of β-glucuronidase-assisted hydrolysis (in order to increase the levels of the parent toxins) (Ahn *et al.*, 2010; Shephard *et al.*, 2013; Solfrizzo *et al.*, 2011, 2014; Wallin *et al.*, 2015), immunoaffinity columns (IAC) (Ahn *et al.*, 2010; Rubert *et al.*, 2011; Shephard *et al.*, 2013; Solfrizzo *et al.*, 2011, 2014; Wallin *et al.*, 2015) and solid-phase extraction (SPE) (Heyndrickx *et al.*, 2015; Njumbe Ediage *et al.*, 2012; Rodríguez-Carrasco *et al.*, 2014a,b; Shephard *et al.*, 2013; Solfrizzo *et al.*, 2011, 2014; Wallin *et al.*, 2015) were applied by some authors as sample preparation and clean-up procedures. However, some studies have also successfully used the so-called dilute and shoot approach by omitting any cleanup procedure (Abia *et al.*, 2013; Ezekiel *et al.*, 2014; Gerding *et al.*, 2014, 2015; Heyndrickx *et al.*, 2015; Shephard *et al.*, 2013; Warth *et al.*, 2012a,b). Although the analysis of mycotoxins in human urine is another important data source for exposure assessment, some challenges are posed to human mycotoxin multibiomarker approaches. These challenges include the lack of toxicokinetic data on mycotoxins in humans resulting in a lot of uncertainties that should be taken into account when perform a risk assessment based on urinary mycotoxin levels; difficulties to correlate human dietary habits, concurrent mycotoxin contamination of food and consequent presence of these mycotoxins in human urine; obstacles to comparison of obtained results between different studies, considering the differences in age, detection limits, number of subjects included in the study and the analytical performances of the used methods (Gerding *et al.*, 2014; Heyndrickx *et*

Table 3. Human mycotoxin exposure assessment using a multibiomarker approach.[1]

Country	No. of analytes	Analytes included[2]	Sample preparation and cleanup[3]	Reference
Austria	4	DON, DON-3-GlcA, DON-15-GlcA, DOM-1	'dilute and shoot'	Warth *et al.*, 2012a
Bangladesh	23	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB_1, FB_2, AFB_1, AFG_2, AFB_2, AFM_1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β-ZAL-14-GlcA, OTA, Otα, enniatin B and DH-CIT	'dilute and shoot'	Gerding *et al.*, 2015
Belgium	18	AFM_1, AFB_1, AFB1-N7-Gua, OTA, OTα, 4-OH-OTA, FB_1, HFB_1, DON, DON-3-GlcA, DOM-1, T-2, HT-2, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL, CIT	liquid-liquid extraction + SPE	Njumbe Ediage *et al.*, 2012
	33	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, FB_1, FB_2, FB_3, HFB_1, OTA, Otα, T-2, HT-2, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, DOM-GlcA, 3-ADON, 3ADON-15-GlcA, 15-ADON, 15ADON-3-GlcA, DAS, FUS-X, ZEA, ZEA-14-GlcA, α-ZAL, α-ZAL-7-GlcA, α-ZAL-14-GlcA, β-ZAL, β-ZAL-14-GlcA, CIT, DH-CIT	'dilute and shoot' or liquid-liquid extraction + SPE	Heyndrickx *et al.*, 2015
Cameroon	15	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, T-2, HT-2, NIV, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL	'dilute and shoot'	Abia *et al.*, 2013; Warth *et al.*, 2012b
Germany	23	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB_1, FB_2, AFB_1, AFG_2, AFB_2, AFM_1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β-ZAL-14-GlcA, OTA, Otα, enniatin B and DH-CIT	'dilute and shoot'	Gerding *et al.*, 2014, 2015
Haiti	23	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB_1, FB_2, AFB_1, AFG_2, AFB_2, AFM_1, ZEA, ZAN, α-ZAL, β-ZAL, ZEA-14-GlcA, ZAN-14-GlcA, α/β-ZAL-14-GlcA, OTA, Otα, enniatin B and DH-CIT	'dilute and shoot'	Gerding *et al.*, 2015
Italy	7	AFM_1, OTA, FB_1, DON, DOM-1, α-ZOL, β-ZOL	IAC + SPE + β-glucuronidase/sulphatase	Solfrizzo *et al.*, 2011
	8	DOM-1, DON, AFM_1, FB_1, β-ZAL, α-ZAL, ZEA, OTA	IAC + SPE + β-glucuronidase/sulphatase	Solfrizzo *et al.*, 2014
Korea	4	AFM_1, OTA, FB_1, FB_2	IAC + SIDA + β-glucuronidase	Ahn *et al.*, 2010
Nigeria	14	AFM_1, FB_1, FB_2, OTA, DON, DON-3-GlcA, DOM-1, NIV, T-2, HT-2, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL	'dilute and shoot'	Ezekiel *et al.*, 2014
South Africa	15	AFM_1, OTA, FB_1, FB_2, DON, DON-3-GlcA, DON-15-GlcA, DOM-1, T-2, HT-2, NIV, ZEA, ZEA-14-GlcA, α-ZAL, β-ZAL	'dilute and shoot' or IAC + β-glucuronidase/sulphatase or SPE	Shephard *et al.*, 2013
Spain	11	AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB[1], FB_2, DON, T-2, HT-2, ZEA	IAC	Rubert *et al.*, 2011
	15	DOM-1, DON, 3-ADON, FUS-X, DAS, NIV, NEO, HT-2, T-2, ZAN, α-ZAL, β-ZAL, ZEA, α-ZOL, β-ZOL	liquid-liquid extraction + SPE	Rodríguez-Carrasco *et al.*, 2014a,b
Sweden	10	AFM_1, DON, FB_1, FB_2, NIV, OTA, ZEA, α-ZOL, β-ZOL, DOM-1	IAC + SPE + β-glucuronidase/sulphatase	Wallin *et al.*, 2015

