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Mycotoxins co-contamination: Methodological aspects and biological relevance of combined toxicity studies.

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Abstract

Mycotoxins are secondary fungal metabolites produced mainly by *Aspergillus*, *Penicillium* and *Fusarium*. As evidenced by large-scale surveys, humans and animals are simultaneously exposed to several mycotoxins. Simultaneous exposure could result in synergistic, additive or antagonistic effects. However, most toxicity studies addressed the effects of mycotoxins separately.

We present the experimental designs and we discuss the conclusions drawn from *in vitro* experiments exploring toxicological interactions of mycotoxins.

We report more than 80 publications related to mycotoxin interactions. The studies explored combinations involving the regulated groups of mycotoxins, especially aflatoxins, ochratoxins, fumonisins, zearalenone and trichothecenes, but also the "emerging" mycotoxins beauvericin and enniatins. Over 50 publications are based on the arithmetic model of additivity. Few studies used the factorial designs or the theoretical biology-based models of additivity. The latter approaches are gaining increased attention. These analyses allow determination of the type of interaction and, optionally, its magnitude. The type of interaction reported for mycotoxin combinations depended on several factors, in particular cell models and the tested dose ranges. However, synergy among *Fusarium* toxins was highlighted in several studies. This review indicates that well-addressed *in vitro* studies remain valuable tools for the screening of interactive potential in mycotoxin mixtures.

Introduction

Mycotoxins are secondary fungal metabolites produced under specific environmental conditions by a variety of molds, mainly *Aspergillus*, *Penicillium* and *Fusarium spp*. As secondary metabolites, they are not essential for life, but may provide the fungus with an ecological advantage in certain environments. Some 300 compounds have been recognized as mycotoxins of which around thirty are considered as threat to human or animal health. Mycotoxin exposure via food and feed may result in many different adverse health effects such as carcinogenicity, immunotoxicity, reproductive toxicity, hepatotoxicity, nephrotoxicity, *etc.* (Bennett and Klich, 2003). Global surveys indicate that more than 70% of the samples of feed and feed raw materials are positive for at least one mycotoxin (Streit *et al.*, 2013a).

Human and animals are simultaneously exposed to several mycotoxins (Schothorst and van Egmond, 2004; Rodrigues and Naehrer, 2012; Streit *et al.*, 2013b); thus, there is a need for an update of the traditional single mycotoxin risk assessment approach (SCF, 2002). Indeed, in the field of toxicological evaluation of chemical mixtures, the consensus is that the customary chemical-by-chemical approach to risk assessment is in danger of underestimating the risk of chemicals to health (Kortenkamp *et al.*, 2009). Simultaneous exposure to different toxins could result in antagonistic, additive or synergistic effects. Although the demonstration of synergism would heighten concerns about health risks, the implications of additive combination effects have not received adequate attention. Sometimes the threshold dose for toxic effects may be exceeded in case of exposure to a mixture although the exposure to each single compound is unlikely to pose risk (Silva *et al.*, 2002). Therefore, an increasing number of mycotoxin studies are devoted to their combined toxicity, especially to the exploration of the type of toxicological interactions.

The toxicity of a mixture is complex. The general principles for such analyses have been thoroughly reviewed elsewhere (ATSDR, 2004; Binderup, 2008). Testing for a possible interaction in mixture toxicity requires a comparison of the actual experimentally determined effects of the mixture with the theoretically expected no interaction effects. This prediction of no interaction, the null hypothesis, is done based on the toxicity of the individual compounds. Stronger-than-expected effects indicate synergism whereas lower-than-expected effects indicate antagonism. Several methods have been proposed but a generally agreed definition of zero interaction does not yet exist (Groten *et al.*, 2001). In this review we present the experimental designs and statistical aspects as well as the main conclusions drawn from experiments exploring interactions in combined toxicity of mycotoxins.

1. The reality of the mycotoxins co-contamination

The reality of mycotoxins co-contamination is confirmed on the one hand by the co-occurrence of these toxins in food and feed stuff and on the other hand by co-exposure monitoring survey.

The co-occurrence of mycotoxins in food and feed is explained by three different reasons: (i) most fungi are able to simultaneously produce several mycotoxins, (ii) commodities can be contaminated by several fungi simultaneously or in quick succession, and (iii) the complete diet comprised different commodities. In practice, the co-occurrence of mycotoxins represents the rule and not the exception.

In a three-year monitoring (2009 - 2011) on the worldwide occurrence of mycotoxins in feedstuffs and feed, Rodrigues and Naehrer (2012) showed that 48% of 7049 analyzed samples sourced in the Americas, Europe and Asia were contaminated with two or more of the tested mycotoxins (aflatoxins, zearalenone, deoxynivalenol, fumonisins and ochratoxin A). A literature review of european multi-mycotoxin contamination studies indicated that 75% to 100% of animal feed samples to contain more than one mycotoxin (Streit *et al.*, 2012), while the co-occurrence of more than two mycotoxins was reported in 95% of spanish barley samples (Ibanez-Vea *et al.*, 2012). Analyzing 83 samples of maize, wheat, barley and silage

issued from Europe, America and Australia by a multi-mycotoxin HPLC-MS/ MS approach Streit *et al.* (2013) have shown that all the samples were co-contaminated by 7 to 69 mycotoxins or other potentially toxic secondary metabolites, mainly produced by the *Fusarium* genus. Moreover, combination of *Aspergillus* and *Fusarium* mycotoxins can be found in the same matrix. The co-occurrence aflatoxin B_1 (AFB_1), ochratoxin A (OTA) and deoxynivalenol (DON) accounted for 55% of the multi-contaminated Spanish barley samples (Ibanez-Vea *et al.*, 2012).The fact that unlike other foodborne toxins or microorganisms, most mycotoxins are resistant to milling, processing and heat treatments, increases the risk of their persistence in the food and feed chains and may participate to the co-contamination (Milicevic *et al.*, 2010, Streit *et al.*, 2012).

These trends depicted by food and feed monitoring for mycotoxins co-contamination are corroborated by exposure data collected in several human bio-monitoring studies. Simultaneous measurement of multiple mycotoxins using advanced LC-MS/MS technique for human exposure assessment surveys in Germany, southern Italy and central Africa showed that 52% to 100% of urine samples contained biomarkers for two or more mycotoxins, and up to five mycotoxins were detected in a severe case of co-exposure (Abia *et al.*, 2013; Gerding *et al.*, 2014; Solfrizzo *et al.*, 2014). Moreover, as shown in Table 1, the exposure data highlight clearly that any kind of combinations involving mycotoxins irrespective of their producing fungi and their known geographical distribution could threaten consumer's health.

2. Experimental designs and statistical aspects to assess mycotoxin toxicological interactions

Interactions are inferred when a mixture of chemicals produces a biological response greater or lower than expected. Thus, the key question remains what is to be expected from a combination of contaminants. The application of Loewe's additivity equation or of Bliss' independence criterion, based on the dose–response curves of single compounds, enables the simulation of a theoretical response that represents the expected behavior of the mixture when interaction is excluded. Some reviews have been written about the subject and several aspects of the problem are still debated, with a particular regard to the biological plausibility of these two different theoretical approaches (Greco *et al.*, 1995; Chou, 2006; Goldoni and Johansson, 2007).

Classically, a two-step approach is recommended when analyzing the pharmacological or toxicological interactions between the different compounds of a mixture (Suhnel, 1996). First, the expected effects of the combination for the case of no interaction have to be predicted. This means a clear statement of what effect size can be expected if the compounds in the mixture do not interact. Then, the data on the effects of the experimental combination have to be compared to the expected ones in order to classify the combination as additive (no interaction *i.e.* as expected), synergistic (*i.e.* interaction resulting in greater effect than expected) or antagonistic (*i.e.* interaction leading to lesser effect than expected). Several experimental designs that denote authors' point of view on this null case have been used in *in vitro* assessment of the combined effects of mycotoxins.

2.1. The arithmetic definition of additivity

In a number of studies, the expected mixture effect size was defined as equal to the arithmetic sum of the sizes of the effects for individual compounds when tested separately (Weber *et al.*, 2005; Kouadio *et al.*, 2007; Ribeiro *et al.*, 2010; Ficheux *et al.*, 2012; Klaric *et al.*, 2012). As an example for the null case, the expected size for the cytotoxic effect of a mixture could be defined as the sum of the cytotoxic effects induced by each mycotoxin alone in mono-exposure experiments, so:

Cytotoxic effect (mycotoxin 1 + mycotoxin 2) = Cytotoxic effect (mycotoxin 1) + Cytotoxic effect (mycotoxin 2)

Then when the measured cytotoxicty values are not significantly different or above or below the expected values the results are interpreted as additive, synergistic or antagonistic respectively.
Although intuitively plausible and very easy to handle, most researchers in the biomedical area seem to agree that combined effects do not simply equal the sum of single effects (Boedeker and Backhaus, 2010). The fallacy of this approach is better perceived when applying it to the combined effect of several doses of the same mycotoxin which by definition cannot behave synergistically, nor antagonistically. As an illustration, dose-response experiments on the cytotoxic effects of DON and fumonisin B_1 (FB_1) were conducted in a study on the *in vitro* myelotoxicity induced by mixtures of *Fusarium* mycotoxins on human hematopoietic progenitors (Ficheux *et al.*, 2012). The authors concluded to an antagonistic effect of both *Fusarium* mycotoxins as the measured values were significantly lower than expected values. If we consider that the 2µM FB_1 dose can be seen as a 1+1µM FB_1 application, the predicted value for 2µM FB_1using the arithmetic approach would be 60±8% cell viability, lower than the measured value (42±5%). Therefore the arithmetic sum model to which so many mycotoxin interaction studies referred to, does not provide a reasonable reference point.

2.2. Factorial design experiments

Factorial design experiments have been employed to assess interaction between mycotoxins (Tajima *et al.*, 2002; Heussner *et al.*, 2006; Lei *et al.*, 2013; Wan *et al.*, 2013a; Wan *et al.*, 2013b). When testing the effects of mixtures of varying combinations and the effects of each individual compound, the effect of any compound could be predicted by subtracting the mean of the groups not containing the compound from the mean of the other groups containing the compound (Groten *et al.*, 1996). In a full factorial design, each chemical in the mixture is studied at all dose levels of the other chemicals. This may require a large number of tested groups and can be very costly. Mycotoxin mixture studies favored the fractional factorials that enable more economy of experimentation because only part of the full factorial is run experimentally. A three-step study was proposed to detect interactions between five *Fusarium* mycotoxins inhibiting DNA synthesis *in vitro*. In stage 1 the combined action (additivity, or departure from additivity) was assessed for the entire mixture, but not for specific pairs of mycotoxins. Stage 2 was specifically meant to economically screen for significant departure from additivity of specific (pairs of) mycotoxins using central composite designs, which allowed to finally apply full factorial design only to two-factor (two mycotoxins) interactions of particular interest (Groten *et al*., 1998).
Despite the fact that interaction is definitely revealed by such statistical methods, the nature of interaction with regard to additivity, synergism or antagonism is not clearly explored and has to be inferred indirectly (Bhat and Ahangar, 2007). Applying a factorial design approach to elucidate the interactions in the combined cytotoxic effects of DON, nivalenol (NIV), zearalenone (ZEA) and FB_1 in swine jejunal epithelial cells, Wan *et al.* (2013a) first conducted dose-response experiments for each mycotoxin individually to select the range limits for subsequent interaction analysis. Then, a central composite design including a fractional factorial part was applied with four factors, i.e. DON, NIV, ZEA and FB1, in order to minimize the number of possible toxin combinations (44 possible combinations of every concentration of each toxin). Nonetheless, 16 more data-points (in addition to the individual dose-response experiments) were required for interaction analysis. Univariate analysis of variance conducted on such data revealed non-additive interactions in all mixtures except DON-ZEA-FB_1, though the type of non-additive interactions (synergy or antagonism) still remained to be established. The factorial design approach could also just point out “a potential for interactive (synergistic) effects of citrinin and ochratoxin A and possibly other mycotoxins in cells of renal origin” (Heussner *et al.*, 2006).

2.3. The theoretical biology models-based definitions of additivity

The most commonly used theoretical biology models-based definitions of zero interaction are Bliss' independent criterion also known as response addition, Loewe's additivity model also named concentration or dose addition (Goldoni and Johansson, 2007) and the median effect principle of the mass action law (Chou, 2006).

Bliss' independence criterion and Loewe's additivity model

The main assumption for Bliss' independent criterion is that the chemical agents act independently from one another. In other words, the mode and possibly the site of action of the compounds in the mixture differ. When no interaction occurs for a combination, the Bliss independent criterion for two toxic agents can be expressed by the following equation:

$E(x,y) = E(x) + E(y) – E(x)*E(y)$

where E is the fractional effect (between 0 and 1), and x and y are the doses of two compounds in a combination experiment.

Loewe's additivity model relies on the assumption that the toxic agents in the mixture of concern act on the same biological sites, by the same mechanisms of action and differ only in their potency. Relatively simple Loewe's additivity model extensions are the isobolographic method and its algebraic variant, the interaction index, particularly useful when assessing two toxic substances *in vitro*. The interaction index can be expressed as (Berenbaum, 1981):

$I = c_1/EC_{x,1} + c_2/EC_{x,2}$

with c_i denoting the applied concentrations (of agent 1 and 2, respectively) and EC_x their individual concentrations that provoke a certain effect x., *e.g.* the effect concentration 50% (EC_{50}). I<1, I>1 and I=1 mean the agents interact synergistically, antagonistically, or are additive. This index has been applied to mycotoxin pairs association (McKean *et al.*, 2006a; McKean *et al.*, 2006b).

Some papers simultaneously tested both Loewe's additivity and Bliss' independence criterion models of zero interaction for mycotoxins combined effects because there is no final agreement on the biological plausibility of these concepts (Tammer *et al*., 2007; Mueller *et al*., 2013). Both Bliss' independence criterion and Loewe's additivity models were used to analyze the inhibition of interferon gamma (IFNγ) production induced by co-exposure to mycotoxins, patulin, gliotoxin, citrinin and ochratoxin A on the human peripheral blood mononuclear cells (Tammer *et al.*, 2007). Dose-response data for the individual inhibition of IFNγ production by each mycotoxin and the mixture inhibition were generated. The dose–response relationships of the individual substances and the mixture were biometrically modelled by fitting the Hill-model to the experimental data set using the best-fit approach. The dose–response functions of individual mycotoxins allowed predictions of the additive responses based either on Bliss' independence criterion or Loewe's additivity models. For the combined effect of the doses of mycotoxin that individually induced a 20% inhibition of IFNγ production the predicted values for additivity were 59% for Bliss independence criterion model and 79% for Loewe's additivity model. Compared to the 69% inhibition of IFN-γ production that was actually induced for the co-exposure, the conclusion is that the combined effect for the four mycotoxins appeared synergistic based on Bliss' independence criterion model and antagonistic based on Loewe's additivity model. There still are ongoing debates on which is the "better" or even the "correct" concept (Boedeker and Backhaus, 2010); however, Loewe's additivity model is slightly preferred because of an overall higher biological plausibility (Goldoni and Johansson, 2007; Kortenkamp *et al.*, 2009). It is likely that most toxic substances exert actions that are not completely different and independent from those of other toxicants due to converging signaling pathways and inter-linked subsystems.

The isobolograms

The isobolographic method can be considered the graphical variant of the interaction index method (Tallarida, 2011). In a Cartesian coordinate system, doses of each agent that give a specified effect e.g. EC_{50} are represented on the x- and y-axes. The straight line connecting the

equally effective doses of the agents is assumed to represent the set of dose pairs that give the specified effect in a situation of no interaction (additivity). Actual dose pairs that give the specified effect are then experimentally determined and reported on the graph. Experimental dose pairs lie below the additivity line in synergistic associations, and above in antagonistic associations.

Isobolograms were drawn to analyze *in vitro* the interactions for *Penicillium* mycotoxins and *Fusarium* mycotoxins as well (Bernhoft *et al.*, 2004; Luongo *et al.*, 2006; Luongo *et al.*, 2008). However, the isobolographic method fails to take into account the variability of the data, and there is a need for further development of statistical methods to characterize accurately the interaction of combination of agents (Gennings *et al*., 1990). As an illustration, in a study investigating the type of interaction for the combined effects of four mycotoxins (fumonisin B1, α-zearalenol, nivalenol and deoxynivalenol) on porcine whole-blood cellular proliferation, dose-response data were generated for a range of six different doses of each mycotoxin individually and for six doses of their binary mixtures at fixed ratios (Luongo *et al.*, 2008). For the analysis of each binary combination, the IC_{50} value with its confidence interval for each mycotoxin alone was determined and represented on the x- or y-axis of an isobologram. The dose of mixture that corresponded to a 50% inhibition of cell proliferation was also estimated and subsequently, the corresponding doses for each mycotoxin and their confidence intervals were reported on the isobologram. An additivity line was drawn to connect the x- and y-axis at the levels of the individual IC_{50}s. However the study could not indicate the confidence band that was associated to the additivity line though the uncertainties for the doses of each mycotoxin in the mixture were represented. Hence, strong conclusions could not be draw concerning the position of the mixture point regarding the additivity line, especially to exclude additivity if any.

The Median Effect Principle of the Mass action law

Another concept that is independent of the mode of action and just considers both the potency (EC_{50}) and the shape of the dose-effect curve for each chemical agent and their mixture has been proposed (Chou, 2011). In the so-called Chou-Talalay method, the mass-action law allows a computer simulation of the individual dose-effect curves and the "no interaction" response that could be expected from the combined effect of several agents (Chou, 2006). Individual agents and their mixtures dose-effect relationship are biometrically modelled using the median-effect equation of the mass action law that is:

$f_a/f_u = (D/D_m)^m$

Where D is the dose of the agent (*e.g.* a cytotoxic mycotoxin), f_a is the fraction affected by D (*e.g.* percentage of viability inhibition/100), and f_u is the fraction unaffected (*i.e.* $f_u = 1 - f_a$). D_m is the median-effect dose (e.g. IC_{50}), and m is the coefficient signifying the shape of the dose-effect relationship ($m = 1$, $m > 1$, and $m < 1$ indicate hyperbolic, sigmoidal, and flat sigmoidal dose-effect curves, respectively).

Then interactions can be analyzed by a combination index- isobologram method derived from the median-effect equation. The combination index (CI) is calculated according to (Chou, 2011):

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j}$$

Where $^{n}(CI)_x$ is the combination index for n agents at x% inhibition, $(D)_j$ are the doses of n agents that exerts x% inhibition in combination, $(D_x)_j$ are the doses of each of n agents alone that exerts x% inhibition. CI = 0.9-1, CI <0.9, and CI >1.1 indicate an additive effect, a synergism, and an antagonism, respectively, regardless of the mechanisms or the units of the agents. Besides indicating the type of interaction (additivity, synergy or antagonism), this index allows a quantitative assessment of the magnitude of the interaction.

The Combination index-Isobologram method also known as the Chou-Talalay method that was tentatively introduced several years ago in the field of mycotoxin mixture assessment, is

gaining the interest of an increasing number of researchers (Koshinsky and Khachatourians, 1992; Jones *et al.*, 1995; Ruiz *et al.*, 2011b; Lu *et al.*, 2013; Tatay *et al.*, 2014; Wang *et al.*, 2014). We used this approach to analyze the interactions for the combined toxicity of *Fusarium* mycotoxins DON, NIV and their acetylated derivatives 3- and 15-acetyldeoxynivalenol (3- and 15-ADON) and fusarenon-X (FX) in human intestinal epithelial cells (Alassane-Kpembi *et al.*, 2013). Dose-response data for individual mycotoxins and their mixtures were generated and dose-response relationships were biometrically modeled using the Median-Effect Equation of the Mass-Action Law. The combination index values were then calculated over the range of the cytotoxicity observed. Binary or ternary mixtures of type B trichothecenes (DON, NIV, and their acetylated derivatives) demonstrated mainly synergistic cytotoxicity at low mycotoxin concentrations (cytotoxic effect between 10 and 30-40 %). At higher concentrations (cytotoxic effect around 50 %), the combinations had an additive or nearly additive effect. The magnitude of the synergistic interaction for 10% cytotoxicity was evaluated to range from 2 to 9.