[1] All studies used urine as biological sample. Liquid chromatography coupled with tandem mass spectrometry was used for analysis in all studies, except for Spain where gas chromatography coupled with tandem mass spectrometry was used.

[2] 15-ADON = 15-acetyldeoxynivalenol; 15ADON-3-GlcA = 15-acetyldeoxynivalenol-3-glucuronide; 3-ADON = 3-acetyldeoxynivalenol; 3ADON-15-GlcA = 3-acetyldeoxynivalenol-15-glucuronide; 4-OH-OTA = hydroxylated form ochratoxin A; AFB_1 = aflatoxin B_1; AFB_1-N7-Gua = aflatoxin B_1-N7Guanine; AFB_2 = aflatoxin B_2; AFG_1 = aflatoxin G_1; AFG_2 = aflatoxin G_2; AFM_1 = aflatoxin M_1; α-ZAL = α-zearalanol; α-ZAL-14-GlcA = α-zearalanol-14-glucuronide; α-ZAL-7-GlcA = α-zearalanol-7-glucuronide; α-ZOL = α-zearalenol; β-ZAL = β-zearalanol; β-ZAL-14-GlcA = β-zearalanol-14-glucuronide; β-ZOL = β-zearalenol; CIT = citrinin; DAS = diacetoxyscirpenol; DH-CIT = dihydrocitrinone; DOM-1 = de-epoxy deoxynivalenol; DOM-GlcA = deepoxy-deoxynivalenol-glucuronide; DON = deoxynivalenol; DON-15-GlcA = deoxynivalenol-15-glucuronide; DON-3-GlcA = deoxynivalenol-3-glucuronide; FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; FB_3 = fumonisin B_3; FUS-X = fusarenone X; HFB_1 = hydrolysed fumonisin B_1; HT-2 = HT-2-toxin; HT-2-4-GlcA = HT-2-toxin-4-glucuronide; NIV = nivalenol; OTA = ochratoxin A; OTα = ochratoxin alpha; T-2 = T-2-toxin; ZAN = zearalanone; ZAN-14-GlcA = zearalanone-14-glucuronide; ZEA = zearalenone; ZEA-14-GlcA = zearalenone-14-glucuronide.

[3] IAC = immunoaffinity column; SIDA = stable isotope dilution assays; SPE = solid phase extraction.

al., 2015; Rubert *et al.*, 2011; Wallin *et al.*, 2015; Warth *et al.*, 2013). For more detailed information on mycotoxin multibiomarker approach, see De Nijs *et al.* (2016).