3. Combined toxicity of mycotoxins

Using either of the methodological approaches described above, several teams have thoroughly examined the combined toxicity of mycotoxins *in vitro* and *in vivo*. The *in vivo* experiments have been reviewed elsewhere (Grenier and Oswald, 2011) and will not be discussed in this review. *In vitro* bioassays have obvious limitations; nonetheless they are less restrictive in the number of test groups which makes the assessment of complex mycotoxin mixtures easier. In particular, *in vitro* experiments allow for dose-response analysis of the individual contaminants and the mycotoxin mixtures. We will now review the *in vitro* experiments investigating the combined toxicity of mycotoxins. In most combined toxicity studies, the mycotoxins tested were grouped based on (i) a shared community in chemical structures (i.e aflatoxins or type B trichothecenes); (ii) toxicological modes of action (i.e mutagenic mycotoxins or carcinogenic mycotoxins), or (iii) their simultaneous production by a given fungi (i.e *Fusarium* mycotoxins or *Aspergillus* mycotoxins).

3.1. Aflatoxins and other mycotoxins

The aflatoxins are a group of closely related highly substituted coumarins containing a fused dihydrofurofuran moiety. Four aflatoxins may occur naturally: the two blue fluorescent toxins (B_1, B_2) that are characterized by fusion of a cyclopentenone ring to the lactone ring of the coumarin moiety, and the two greenish yellow fluorescent toxins (G_1, and G_2) that contain a fused lactone ring. AFB_2 and AFG_2 are considered relatively nontoxic unless they are first metabolically oxidized to AFB_1 and AFG_1*in vivo*. The metabolism of aflatoxin B_1 and B_2 in the mammalian body may result in two metabolites M_1 and M_2 as hydroxylated derivatives of the parent compound. Aflatoxins are hepatocarcinogenic agents in numerous animal species and have been implicated in the etiology of human hepatocellular carcinoma (Wild and Montesano, 2009).

In association with other mycotoxins, the mutagenic and cell viability effects of aflatoxins have been frequently questioned. Nearly all the papers addressing mutagenic activity of these mycotoxin combinations referred to the well-known Ames test using *Salmonella* Typhimurium strains TA 100 and TA 98 (Sedmikova *et al.*, 2001; Kuilman-Wahls *et al.*, 2002; Vilar *et al.*, 2003). However, a bioluminescence test using the marine bacterium *Photobacterium phosphoreum* strain NCMB 844 was also proposed (Yates *et al.,* 1987). Mutagenicity was analyzed with a dark mutant of this organism whose reversion to the bioluminescent condition is stimulated by compounds attacking guanine sites in desoxyribonucleic acids. Aflatoxins combinations have been assessed for their cytotoxic and genotoxic effects mainly in human and animal primary hepatocytes or transformed cell lines (Friedman *et al.*, 1997; He *et al.*, 2010; Ribeiro *et al.*, 2010; Corcuera *et al.*, 2011). Aflatoxin combinations are also considered potentially immunotoxic, thus they have been evaluated for

their combined effects on the viability and functionality of immune system cells (Theumer *et al.*, 2003; Herzog-Soares and Freire, 2004; Russo *et al.*, 2010; Theumer *et al.*, 2010; Russo *et al.*, 2011).
Publications related to mycotoxin mixtures involving aflatoxins have been grouped in studies presenting the combined effects of (i) the different aflatoxins, (ii) aflatoxin B_1 and other possibly carcinogenic mycotoxins, (iii) aflatoxin B_1 and other mycotoxins from *Aspergillus* and (iv) aflatoxin B_1 and mycotoxins from *Fusarium*.

Combined toxicity of the different aflatoxins
Arithmetic definition of additivity was used in a large part of the combined toxicity studies for the different aflatoxins that are presented in Table 2. Cell viability as an endpoint showed synergy for the combined toxicity of AFB_1 and AFB_2 in human umbilical vein endothelial cells while additivity was observed for the same endpoint in human lung fibroblast and in human ovarian cancer cell line A 2780 (Braicu *et al.,* 2010). No interaction was reported between AFB_1 and AFB_2 for RNA synthesis and membrane integrity in rat hepatocytes primary culture, while an undetermined interaction was revealed between AFB_1 and AFG_1 (Friedman *et al.,* 1997). The immunotoxic interactions between aflatoxin metabolites AFM_1 and AFM_2 excreted in milk and between these metabolites and their parent-compounds AFB_1 and AFB_2 have been investigated (Russo *et al.*, 2010; Russo *et al.*, 2011; Bianco *et al.*, 2012b). No interaction could be detected when macrophages were co-exposed to AFM_1 and AFM_2, while their combinations with the parent-compounds AFB_1 and AFB_2 resulted in stronger toxicity compared to individual toxins, suggesting a synergism. Naturally-occurring mixtures of aflatoxins, i.e. aflatoxins B_1, B_2, G_1 and G_2 have been rated as carcinogenic to humans (group 1) and the metabolite AFM_1 possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer (IARC). However we reported no publication analyzing *in vitro* the combined genotoxicity of the aflatoxins.

Combined toxicity of aflatoxins and other possibly carcinogenic mycotoxins
Besides aflatoxins, OTA and FB_1 are the only other mycotoxins that have been demonstrated to be carcinogenic in laboratory animals and rated as possible human carcinogens (group 2B). Co-exposure to these carcinogenic mycotoxins is not uncommon. Dietary exposure assessment in several villages in Tanzania showed that up to 82% of children tested were positive for blood AFB_1-albumin adducts and urinary FB_1 (Shirima *et al.,* 2013). Co-occurrence of AFB_1, OTA and FB_1 was also detected in 20% of randomly collected cereal and feed samples from households of an endemic nephropathy area in Croatia (Klaric *et al.,* 2009). This explains the interest to investigate the interaction between aflatoxins and these mycotoxins especially in term of carcinogenic effect (Table 3).
Arithmetic definition of additivity was mainly used in these combined toxicity studies. Antagonistic cytotoxicity in human hepatoma cells HepG2 has been strongly demonstrated for AFB_1 and FB_1 by the calculation of their interaction index (McKean *et al.*, 2006b). Conflicting conclusions for the clastogenic effect of AFB_1-OTA association exist. A quantitative analysis of DNA fragmentation in monkey kidney Vero cells exposed to both mycotoxins simultaneously, suggested an additive effect (El Golli-Bennour *et al.,* 2010). This conclusion was made based on the calculation of a ratio of expected to observed IC_{50} values for the mycotoxin mixture. However, the authors did not specify how the expected IC_{50} value was obtained. On the contrary, OTA was shown to reduce the DNA damage caused by AFB_1 alone in HepG2 cell line, while an increase of the mutagenic effect of AFB_1 in presence of OTA was reported using the *S.* Typhimurium mutagenicity test (Sedmikova *et al.,* 2001; Corcuera *et al.,* 2011). The authors speculated that AFB_1 and OTA could compete for the same CYP enzymes that represent a bio-activation route for AFB_1, and a higher affinity of OTA for the CYPs involved could result in less AFB_1 bio-activated molecules (AFB_1-epoxide) to attack and damage DNA.

No enhancement of the clastogenic effect has been noted when combining FB_1 and AFB_1 in rat primary hepatocyte and spleen mononuclear cell culture, whereas biomarkers of oxidative stress were lowered by the mixture compared to the individual AFB_1 effect (Ribeiro *et al.,* 2010; Theumer *et al.,* 2010).
With respect to immunotoxic effects, the AFB_1- FB_1 mixture was more effective in reducing the mitogenic response and cytokine production of mononuclear cells on the one hand and H_2O_2 release of adherent peritoneal cells on the other, compared to the individual mycotoxins (Theumer *et al.,* 2003).

Combined toxicity of aflatoxins and other mycotoxins from Aspergillus species
Citrinin (CIT) and cyclopiazonic acid (CPA) are mycotoxins produced by *Aspergillus* and/or *Penicillium* strains that have been frequently associated to AFB_1 for mixture toxicity studies (Table 4). A number of *Aspergillus* strains that produce B- and G-type aflatoxins may also produce CPA (Lee and Hagler, 1991; Pildain *et al.,* 2008). As a consequence CPA and aflatoxins often co-contaminate crops (Urano *et al.*, 1992; Chang *et al.*, 2009). Likewise, aflatoxins and citrinin have been simultaneously detected in various food and feed commodities (Kpodo *et al.*, 1996; Garon *et al.*, 2006; Nguyen *et al.*, 2007; Richard *et al.*, 2009).
All the studies reported for the combined toxicity of aflatoxins and CPA or CIT defined their reference point using the arithmetic definition of additivity. Following metabolic activation by either human S-9 mix or rat S-9 mix, the mutagenic activity of AFB_1 and CPA combination assessed by reverse mutation of *S.* Typhimurium TA 98 and TA 100 strains constantly resulted in a reduction compared to AFB_1individual effect (Kuilman-Wahls *et al.*, 2002; Vilar *et al.*, 2003). This reduction of the AFB_1 mutagenicity by CPA was attributed to the inhibitory effect of CPA on cytochrome P450 (CYP450) 3A4 activity. On the opposite, the marine bacterium *P. phosphoreum* reverse mutation test revealed an enhanced genotoxic effect for AFB_1 in mixture with CPA (Yates *et al*., 1987).

Combined toxicity of aflatoxin B_1 and mycotoxins from Fusarium species
The simultaneous spoilage of food commodities by *Aspergillus* and *Fusarium* strains is not uncommon and may be associated to natural co-occurrence of aflatoxins and various *Fusarium* mycotoxins, including DON, NIV and ZEA (Ali *et al*., 1998; Almeida *et al*., 2012). Data on the combined toxicity of aflatoxins and these fusariotoxins are presented in Table 5.
Interactive cytotoxicity between AFB_1 and *Fusarium* toxins ZEA and DON has been demonstrated at low doses and high doses in porcine kidney cells using the factorial design approach (Lei *et al.*, 2013). Synergy for cytotoxicity has been previously shown between AFB_1 and T-2 toxin by calculation of their interaction index (McKean *et al.*, 2006a). It is noteworthy that the type of toxic interaction in cell viability between AFB_1 and *Fusarium* toxins, especially trichothecenes, may depend on the cell model as additivity was reported in fish primary hepatocytes and human hepatoma cells (HepG2) while synergy was observed in human bronchial epithelial cells (BEAS-2B) (McKean *et al.*, 2006a; He *et al.*, 2010).
Surprisingly, the mutagenic activity of AFB_1 was significantly enhanced by the trichothecene mycotoxins DON and T-2 toxin which demonstrated no individual effect by their own in the *Salmonella* prokaryote mutagenicity test (Smerak *et al*., 2001). However, the authors reported a significant clastogenic effect for the trichothecene mycotoxins that may explain the enhanced mutagenic outcomes of the activity of AFB_1 in presence of either or both trichothecenes.

3.2. Ochratoxins and other mycotoxins

Ochratoxins are produced by several species belonging to both *Aspergillus* and *Penicillium* genera. Ochratoxin A (OTA) is toxic to several organs, especially the kidney, whereas its dechloro-analogue ochratoxin B only displays limited toxicity (Roth *et al.*, 1989; Heussner *et*

al., 2006). Studies addressing the toxicity of ochratoxins in association with other mycotoxins mainly concern OTA.
As already mentioned, OTA is a nephrotoxic compound, and as a consequence, most of the studies involved renal cell lines or renal primary cells cultures. Cytotoxicity is the main endpoint explored for the mycotoxin combinations. Mycotoxins associations including ochratoxins have also been screened for genotoxicity via DNA damages, clastogenic effects and mutagenic activity (Knasmuller *et al.,* 2004). As far as the immune system is concerned, mitogen-induced lymphocyte proliferation and cytokine production were the main endpoints for papers addressing the combined toxicity of ochratoxin (Table 6 and Table 7).

Combined toxicity of ochratoxins and other mycotoxins from Aspergillus or Penicillium
Ochratoxins may co-occur with other mycotoxins produced by species from *Aspergillus* and *Penicillium* genera. Among these mycotoxins, citrinin (CIT) is the most frequently associated with OTA, as illustrated by several studies undertaken in Bulgaria, Croatia and Serbia that showed that populations in endemic nephropathy regions were more frequently exposed to OTA and CIT due to microclimatic conditions (Klaric *et al.*, 2013). The combined toxicity of ochratoxins with patulin (PAT), cyclopiazonic acid (CPA), gliotoxin (GLIO), roquefortin (ROQ), penicillic acid (PA) and sterigmatocystin (STER) was also studied (Bernhoft *et al.*, 2004; Heussner *et al.*, 2006; Tammer *et al.*, 2007; Anninou *et al.*, 2014). The publications related to ochratoxins and other mycotoxins from *Aspergillus* and *Penicillium* are presented in Table 6.
OTA and CIT have mostly been reported to act in a synergistic manner for their cytotoxic (Roth *et al.*, 1989; Bouslimi *et al.*, 2008a; Bouslimi *et al.*, 2008b; Klaric *et al.*, 2012) and their genotoxic effects (Knasmuller *et al.,* 2004). The co-exposure of human kidney cells (HK-2) with both mycotoxins increased DNA adduction and CYP 450 and peroxydase enzymes expression (Manderville and Pfohl-Leszkowicz, 2008; Pfohl-Leszkowicz *et al.,* 2008). However, both mycotoxins failed to induce reverse mutation in the Ames test, and showed antagonism in porcine kidney PK15 epithelial cells (Wurgler *et al.*, 1991; Klaric *et al.*, 2012). Considering immunotoxicity endpoints a synergy between OTA and CIT was observed for mitogen-induced lymphocyte proliferation, while an additive effect was observed for the inhibition of IFN-γ production by peripheral blood mononuclear cells (Bernhoft *et al*., 2004; Tammer *et al*., 2007). Except CPA that showed an antagonistic immunotoxicity, the combinations of OTA with other *Aspergillus* and *Penicillium* mycotoxins were reported as additive. However, most of these studies rely on an arithmetic definition of additivity.

Combined toxicity of ochratoxins and mycotoxins from Fusarium species
Publications related to the combined toxicity of ochratoxins and mycotoxins produced by *Fusarium* species mainly concern OTA and fumonisin B_1 (FB_1), and in a lesser extent OTA and the emerging mycotoxin beauvericin (BEA) (Table 7). All of these studies considered the arithmetic definition of additivity as the reference point. Conflicting conclusions have been reported for the interaction between OTA and FB_1 for cytotoxicity including synergism (Creppy *et al.*, 2004; Carratu *et al.*, 2005; Mwanza *et al.*, 2009) and addition (Klaric *et al.*, 2007; Klaric *et al.*, 2008b). Genotoxic potential for binary combinations of OTA, FB_1 and BEA were mainly depicted as additive (Klaric *et al.*, 2007; Klaric *et al.*, 2008a).

3.3. Fusarium mycotoxins
Fusarium species can produce a wide variety of mycotoxins. The most common *Fusarium* mycotoxins that occur at biologically significant concentrations in food chain are fumonisins, zearalenone and trichothecenes (Placinta *et al.*, 1999). The mycotoxin association patterns involved the "major" mycotoxins from *Fusarium*, the trichothecenes, FB_1 and ZEA, although increasing attention is being paid to combinations including the emerging mycotoxins beauvericin (BEA) and enniatins (ENN) (Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b; Ficheux *et al.*,

2012; Kolf-Clauw *et al.*, 2013; Lu *et al.*, 2013). Combined toxicity studies mainly investigated the cytotoxicity or immunotoxicity of *Fusarium* mycotoxins. Due to its estrogenic activity, mixtures including ZEA have also been tested on the reproductive system cells (Malekinejad *et al.*, 2007; Ranzenigo *et al.*, 2008). Among publications concerning the combined effect of *Fusarium* mycotoxins, we have separately considered (i) those concerning the combined effects of trichothecene mycotoxins, (ii) those concerning the major mycotoxins from *Fusarium*, and (iii) other studies on combined toxicity of mycotoxins from *Fusarium*.

Combined toxicity of trichothecenes

Publications analyzing the combined toxicity of trichothecenes are presented in Table 8. Not all the trichothecenes involved in association studies are *Fusarium* mycotoxins. The non-macrocyclic trichothecenes produced by *Fusarium* species have been combined to the macrocyclic trichothecenes roridin A and verrucarin A produced by *Myrothecium* species (Koshinsky and Khachatourians, 1992; Jones *et al.*, 1995).

Combination of the type B trichothecenes DON or NIV to the type A T-2 toxin or DAS resulted in additive or antagonistic response either for the cytotoxic or the immunotoxic endpoints (Thompson and Wannemacher, 1986; Thuvander *et al.*, 1999; Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b). However, the striking fact of the combined toxicity of this group of mycotoxins is that the type and intensity of interactions vary accordingly with the tested doses and the combination ratios. Using human and porcine intestinal cells as well, we observed a synergistic cytotoxicity when combining DON, NIV and their acetyl derivatives at low doses while the interaction was additive to antagonistic for higher mycotoxin doses (Alassane-Kpembi *et al.*, 2013; Alassane-Kpembi *et al.*, 2015). Likewise, the interactive immunotoxicity of DON and NIV mixture is thought to be limited to low doses (Severino *et al.*, 2006). Earlier, it has also been shown that the interaction between the type A trichothecenes T-2 toxin and HT-2 toxin and the type D trichothecene roridin A changes from antagonistic to synergistic for graded toxicity levels towards the yeast *Kluyveromyces marxianus* (Koshinsky and Khachatourians, 1992).

Except factorial designs, all kinds of methodological approaches have been used for the elucidation of the type of interaction for mixtures involving trichothecenes. However, unlike early discussed mycotoxin groups, a number of studies in this group can be considered reliable enough for their conclusions since they are not built on mistaken interaction analysis approaches.

Combined toxicity of the "major" mycotoxins from Fusarium

Most joint toxicity studies are related to simultaneous contamination by type B trichothecenes (DON and or NIV), FB_1 and ZEA or its alcohol metabolite α-zearalenol (Table 9). Using the factorial designs the combination between the main *Fusarium* mycotoxins was shown to act additively on the porcine Ipec J2 cell viability reduction while interaction occurred for pro-inflammatory cytokines mRNA expression and the modulation of the expression of β-defensins 1 and 2 (Wan *et al.*, 2013a; Wan *et al.*, 2013b; Wan *et al.*, 2013c). Their toxicity was also found additive for the inhibition of DNA synthesis in mouse fibroblast by the same methodological approach (Groten *et al.*, 1998; Tajima *et al.*, 2002). In binary association, synergy was reported between ZEA or its alcohol metabolite α-zearalenol and FB_1 for various endpoints and cell systems (Groten *et al.*, 1998; Tajima *et al.*, 2002; Luongo *et al.*, 2006; Kouadio *et al.*, 2007; Luongo *et al.*, 2008). The synergy may also exist for the combined anti-proliferative effect of ZEA and DON on porcine granulosa cell but it was not confirmed for other endpoints in pig reproductive toxicology (Malekinejad *et al.*, 2007; Ranzenigo *et al.*, 2008).

Other studies on combined toxicity of mycotoxins from Fusarium

The emerging *Fusarium* toxins beauvericin (BEA) and enniatins (ENN) have been involved in mycotoxins combined effects studies for their cytotoxic and genotoxic potential (Table 10). Binary and ternary mixtures of ENN A, A_1, B and B_1 clearly exerted synergistic cytotoxicity on ovarian cells and intestinal cells (Lu *et al.*, 2013; Prosperini *et al.*, 2014). On the contrary, the toxicity of T-2 toxin was down-modulated by ENN B_1 in pig intestinal epithelial cells and explants culture (Kolf-Clauw et al., 2013). Assuming the arithmetic definition of additivity, no interaction could be detected in combined myelotoxicity for ENN B and BEA, while synergy was shown in BEA and DON mixture (Ficheux *et al.*, 2012). In other cell lines and by means of the Chou-Talalay method antagonism was observed for the latter association (Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b). This cell line–related discrepancy was also noted for the combined toxicity of BEA and the type A trichothecene T-2 toxin (Ruiz *et al.*, 2011a; Ruiz *et al.*, 2011b). The combination of BEA to FB_1 led to an additive induction of apoptosis in mononuclear cells (Dombrink-Kurtzman, 2003).