4. Risk characterisation of multiple mycotoxins

Different approaches for risk characterisation of toxic compounds

Risk characterisation is the last step of the risk assessment process, integrating information obtained in hazard assessment and exposure assessment steps. Risk characterisation aims to produce scientific advice for risk managers and has been defined as the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterisation and exposure assessment (FAO/WHO, 2009). Within the risk characterisation step, a comparison between the dietary exposure and the relevant health-based guidance value is performed. It should be highlighted that reference doses are only defined for the adult population and this renders difficulty in the children and infants risk assessment for which the available reference doses are not suitable.

Different approaches have been used for risk characterisation of toxic compounds, according to their genotoxic and carcinogenic potential. In the risk characterisation for non-genotoxic and carcinogenic substances, a health-based guidance value is compared with estimates of dietary exposure. The Hazard Quotient (HQ) is derived by comparing their respective reference dose (e.g. tolerable daily intake (TDI)) with the exposure to evaluate whether the exposure level is tolerable or not, and a ratio of HQ<1 indicates a tolerable exposure level and a ratio of HQ>1 indicates a non-tolerable exposure level (EFSA, 2013). For those substances that are genotoxic and carcinogenic, the traditional assumption is that some degree of risk may exist at any level of exposure and it is recommended that the exposure should be as low as reasonably achievable (ALARA). However, this approach presents limited value, because it does not allow risk managers to prioritise different contaminants or to target risk management actions. The Margin of Exposure (MOE) approach, which is the ratio between an amount of a substance producing a small but measurable effect in laboratory animals or humans and the estimated human exposure, has been proposed by WHO and EFSA as the methodology for risk characterisation of compounds that are genotoxic and carcinogenic (EFSA, 2013; FAO/WHO, 2009). The Scientific Committee of EFSA considers that MOE values of 10,000 or more, when based on a benchmark dose lower confidence limit 10 ($BMDL_{10}$) from an animal study and taking into account overall uncertainties in the interpretation are considered 'of low concern from a public health point of view'. Benchmark dose lower confidence limit corresponds to the lower boundary of the confidence interval on the benchmark dose. EFSA's Scientific Committee notes that the magnitude of a MOE only indicates a level of concern and does not quantify risk (EFSA, 2012, 2013).

Given the number of chemicals to which humans are potentially exposed, the risk characterisation to this exposure should be also addressed. As referred in the hazard assessment section, combination effects could occur as a result of different chemicals present in food and consequently different combined effects could happen. Diverse approaches have been used for multiple chemicals risk characterisation, most of these are based on the concepts of CA and IA. Examples of risk characterisation methods include the Hazard Index (HI), Point of Departure Index (PODI), Combined Margin of Exposure Index (MOET), Toxic Unit Summation (TUS) and Relative Potency Factors/Toxic Equivalency Factors (RPF/TEF) (Sarigiannis and Hansen, 2012; WHO, 2009). The Hazard Index, the mostly used for non-genotoxic and carcinogenic compounds, is defined as the sum of the respective Hazard Quotients (HQs) for individual mixture components, calculated as the ratio between exposure and a reference dose and has been put forward as the preferred approach when extensive mechanistic information of the mixture components is not available. The HI does not predict the overall health effect of the mixture, but provide a measure of the total risk based on the individual risk of each component. Thus, the HI can be used also for identification of the largest contributors to the risk (EFSA, 2013; Sarigiannis and Hansen, 2012). The combined MOE is called the MOET, and is calculated as the reciprocal of the sum of the reciprocals of the individual MOEs (EFSA PPR, 2008). MOET is usually used for the mixtures of chemicals that have genotoxic and carcinogenic potential. According to the PPR Panel of EFSA, no established criteria has been set yet to define the magnitude of an acceptable MOE for mixtures of chemicals with a threshold effect. However, it is widely accepted that for MOEs above the uncertainty factor of 100, the combined risk is considered acceptable (EFSA PPR, 2008; Sarigiannis and Hansen, 2012).

Characterisation of risk from exposure to multiple mycotoxins in food

Several studies were performed to evaluate the dietary exposure to mycotoxins (Table 2), although the characterisation of risk resulting from that exposure it is not usually done. In the last years, few works were published applying methodologies of evaluation of risk using approaches that consider the simultaneous exposure to different mycotoxins. Assunção *et al.* (2015) performed a risk assessment of single and 12 mycotoxins present in breakfast cereals consumed by children (1-3 years old) from Lisbon region (Portugal). The daily exposure of children

to AFs, OTA, FBs and trichothecenes were determined using deterministic and probabilistic approaches. For the non-carcinogenic mycotoxins, the authors used the HI to characterise the risk of mycotoxins from the same family group. For the AFs, as carcinogenic compounds, MOET was determined to characterise the risk. García-Moraleja *et al.* (2015) studied the presence of 21 mycotoxins in coffee and calculated the daily intake of mycotoxins from coffee consumption using deterministic approach at various scenarios of food consumption in Spanish adolescents and adults. The risk was characterised via comparison with the TDI or the provisional tolerable weekly intake (PTWI) proposed by The Joint FAO/WHO Expert Committee on Food Additives (JECFA). Han and collaborators (2014) assessed the cumulative health risks of concomitant exposure via dietary intake to multiple mycotoxins, namely DON and its acetyl derivatives of 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol. Sirot *et al.* (2013) evaluated the exposure to 25 mycotoxins of the general French population within the second French total diet study. The health risk assessment was performed via comparison of the dietary exposure with international health-based guidance values (TDI, PMTDI, or PTWI), and the population rate exceeding the health-based guidance value was also estimated for adults and children. Cano-Sancho *et al.* (2013) assessed the exposure of Catalonian (Spain) population to aflatoxins (AFB_1, AFB_2, AGB_1 and AFG_2) and individual AF risk characterisation was calculated by estimating the MoEs, dividing the $BMDL_{10}$ by the average and percentile 95 of the exposure estimates. De Boevre *et al.* (2013) assessed the quantitative dietary exposure of mycotoxins and their modified or masked forms (13 in total) through consumption of cereal-based food products of the Belgian population. The output of exposure (individual and family groups) was compared to the mycotoxins TDI.

Main challenges in risk characterisation of multiple mycotoxins

As referred previously, humans are naturally and frequently exposed to a multitude of mycotoxins, but health risk assessments are usually performed on individual mycotoxins, which may underestimate the total risks. This could be explained by the fact that evaluation of all possible combinations of mycotoxins that can occur in food and consequently their potential combined toxic effects are virtually impossible (FAO/WHO, 2009). One of the main challenges posed to risk characterisation of multiple mycotoxins is the absence of toxicological data that could be used to characterise the risk. The use of toxicological data is mandatory to risk characterisation, and independently of the mechanisms of combined effects or interactions, data of multiple mycotoxins are not yet complete for all the toxins potential present in food.

The use of harmonised terminology is an important step for a common understanding of the key terms and concepts that are used when dealing with combined exposure to multiple chemicals for risk assessment purposes (EFSA, 2013). This fact constitutes another challenge for risk characterisation of multiple mycotoxins in food. It is visible that researchers used similar approaches, however not always using the same terminology. An improvement in the quality of the obtained results is expected if a harmonised approach and methodology for risk characterisation is achieved.

5. Conclusions and future perspectives

In the context of food safety, risk assessment is a conceptual framework that aims to estimate the risk of occurrence of adverse health effects after exposure to mycotoxins present in food. Risk assessment of multiple mycotoxins is a very challenging domain integrating knowledge from different scientific areas and demanding a big effort from scientific community, risk assessors and managers. The challenges posed to risk assessment of multiple mycotoxins were reviewed in the present work, and Figure 1 reflects the interrelation between different risk assessment steps including the identified challenges for each step.

This study gathers, for the first time, an overview of the main challenges associated to the human health risk assessment of multiple mycotoxins present in food. Additionally, a physiologically-based framework for the hazard assessment of multiple mycotoxins in food is proposed. Hazard assessment pointed challenges related to the prioritisation of mixtures for risk assessment purposes; harmonisation of the experimental approaches for toxicity testing and mathematical models to analyse interactions, among others. Future *in vitro* and *in vivo* studies on combinatory toxicology are still needed and should be directed to cover: (1) mixtures of mycotoxins that are more likely to occur; (2) realistic low concentrations, considering the range of human exposure measured, e.g. in biomonitoring studies; (3) prolonged exposure times to better mimic long-term human exposure and (4) several relevant endpoints, including genotoxicity and immunotoxicity using high throughput methodologies. Furthermore, modelling of data generated in such studies using adequate mathematical models is central to uncover interactions. On the other hand, exploring genotoxic effects and interactions at the mechanistic level remains a challenging issue because information on the mechanism of action of single mycotoxins within a mixture may be somewhat limited, making it difficult to understand their interactions. Such data can be generated from toxicity studies using predictive and high throughput methodologies, including toxicogenomic approaches. Exposure assessment highlights the importance concerning the availability of harmonised food consumption data to perform exposure assessments as well as the development of multianalyte methods for multiple mycotoxins quantification in different