Conclusion

For the main mycotoxin groups, reference doses for regulatory purpose already exist. Exposure below these levels is usually considered safe. Whether the consumer is also protected against combined exposure to mycotoxins if each component is present below its individual threshold dose is gaining increasing interest. The present review analyzed the methodological aspects and main conclusions for the publications related to the toxicological interactions of mycotoxins.

More than eighty publications have been dedicated to the combined toxicity of mycotoxins, especially *Fusarium* toxins. Besides the regulated mycotoxins, an increasing number of studies are paying attention to mixtures involving the “emerging” ones. Considering the increasing attention given to modified mycotoxins; we can anticipate that their combined toxicity will be studied (Alassane-Kpembi *et al.,* 2015; Pierron *et al.,* 2015). Many methodological approaches have been used to explore the interactions in combined toxicity of mycotoxins. The main approaches are (i) the arithmetic definition of additivity, (ii) the factorial designs and (iii) the theoretical biology-based Combination index-isobologram method. A crucial issue for toxicodynamic interaction analysis is the statement of the non-interaction response. Factorial designs allow a reliable detection of departure from the additive response, while the Combination index-isobologram method makes it possible to determine the type of the interaction and to optionally quantify its magnitude. Only a few papers used these approaches for mycotoxin interaction analysis and most of them concern the combined toxicity of *Fusarium* toxins. Out of 35 publications only 13 used the isobologram approach and 4 used factorial designs.

Many biological models with different metabolic abilities along with various mycotoxin association patterns have been used. The biological models include human or animal primary cells or non-transformed or immortalized cell lines as well as prokaryote models. This review gathered the mycotoxins according to their producing fungi and indicates that *Fusarium* mycotoxins were the most studied. However, other mycotoxin combination strategies could be considered, as the mycotoxin co-occurrence patterns in commodities and the co-exposure patterns reported in bio-monitoring studies indicate that humans and animals are exposed to a wide variety of mycotoxin combinations in real life.

The main conclusion from all these studies is that very few studies used a robust methodological approach for the analysis of the combined effect of mycotoxins, and the type of interaction in terms of additivity, synergy or antagonism varies accordingly with the mycotoxin combinations, and even with the concentrations tested. More studies employing the isobologram approach are needed to feed a reliable database for the interactions between mycotoxins. Several publications reported synergy, especially for *Fusarium* toxins, using the Combination index-isobologram method. These *in vitro* synergistic interactions should be confirmed *in vivo*.

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Table 1: Selected mycotoxins' co-exposure/co-occurrence patterns reported worldwide

Co-exposure/ Co-contamination patterns	Sampling	Methodological approach	References
DON-ENNB-ZEA ; DON-CIT-T2 ; DON-CIT ; DON-ZEA ; DON-ENNB	Urine samples from 101 German adult volunteers	LC-MS/MS urinary multi-biomarker approach	**(Gerding *et al.*, 2014)**
DON-CIT-OTA-FB_1; DON-CIT-OTA; DON-OTA-ENNB CIT-OTA-ENNB ; CIT-OTA-FB_1 ; AFM_1-CIT-OTA ; AFM_1-CIT-DON ; ENNB-OTA ; DON-OTA ; CIT-OTA ; CIT-FB_1 ; CIT-ENNB AFM_1-CIT	Urine samples from adult volunteers: - 95 Bangladeshis - 50 Germans - 142 Haitians	LC-MS/MS urinary multi-biomarker approach	**(Gerding *et al.*, 2015)**
DON-ZEA-FB_1-OTA-AFB_1	Urine samples from 52 Italian adult volunteers	LC-MS/MS urinary multi-biomarker approach	**(Solfrizzo *et al.*, 2014)**
AFB_1-FB_1-DON	Blood and urine samples from 148 Tanzanian children aged 12-22 months	Albumin ELISA, HPLC and HPLC/MS	**(Shirima *et al.*, 2013; Srey *et al.*, 2014)**
AFM_1-FB_1-OTA-DON-NIV ; FB_1-FB_2-OTA-NIV ; FB_1-DON-NIV ; DON-ZEA-NIV ; OTA-NIV	Urine samples from 175 Cameroonian HIV-positive and HIV-negative adult volunteers	LC-MS/MS urinary multi-biomarker approach	**(Abia *et al.*, 2013)**
AFB_1-AFB_2-AFG_1-AFG_2-FB_1-FB_2-…-BEA-DON-NIV-ZEA-CIT-FA-$ENNB_1$; AFB_1-AFB_2-…-FB_1-…-OTA-BEA-STER-ZEA	122 maize, millet, infant food and feed samples from Burkina Faso and Mozambique	LC-MS/MS multi-toxin method	**(Warth *et al.*, 2012)**
FBs-DON-ZEA-AFs-OTA	92 commercial compound feeds from South Africa	LC-MS/MS multi-toxin method	**(Njobeh *et al.*, 2012)**
AFs-ZEA; AFs-OTA; OTA-ZEA; FBs-ZEA	37 randomly collected cereal and feed samples from households in endemic nephropathy areas (Croatia)	ELISA and Thin-layer chromatography	**(Klaric *et al.*, 2009)**
DON-NIV-BEA-ENNs	93 oat samples collected in 2010 and 2011 from field trials and grain delivery stations in central and southern Sweden	HPLC/ESI-MS/MS	**(Fredlund *et al.*, 2013)**

*Abbreviations used: AFs= aflatoxins,BEA= beauvericin, CIT= citrinin, DON= deoxynivalenol, ENN= enniatin, FA= fusaric acid, FBs= fumonisins, NIV= nivalenol, OTA= ochratoxin A, STER= sterigmatocystin, ZEA= zearalenone

Table 2: Interactions between aflatoxins

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint	Combined effect	Reference
AFB_1-AFB_2-AFG_1	Arithmetic definition of additivity	J774A.1 murine macrophages	Comparison of combination treatment with 0.01 ng/mL AFB_1 + 0.01 ng/mL AFB_2 to treatment with AFB_1 or AFB_2 alone	Cytokine secretion	Alleged synergy AFB_1-AFB_2 at the lower dose for increase in IL-6 secretion	**(Bruneau *et al.*, 2012)**
AFB_1-AFB_2- AFG_1-AFG_2	Arithmetic definition of additivity	Human umbilical vein endothelial cells (HUVEC), human lung fibroblasts (HFL), and A2780	Comparison of IC_{50} values for mixtures and individual toxins	Cell viability	Alleged synergy AFB_1-AFB_2 in HUVEC cells, additivity (AFB_1-AFB_2-AFG_1-AFG_2) in HFL and A2780	**(Braicu *et al.*, 2010)**
AFB_1-AFB_2-AFG_1	Two-way ANOVA	Rat hepatocytes, rat liver slices	Comparison of the effects of serial dilutions of AFB_1 (0-480 ng/mL) in presence or absence of AFB_2 at 120 ng/ mL, and comparison of the effects of serial dilutions of AFB_1 (0-480 ng/mL) in presence or absence of AFG_1 at 120 ng/ mL or 240 ng/mL	RNA synthesis, membrane integrity	No interaction AFB_1-AFB_2, undetermined interaction AFB_1-AFG_1	**(Friedman *et al.*, 1997)**
AFB_1-AFB_2-AFM_1-AFM_2	Arithmetic definition of additivity	J7741.A murine macrophages	Comparison of the effects of serial dilutions of individual toxins and their combination	Cell viability, activation of macrophagic functions (Nitric oxide production)	Stronger effects of mixtures compared to individual toxins suggesting interactions	**(Russo *et al.*, 2011)**
AFB_1-AFB_2-AFM_1-AFM_2	Arithmetic definition of additivity	J774A.1 murine macrophages	Comparison of IC_{30} values for viability reduction for individual toxins and mixtures, comparison of nitric oxide production inhibition by graded levels of individual toxins and mixtures	Cell viability, apoptosis, inhibition of nitric oxide production	Stronger effects of mixtures compared to individual toxins suggesting interactions	**(Bianco *et al.*, 2012b)**
AFM_1-AFM_2	Arithmetic definition of additivity	J7741.A murine macrophages	Comparison of the effects of serial dilutions of individual toxins and their combination	Cell viability, activation of macrophagic functions (Nitric oxide production)	No interaction	**(Russo *et al.*, 2010)**

*Abbreviations used: AFB_1= aflatoxin B_1, AFB_2= aflatoxin B_2, AFG_1= aflatoxin G_1, AFG_2=aflatoxin G_2, AFM_1=aflatoxin M_1, AFM_2=aflatoxin M_2

Table 3: Interactions between Aflatoxin B_1 and carcinogenic or possibly carcinogenic mycotoxins

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint*	Combined effect	Reference
AFB_1-FB_1	Interaction index metric	Human hepatoma cells (HepG2), Human bronchial epithelial cells (BEAS-2B)	Dose-response curves and determination of IC_{50} values for individual toxins and their mixture	Cell viability	Demonstrated additivity BEAS-2B, antagonism HepG2	**(McKean *et al.*, 2006b)**
AFB_1-FB_1	Arithmetic definition of additivity	Rat primary hepatocytes culture	Comparison of the toxic effects of individual mycotoxin and mixture doses	Cell viability, DNA fragmentation and apoptosis	No toxicity enhancement	**(Ribeiro *et al.*, 2010)**
AFB_1-FB_1	Arithmetic definition of additivity	Rat Wistar spleen mononuclear cells (SMC) and adherent peritoneal cells (APC)	Comparison of the effects of 20 µmol/L AFB_1 and 10 µmol/L FB_1, to the effects of a mixture 20µmol/L AFB_1 + 10 µmol/L FB_1	Mitogenic response and cytokines (IL-2, IL-4, IL-10) production of SMC and H_2O_2 release of APC	Differences in the effects produced by a mixture of mycotoxins in comparison to the individual action of the same toxins	**(Theumer *et al.*, 2003)**
AFB_1-FB_1	Arithmetic definition of additivity	Rat Wistar spleen mononuclear cells (SMC)	Comparison of the individual effects of 20 µg/mL FB_1 and 10 µg/mL AFB_1 to the effects of a mixture of 20 µg/mL FB_1 + 10 µg/mL AFB_1	Genotoxicity (alkaline comet assay and micronuclei assay) and oxidative stress (malondialdehyde (MDA) levels, catalase (CAT) and superoxide dismutase (SOD) activities)	No difference in DNA injury, no difference in MDA levels, higher CAT and SOD activities in AFB_1 individual treatment compared to FB_1, and the mixture	**(Theumer *et al.*, 2010)**