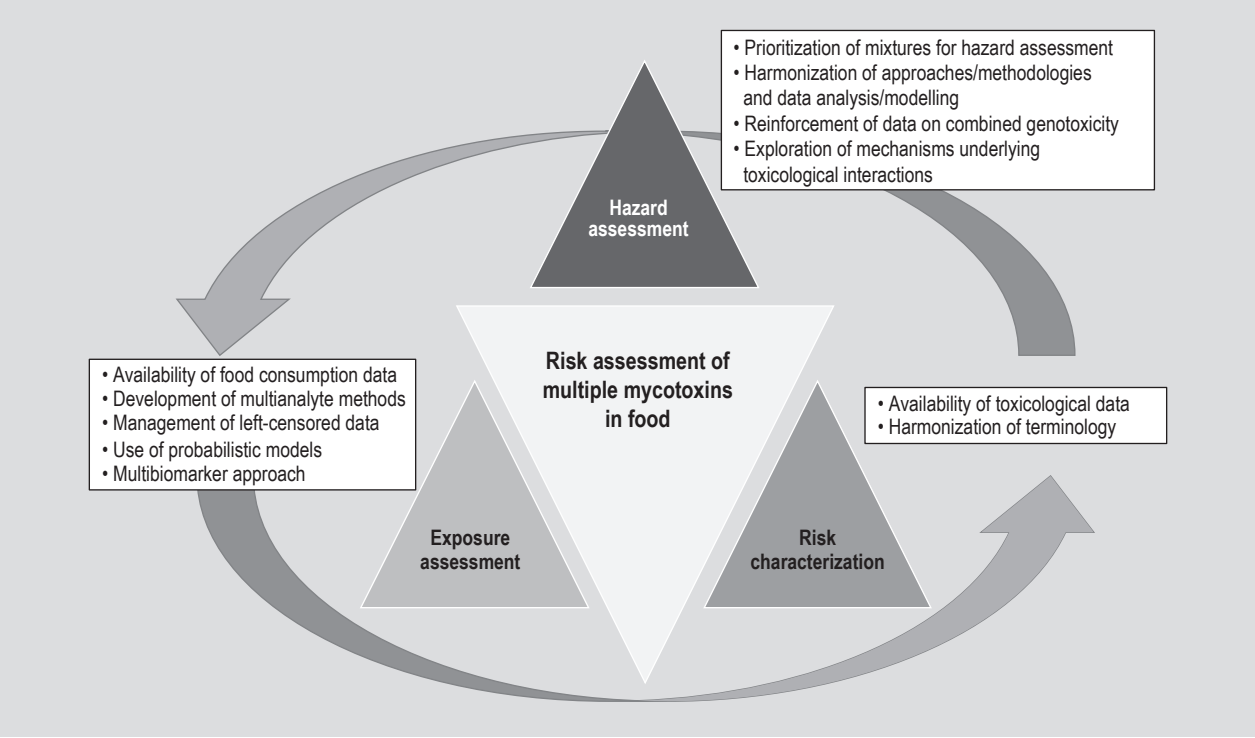

Figure 1. Holistic overview reflecting the interrelation between different steps of multiple mycotoxins health risk assessment and respective challenges.

food matrices. The management of left-censored data is crucial for the quantification of low levels of mycotoxins as well as the use of probabilistic methods allowing a more realistic risk assessment and considering different exposure scenarios. Considering that risk characterisation is the last step of the risk assessment process, the quality of data obtained in the hazard characterisation and exposure assessment steps are crucial to evaluate properly the risk associated to the exposure to multiple mycotoxins. At this level, the availability of toxicological data and the use of harmonised terminology pose the main challenges for an accurate risk characterisation.

A multidisciplinary effort should be developed to perform the human health risk assessment of multiple mycotoxins present in food, considering that the information obtained from the risk assessment process will be used by risk managers to prioritise risk and to develop actions towards disease prevention. The present work reinforces the urgent need to perform more research studies to clarify the nature of interactions among mycotoxins and derive new health-based guidance values for grouped mycotoxins and/or co-occurring mycotoxins in order to protect human health.

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