Table 3 continued: Interactions between Aflatoxin B_1 and carcinogenic or possibly carcinogenic mycotoxins

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint*	Combined effect	Reference
AFB_1-OTA	Arithmetic definition of additivity	Human hepatoma HepG2 cells	Comparison of IC_{50} values for mixtures and individual toxins	Cytotoxicity and genotoxicity	Alleged additive effect for cytotoxicty, antagonism for genotoxicity	**(Corcuera *et al*., 2011)**
AFB_1-OTA	Brown Interaction index	Monkey kidney Vero cells	Calculation of a ratio of expected to observed IC_{50} for the mixture	Cytotoxicity and genotoxicity	Alleged additivity	**(El Golli-Bennour *et al*., 2010)**
AFB_1-OTA	Arithmetic definition of additivity	*Salmonella* Typhimurium strains TA 100 and TA 98	Comparison of the mutagenic activity for serial dilutions of individual toxins and their mixture.	Mutagenic activity	Significant increase of the mutagenic activity of AFB_1	**(Sedmikova *et al*., 2001)**
AFB_1-OTA-FB_1	Arithmetic definition of additivity	Madin-Darby Bovine Kidney (MDBK) cells	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Cell viability MTT, NR	Alleged additivity for AFB_1-$FB_1$and AFB_1-OTA. Alleged synergy for AFB_1-FB_1-OTA ternary mixture	**(Clarke *et al*., 2014)**
AFB_1-OTA-FB_1	Arithmetic definition of additivity	Madin-Darby Bovine Kidney (MDBK) cells	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Cell viability: high content analysis, MTT, NR	Alleged additivity for AFB_1-$FB_1$and AFB_1-OTA. Alleged synergy for AFB_1-FB_1-OTA ternary mixture	**(Clarke *et al*., 2015)**

Table 4: Interactions between Aflatoxin B_1 and other mycotoxins from *Aspergillus* species

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint	Combined effect	Reference
AFB_1-CIT	Arithmetic definition of additivity	Mice macrophage	Comparison of infectivity and proliferation in control, individual and combined toxin groups	Infectivity and proliferation of *Toxoplasma gondii*	Increased infectivity and proliferation of *T. gondii* for the combined exposure compared to control	**(Herzog-Soares and Freire, 2004)**
AFB_1-CPA	Arithmetic definition of additivity	*Salmonella* Typhimurium strains TA 100 and TA 98	Comparison of the mutagenic activity for serial dilutions of individual toxins and their mixture	Mutagenic activity following metabolic activation by rat S-9 mix	Reduction of AFB_1 mutagenic activity	**(Kuilman-Wahls *et al*., 2002)**
AFB_1-CPA	Arithmetic definition of additivity	*Salmonella* Typhimurium strains TA 100 and TA 98	Comparison of the mutagenic activity for serial dilutions of individual toxins and their mixture	Mutagenic activity following metabolic activation by human S-9 mix	Reduction of the mutagenic activity of AFB_1.	**(Vilar *et al.*, 2003)**
AFB_1-CPA	Arithmetic definition of additivity	Marine bacterium *Photobacterium phosphoreum* strain NCMB 844 and strain NRRLB 1177	Comparison of the effects for individual toxin doses and dose pairs in mixture	Genotoxicity and cytotoxicity	Enhanced genotoxic effect of AFB_1 by CPA	**(Yates *et al*., 1987)**

*Abbreviations used: AFB_1= aflatoxin B_1, CIT= citrinin, CPA= cyclopiazonic acid

Table 5: Interaction between aflatoxin B_1 and mycotoxins from *Fusarium* species

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint*	Combined effect	Reference
AFB_1-DON	One-way ANOVA	*Cyprinus carpio* primary hepatocytes	Comparison of the effects of the mixture and the effects of individual toxins	Cell viability (MTT test), enzyme (Aspartate aminotransferase AST, Alanine transferase ALT, Lactate dehydrogenase LDH) activity in cell supernatant	Alleged additivity	**(He *et al.*, 2010)**
AFB_1-T-2 toxin	Interaction index metric	Human hepatoma HepG2cells , Human BEAS-2B bronchial epithelial cells	Dose-response curves and determination of IC_{50} values for individual toxins and their mixture	Cell viability	Demonstrated synergy in BEAS-2B, additivity in HepG2	**(McKean *et al.*, 2006a)**
AFB_1-DON-T-2 toxin	Arithmetic definition of additivity	Prokaryote model (*Salmonella* Typhimurium, strains TA98 and TA100)	Comparison of the effects of individual toxins and their mixtures	Mutagenic activity	Significant enhancement of the mutagenic effect of AFB_1 (no activity for T-2 and DON alone, but greater activity for the combinations with AFB_1)	**(Smerak *et al.*, 2001)**
AFB_1-DON-ZEA	Factorial design	Immortalized BRL 3A rat liver cells	Central composite design for binary and ternary mixtures experiments, with the IC_{30} of cell viability of each mycotoxin chosen as the center point	Cell viability (MTT)	Demonstrated interactive cytotoxicty. Alleged synergy for AFB_1-ZEA and AFB_1-DON.	**(Sun *et al.*, 2015)**
AFB_1-DON-ZEA	Factorial design	Porcine Kidney PK15 cells	Central composite design for binary and ternary mixtures experiments, with the IC_{30} of cell viability of each mycotoxin chosen as the center point	Cell viability (MTT), membrane damage (LDH), apoptosis and oxidative stress	Demonstrated interactive cytotoxicity. Alleged synergism for AFB_1-ZEA, AFB_1-DON, low dose antagonism and high dose synergism ZEA-AFB_1, and alleged synergism DON-AFB_1 for oxidative damage	**(Lei *et al.*, 2013)**

*Abbreviations used: AFB_1= aflatoxin B_1, DON= deoxynivalenol, ZEA= zearalenone, MTT= (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium, LDH= lactate deshydrogenase

Table 6: Interactions between ochratoxins and other mycotoxins from *Aspergillus* and *Penicillium*

Mycotoxin association*	Interaction model	Cell model	Study design*	Endpoint	Combined effect	Reference
OTA-CIT	Arithmetic definition of additivity	Human HepG2 hepatoma cells	Comparison of the toxic effect of the mixture at a dose of 20% of the IC_{50} of each toxin to that produced by either of the toxins at its IC_{50}.	Cell viability	Alleged synergy	**(Gayathri *et al.*, 2015)**
OTA-CIT	Arithmetic definition of additivity	Porcine PK15kidney epithelial cells	Comparison of the toxic effect of the mixture to the sum of the toxic effects of individual compounds at their concentration in the mixture	Cell viability, apoptosis, necrosis, genotoxicity	Alleged additive effect for cell viability, synergy for apoptosis and necrosis, antagonism for genotoxicity	**(Klaric *et al.*, 2012)**
OTA-CIT	Arithmetic definition of additivity	Monkey kidney Vero cells	Cytotoxicity comparison of IC_{50} values for individual toxins and their mixture, DNA damage comparison of the effects for several concentrations of individual toxins and their mixture	Cell proliferation, DNA damage	Alleged synergy	**(Bouslimi *et al.*, 2008a)**
OTA-CIT	Arithmetic definition of additivity	Monkey kidney Vero cells	Cytotoxicity comparison of IC_{50} values for individual toxins and their mixture, DNA damage comparison of the effects for several concentrations of individual toxins and their mixture	Cell proliferation, oxidative cell damage	Alleged synergy	**(Bouslimi *et al.*, 2008b)**
OTA-CIT	Arithmetic definition of additivity	Human HK2 kidney cells	Comparison of the toxic effect of the mixture to the toxic effects of individual compounds	DNA adduction, expression of CYP3A4, COX and LOX	Two-fold increase of the OTA-related DNA adduction and significant increase of the expression of COX and LOX	**(Manderville and Pfohl-Leszkowicz, 2008)**
OTA-CIT	Arithmetic definition of additivity	Human proximal tubule-derived cells (IHKE cells)	Comparison of the toxic effect of the mixture to the sum of the toxic effects of individual compounds at their concentration in the mixture	Cytotoxicity, apoptosis	Alleged antagonism for apoptosis at concentration of 2.5-5 µM CIT, additivity at concentration 7.5-15 µM	**(Knecht *et al.*, 2005)**

Table 6 continued: Interactions between ochratoxins and other mycotoxins from *Aspergillus* and *Penicillium*

Mycotoxin association*	Interaction model	Cell model*	Study design*	Endpoint	Combined effect	Reference
OTA-CIT	Theoretical biology model-based definition of additivity	Pig renal cortical cubes	Logistic function analysis of the dose-response curve for the individual compounds and their mixture	Protein synthesis, organic ions tetraethylammonium (TEA) and paraminohippurate (PAH) transport	Demonstrated synergy and additivity for TEA and PAH ions transport and protein synthesis	**(Braunberg *et al*., 1994)**
OTA-OTB-CIT	Arithmetic definition of additivity	Hepatoma tissue culture cells	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Protein synthesis	Alleged slight synergy	**(Roth *et al*., 1989)**
OTA-OTB-CIT-PAT	Factorial design	Porcine LLC-PK1 renal cell line	Step-wise approach :Full factorial design , then inscribed central composite design	Cell viability	Demonstrated potential synergy OTA CIT	**(Heussner *et al.*, 2006)**
OTA-CIT-PAT-GLIO	Loewe additivity and Bliss independence criterion models	Peripheral blood mononuclear cells	Comparison of actual mixture toxicity data to predicted ones based on concentration addition and response addition concepts	Cell viability, cytokine production	Demonstrated additivity	**(Tammer et al., 2007)**
OTA-CIT-PAT-CPA-ROQ-Pen Ac	Isobologram method	Piglet Lymphocytes	Dose-response curves and isobologram drawing at IC_{20}	Mitogen-induced lymphocyte proliferation	Demonstrated synergy OTA-CIT, additivity OTA-CPA, Pen Ac-RQ, PAT-RQ, PAT-Pen Ac, antagonism CIT-CPA	**(Bernhoft *et al.*, 2004)**
OTA-CIT-STER	Arithmetic definition of additivity	Human Hep3B hepatocellular cell line	Comparison of the expected and the observed effects for mycotoxin mixtures and calculation of the Coefficients of Drug Interaction	Cytotoxicity, cytostaticity and genotoxicity	Alleged additive to antagonistic cytotoxic and genotoxic effects	**(Anninou *et al*., 2014)**

*Abbreviations used: CIT= citrinin, COX= cyclooxygenase, CPA= cyclopiazonic acid, CYP3A4= cytochrome P450 3A4, GLIO= gliotoxin, IC_{20}-IC_{50}= inhibitory concentration 20-50%,LOX= lipoxygenase, OTA= ochratoxin A, OTB= ochratoxin B, PAT= patulin, Pen Ac= penicillic acid, ROQ= roquefortin, STER= sterigmatocystin

Table 7: Interactions between ochratoxins and *Fusarium* mycotoxins

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint*	Combined effect	Reference
OTA-FB_1	Arithmetic definition of additivity	Madin-Darby Bovine Kidney (MDBK) cells	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Cell viability: high content analysis, MTT, NR	Alleged synergy	**(Clarke *et al.*, 2015)**
OTA-FB_1	Arithmetic definition of additivity	Madin-Darby Bovine Kidney (MDBK) cells	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Cell viability: MTT, NR	Alleged additivity	**(Clarke *et al.*, 2014)**
OTA-FB_1	Arithmetic definition of additivity	Pig lymphocytes, human lymphocytes	Comparison of the toxic effect of the mixture to the sum of the toxic effects of individual compounds at their concentration in the mixture	Cell viability	Alleged synergy	**(Mwanza *et al.*, 2009)**
OTA-FB_1	Arithmetic definition of additivity	Porcine PK15 kidney epithelial cells	Combination of equal concentrations of two or all three mycotoxins	Clastogenic effect	Alleged additivity for presence of micro nuclei and for presence of nucleoplasmic bridges	**(Klaric *et al.*, 2008a)**
OTA-FB_1	Arithmetic definition of additivity	Human intestinal Caco-2 cells	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Protein synthesis inhibition	Alleged synergy	**(Carratu *et al.*, 2005)**
OTA-FB_1	Arithmetic definition of additivity	Monkey kidney Vero cells, human intestinal caco-2 cells, rat C6 glioma cells	Comparison of the toxic effects of mixtures to the sums of the toxic effects of individual compounds at their concentrations in the mixtures	Cell viability	Alleged synergy	**(Creppy *et al.*, 2004)**

Table 7 continued: Interactions between ochratoxins and *Fusarium* mycotoxins

Mycotoxin association*	Interaction Model	Cell model	Study design	Endpoint*	Combined effect	Reference
OTA-FB_1-CIT	Arithmetic definition of additivity	Human peripheral blood mononuclear cells	Comparison of the toxic effect of the mixture to each of the toxic effects of individual compounds at their concentration in the mixture	Mitogen-induced cell proliferation, cell viability MTT	Stronger effect of the mixture compared to any individual compound	**(Stoev *et al.*, 2009)**
OTA-FB_1-BEA	Arithmetic definition of additivity	Porcine PK15kidney epithelial cells	Comparison of the toxic effects of individual mycotoxin and binary and ternary mixture of equal concentrations of the toxins	Cell viability, apoptosis	Alleged additivity for cell viability, additivity and synergy for apoptosis	**(Klaric *et al.*, 2008b)**
OTA-FB_1-BEA	Arithmetic definition of additivity	Porcine PK15kidney epithelial cells	Combination of equal concentrations of two or all three mycotoxins	Cell viability, lipid peroxidation (TBARS) and GSH depletion	Alleged additivity, possibly synergy and antagonism	**(Klaric *et al.*, 2007)**
OTA-BEA	Arithmetic definition of additivity	Porcine PK15 kidney epithelial cells, Human leukocytes (HL)	Combination of two concentrations	Genotoxic potential	Alleged additivity and synergy in PK15, additivity in HL	**(Klaric *et al.*, 2010)**
OTA-ZEA	Loewe additivity and Bliss independence criterion models	Human HepG2 hepatoma cells and Immortalized murine ovarian granular KK-1 cells	Comparison of actual mixture toxicity data to predicted ones based on concentration addition and response addition concepts	Cell viability and intracellular ROS production	Demonstrated additivity for cell viability, departure from additivity for ROS production	**(Li *et al.*, 2014)**
OTA-ZEA-α-ZOL	CI-isobologram method	Human HepG2 hepatoma cells	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cell viability MTT	Demonstrated antagonism for OTA-ZEA, OTA-α-ZOL and OTA-ZEA-α-ZOL mixtures	**(Wang *et al.*, 2014)**

*Abbreviations used: BEA= beauvericin, CIT= citrinin, FB_1= fumonisin B_1, GSH= glutathione, MTT= (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium, NR= Neutral Red, OTA= ochratoxin A, ROS= reactive oxygen species, TBARS= Thiobarbituric acid reactive substances, ZEA= zearalenone, α-ZOL= α-zearalenol

Table 8: Interaction between trichothecenes

Mycotoxin association*	Interaction Model	Cell model	Study design	Endpoint*	Combined effect	Reference
DON-NIV	Arithmetic definition of additivity	Rat IEC-6 intestinal epithelial cells	Incubation with graded levels of DON or NIV alone or in combination	Cell viability, apoptosis, cell migration	No additive or synergistic effects	**(Bianco *et al.*, 2012a)**
DON-NIV	Arithmetic definition of additivity	Murine J7741.A macrophages	Comparison of the IC_{50} values of the toxins and their mixture	Cell viability, Pro-apoptotic activity	No synergy	**(Marzocco *et al.*, 2009)**
DON-NIV	Arithmetic definition of additivity	Human Jurkat T cells	Incubation with graded levels of DON or NIV alone or in combination ratio 1:1 and 10:1 for DON:NIV	Lymphocyte proliferation and cytokines expression	Alleged interactive effect for lymphocyte proliferation, and interactive effects at lower concentrations (0.06-4µM) for IFNgamma and IL-2 mRNA transcription	**(Severino *et al.*, 2006)**
DON-NIV-3-DON-15-ADON -FX	CI-Isobologram method	Porcine IPEC-1 intestinal epithelial cells	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cytotoxicity MTT	Demonstrated synergy for all binary combinations, excepted for NIV-FX (additivity), and for DON-FX (antagonism)	**(Alassane-Kpembi *et al.*, 2015)**
DON-NIV-3-DON-15-ADON -FX	CI-Isobologram method	Human intestinal Caco-2 cells	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cytotoxicity NR MTT	Demonstrated low-dose synergies, antagonism for ternary mixture DON-NIV-FX	**(Alassane-Kpembi *et al.*, 2013)**
DON-NIV-T-2 toxin-DAS	Arithmetic definition of additivity	Human lymphocytes	Comparison of the mixture toxicity and the individual toxicity of 2× the concentration of toxin used at the combined exposure	Mitogen-induced lymphocyte proliferation Cell viability, Immunoglobulin production	Alleged additivity (NIV - T2, NIV - DAS, NIV - DON), and slight antagonism (DON - T2, DON - DAS) for lymphocytes proliferation	**(Thuvander *et al.*, 1999)**

Table 8 continued: Interaction between trichothecenes

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint	Combined effect*	Reference
T-2 toxin-Verrucarin A	CI-Isobologram	Yeast *Kluyveromyces marxianus*	Comparison of actual toxicity data for binary mixture at various ratios to predicted ones based on the Mass action law concept	Growth inhibition	The type and intensity of interactions varied with the combination ratios, growth percent inhibition and the growth medium: In a rich medium synergy over a 2-3 log value concentration range for a 1.0 µg/ml T-2 toxin:0.75µg/ml verrucarin A ratio	**(Jones *et al.*, 1995)**
T-2 toxin-HT-2 toxin-Roridin A	CI-Isobologram	Yeast *Kluyveromyces marxianus*	Comparison of actual toxicity data for binary mixture at various ratios to predicted ones based on the Mass action law concept	Growth inhibition	The type of interaction varied accordingly to the mixture ratios and the percent of inhibition of growth: from antagonism to synergy for increasing percent inhibition of yeast growth	**(Koshinsky and Khachatourians, 1992)**
12-13 epoxytrichothecenes	Arithmetic definition of additivity	Monkey Vero cells, rat spleen lymphocytes	Comparison of IC_{50} values of different ratios binary mixtures to the IC_{50} values of the individual toxins	Protein synthesis inhibition	Alleged additivity	**(Thompson and Wannemacher, 1986)**

*Abbreviations used: DAS= diacetoxyscirpenol, DON= deoxynivalenol, FX= fusarenon-X, IC_{50}=inhibitory concentration 50%, IFN= interferon, IL-2= interleukin 2, MTT= (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium, NIV= nivalenol, NR= neutral red, 3-ADON= 3-acetyldeoxynivalenol, 15-ADON= 15-acetyldeoxynivalenol

Table 9: Interactions between the "major" mycotoxins from *Fusarium*

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint*	Combined effect	Reference
DON-ZEA	Arithmetic definition of additivity	Human HCT 116 colon carcinoma cells	Comparison of the effects of 100 µM DON, 40 µM ZEA and 100+40 µM DON+ZEA	Cell viability, cell cycle, trans-membrane potential and permeability transition pore opening	Alleged sub-additive response	**(Bensassi *et al*., 2014)**
DON ZEA	Arithmetic definition of additivity	Porcine granulosa cells	Comparison of the effects of 30 ng/mL of DON, 30 ng/mL of ZEA and both	Granulosa cell proliferation, steroidogenesis, gene expression	Alleged possibly synergy for alteration of GC proliferation	**(Ranzenigo *et al*., 2008)**
DON-ZEA	Arithmetic definition of additivity	Pig oocytes	Comparison of the effects of various ratios leading to 3.12 µM mixture DON-ZEA to the effects of 3.12 µM of each toxin alone of DON ZEA mixture	Abnormalities in formation of the meiotic spindle, Inhibition of oocyte maturation, developmental competence of matured oocytes after *in vitro* fertilization	Alleged no synergy	**(Malekinejad *et al*., 2007)**
DON-ZEA-FB_1	Arithmetic definition of additivity	Human intestinal cell line Caco-2	Comparison of the effects of Binary and tertiary mixtures to the sum of the effects of each toxin alone	Malonedialdehyde (MDA) production, DNA and protein synthesis inhibition, DNA methylation, DNA fragmentation, cell viability, lipid peroxidation	Alleged antagonism FB_1-ZEA for cell viability, synergy ZEA-FB_1 and ZEA-DON for lipid peroxidation, far less than additivity in DNA synthesis inhibition for binary mixtures of DON, FB_1 and ZEA	**(Kouadio *et al*., 2007)**
DON-ZEA-FB_1	Arithmetic definition of additivity	Brewing yeast strains (*Saccharomyces cerevisiae*, *Kluyveromyces marxianus*, *Pichia membranaefaciens*, *Hansenula anomala*, and *Schizosaccharomyces pombe*)	Comparison of the growth inhibition for combinations and the sum of the inhibition for toxins alone	Yeast growth	Alleged interaction at high concentration: synergism or antagonism depending on toxin combination ratios	**(Boeira *et al*., 2000)**

Table 9 continued: Interactions between the "major" mycotoxins from *Fusarium*

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint*	Combined effect	Reference
DON-ZEA-FB_1-NIV	Factorial design	Porcine intestinal Ipec J-2 cells	Inscribed central composite design with four toxins and two concentrations for each toxin	Cell viability	Demonstrated non-additive interactions except DON-FB_1-ZEA	**(Wan *et al.*, 2013a)**
DON-ZEA-FB_1-NIV	Factorial design	Porcine intestinal Ipec J-2 cells	Inscribed central composite design with four toxins and two concentrations for each toxin	Pro-inflammatory cytokines IL-1α, IL-1β, IL-6, IL-8, TNF-α and MCP-1 mRNA expression	Demonstrated non-additive interactions	**(Wan *et al.*, 2013b)**
DON-ZEA-FB_1-NIV	Factorial design	Porcine intestinal Ipec J-2 cells	Inscribed central composite design with four toxins and two concentrations for each toxin	Modulation of the expression of beta-defensin 1 & 2	Alleged non-additive interactions	**(Wan *et al.*, 2013c)**
DON-ZEA-FB_1-NIV	Arithmetic definition of additivity	Human Jurkat cells and porcine lymphocytes	Increasing concentrations of FB_1 with constant concentrations of ZEA or co-incubation with DON and NIV	Mitogen-activated lymphocyte proliferation	Alleged interaction	**(Severino *et al.*, 2008)**
DON-ZEA-FB_1-NIV-T-2 toxin	Factorial design	L929 mouse fibroblasts	Central composite design with five toxins and five concentrations for each toxin, then full factorial design for two-factor interactions of particular interest	DNA synthesis inhibition	Demonstrated mainly additive combinations and synergy for ZEA-FB_1, NIV-T2	**(Tajima *et al.*, 2002)**
DON-ZEA-FB_1-NIV-T-2 toxin	Factorial design	L929 mouse fibroblasts	Central composite design with five toxins and five concentrations for each toxin, then full factorial design for two-factor interactions of particular interest	DNA synthesis inhibition	Demonstrated mainly additive combinations and synergy for ZEA-FB_1, NIV-T2	**(Groten *et al.*, 1998)**

Table 9 continued: Interactions between the "major" mycotoxins from *Fusarium*

Mycotoxin association*	**Interaction model**	Cell model	Study design	Endpoint*	Combined effect	Reference
DON-α-ZOL-FB_1	Arithmetic definition of additivity	Porcine granulosa cells	Comparison of the effects of 3.4µM DON, 9.4µMα-ZOL, and 10µM $FB_1$to 3.4+10µM DON+FB_1 and 9.4+10µM α-ZOL +FB_1	Proliferation of granulosa cells, and their steroid (progesterone and estradiol) production	Alleged significant interaction for progesterone production, no significant interaction for cell proliferation and estradiol production	(Cortinovis *et al*., 2014)
DON-α-ZOL-FB_1-NIV	Isobologram method	Swine whole-blood culture	Analysis of the effects for serial dilutions of the mycotoxins	Cell proliferation	Demonstrated synergy (FB_1 α-ZOL), no interaction (DON NIV)	(Luongo *et al*., 2008)
ZEA-T-2 toxin	Arithmetic definition of additivity	Monkey kidney Vero cells	Combination of toxins at equimolar concentration	Cytotoxicity MTT ROS production, and expression of Heat shock protein HSP70	Increased toxicity compared to each toxin alone	(Bouaziz *et al*., 2013)
ZEA-α-ZOL	CI-isobologram method	Human HepG2 hepatoma cells	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cell viability MTT	Demonstrated antagonism for low doses, additivity for medium doses and synergy at high doses	(Wang *et al*., 2014)
ZEA-α-ZOL-β-ZOL	CI-Isobologram method	Hamster ovarian cells CHO-K1	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cytotoxicity MTT	Demonstrated additive effects for ZEA-α-ZOL and ZEA-β-ZOL, antagonism at high concentration for α-ZOL-β-ZOL and synergy at low concentration for ZEA-α-ZOL-β-ZOL	(Tatay *et al*., 2014)
FB_1-α-ZOL	Isobologram method	Human Jurkat T cells	Analysis of the effects for serial dilutions of the mycotoxins	Lymphocyte proliferation, cytokine (IL-2 and INF-gamma) expression	Demonstrated synergy for lymphocyte proliferation, interactive effect cytokine expression	(Luongo *et al*., 2006)

*Abbreviations used: DON= deoxynivalenol, FB_1= fumonisin B_1, IL= interleukin 2, IFN= interferon, NIV= nivalenol, MCP-1= monocyte chemoattractant protein-1, MTT= (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium, NIV= nivalenol, ROS= reactive oxygen species, TNF-α= tumor necrosis factor-α, ZEA= zearalenone, α-ZOL= α-zearalenol, β-ZOL= β-zearalenol

Mycotoxin association*	Interaction Model	Cell model	Study design	Endpoint*	Combined effect	Reference
BEA-DON-T-2 toxin	CI-Isobologram method	Chinese hamster ovarian CHO-K1cells	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cell viability MTT and NR	Demonstrated antagonism DON-BEA, DON-T2 antagonism, BEA-T2 synergism, DON-BEA-T2 synergism and low dose antagonism	**(Ruiz *et al.*, 2011a)**
BEA-DON-T-2 toxin	CI-Isobologram method	Monkey kidney Vero cells	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cell viability NR	Demonstrated antagonism DON-BEA, T2-BEA antagonism, DON-T-2 antagonism, DON-BEA-T-2 antagonism	**(Ruiz *et al.*, 2011b)**
BEA-DON-T-2 toxin	Arithmetic definition of additivity	Chinese hamster ovary CHO-K1 cells, monkey kidney Vero cells	Comparison of the tested and predicted toxicities for mycotoxin mixtures (simple additive)	Cell viability NR	Potential of interactive effects	**(Font *et al.*, 2009)**
BEA-DON-T-2 toxin-ZEA-ENN	Arithmetic definition of additivity	Human Colony Forming Unit-Granulocyte and Macrophage (CFU-GM)	Comparison of the toxic effect of mixtures to the sum of the toxic effects of individual compounds at their concentration in the mixture	Myelotoxicity	Alleged synergy DON-BEA, antagonism DON-FB_1, synergy or additivity DON-T-2, additivity DON-ZEA, T2-ZEA, BEA-ENN B,	**(Ficheux *et al.*, 2012)**
BEA-FB_1	Arithmetic definition of additivity	Turkey peripheral blood mononuclear cells	Comparison of 8 µM FB_1, 8 µM BEA, 8+8 µM FB_1 & BEA	Apoptosis assessed by nuclear DNA fragmentation	Alleged slightly additive effect	**(Dombrink-Kurtzman, 2003)**
ENN A-A_1-B-B_1	CI-Isobologram method	Caco-2 cells	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cell viability MTT	Demonstrated synergy for ENN B – ENN A_1, ENN B_1 – ENN A_1, ENN A – ENN A_1 – ENN B ; antagonism for ENN B – ENN B_1; additivity for all other combinations	**(Prosperini *et al.*, 2014)**

Table 10 continued: Interactions involving other mycotoxins from *Fusarium*

Mycotoxin association*	Interaction model	Cell model	Study design	Endpoint*	Combined effect	Reference
ENN A-A_1-B-B_1	CI-Isobologram method	Hamster ovarian cells CHO-K1	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cell viability MTT	Demonstrated synergistic effect of combined ENs A+A_1, A+B, A_1+B_1, A+A_1+B, A+A_1+B_1, A+B+B_1 and A_1+B+B_1	**(Lu *et al.*, 2013)**
ENN B_1-T-2 toxin	CI-Isobologram method	Porcine intestinal IPEC 1 cells and porcine intestinal tissue explants	Comparison of actual mixture toxicity data to predicted ones based on the Mass action law concept	Cytotoxicity and morphological and histopathological scoring	Demonstrated less than additivity with decreasing concentrations of toxins	**(Kolf-Clauw *et al.*, 2013)**
FA-DON-FB_1	Arithmetic definition of additivity	Pineal cell cultures	Comparison of 1 µM FA, 1µM DON, 1 µM FB_1, 1+1 µM DON+FA, and 1+1 µM FA+FB_1	Levels of pineal 5-HT and 5-HTP	Alleged possibly synergy or antagonism	**(Rimando and Porter, 1999)**

*Abbreviations used: BEA= beauvericin, DON= deoxynivalenol, ENN= enniatin, FA= fusaric acid, FB_1= fumonisin B_1, MTT= (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium, NR= neutral red, 5HT= 5-hydroxytryptamine, 5-HTP= 5-hydroxy-l-tryptophan, ZEA= zearalenone