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Abstract

This paper describes a methodology for hazard assessment of groups of related substances for which toxicity data are insufficient, and which utilises, next to conventional toxicological assessments and mechanistic information, the derivation of relative toxicity potency factors (RPFs). Zearalenone (ZEN) and T-2 toxin (T2) and HT-2 toxin (HT2) and their modified forms have been used as examples. A tolerable daily intake (TDI) for ZEN of 0.25 µg/kg bw was established. *In vitro* and *in vivo* studies suggested that modified forms of ZEN act via the same mode of action as ZEN (oestrogenicity). Results from *in vivo* uterotrophic assays were used to establish RPFs, allowing inclusion the different modified forms in a group TDI with ZEN. A TDI for the sum of T2/HT2 of 0.02 µg/kg bw per day and an acute reference dose (ARfD) of 0.3 µg/kg bw for the sum of T2/HT2 was established. *In vitro* studies show that phase I metabolites of T2/HT2 act via a similar mode of action as their parent compounds, namely protein synthesis inhibition with immune- and haematotoxicity. The phase I

metabolites as well as conjugates of T2/HT2 and their phase I metabolites can be included in a group TDI with T2/HT2 applying RPFs.

Key words: zearalenone (ZEN), T2-toxin (HT2), modified forms, toxicity assessment, relative potency factors (RPFs);

Abbreviations:

Ac, acetyl; AP, alkaline phosphatase; ARfD, acute reference dose; BMD, benchmark dose; $BMDL_{05}$, 95% lower confidence limit of the BMD_{05}, the dose associated with a 5% change in response relative to control; $BMDL_{10}$, 95% lower confidence limit of the BMD_{10}, the dose associated with a 10% change in response relative to control; CONTAM Panel, EFSA Panel on Contaminants in the Food Chain; DE, de-epoxy; E2, 17ß-estradiol; EC_{50}, half maximal effective concentration; EFSA, European Food Safety Authority; ER, oestrogen receptor; Fer, feruloyl; Glc, glucoside; Glcp, glucopyranoside; GlcA, glucuronic acid; HBGV, health based guidance value; 5-HT, 5-hydroxytryptamine; HT2, HT2-toxin; IL, interleukin; JMPR, Joint FAO/WHO Expert Committee on Food Additives; LC_{100}, absolute lethal concentration; LD_{50}, median lethal dose; LOAEL, lowest observed adverse effect level; MAPK, mitogen-activated protein kinase; MCF-7, human breast adenoma cell line; mRNA, messenger ribonucleic acid; Mal, malonyl; NEO, neosolaniol; NOAEL, no observed adverse effect level; NOEL, no observed effect level; PMTDI, provisional maximum tolerable daily intake; PYY3–36, pancreatic peptide YY3-36; *s.c.,* subcutaneous; SCF, Scientific Committee on Food; Sulf, sulfate; T2, T2-toxin; RAL, resorcyclic acid lactone; RPF, relative potency factor; Sulf, sulfate; TDI, tolerable daily intake; t-TDI, temporary tolerable daily intake; TNF, tumour necrosis factor; UDPGA, uridine 5-diphosphate glucuronic acid; UF, uncertainty factor; WHO, World Health Organization; ZAL, zearalanol; ZAN, zearalanone; ZEL, zearalenol; ZEN, zearalenone.

1. Introduction

Fusarium toxins are produced by fungi of the genus *Fusarium* that grow in the field and frequently contaminate crops used for food and feed. Zearalenone (ZEN), T2-toxin (T2) and HT2-toxin (HT2) are major representatives this toxin class. Both infected plants and the fungi themselves can form metabolites of these toxins, which can occur in considerable amounts in plants together with their parent compounds (EFSA CONTAM Panel, 2016; 2017). In addition, metabolites of ZEN have been shown to occur in animal derived food products such as cow's

milk (Xia et al., 2009; Huang et al., 2014; Dänicke and Winkler, 2015). These mycotoxin derivatives have been defined as "modified forms" of mycotoxins (Rychlik et al., 2014). Modified forms include both phase I metabolites formed by oxidation, reduction or hydrolysis as well as phase II metabolites, i.e. glucose or sulfate conjugates of parent compounds or their phase I metabolites (EFSA CONTAM Panel, 2016; 2017). Although some modified fusarium toxins may still possess biological activity and exert considerable toxicity, to date, modified forms of fusarium toxins are neither considered in dietary risk assessments nor in the EU legislative framework on contaminants. The data collected and evaluated in two recent EFSA opinions (EFSA CONTAM Panel, 2016; 2017) were used as a starting point for the present scientific review. The objective of this scientific review is the introduction of a methodology for hazard assessment of groups of structurally related mycotoxins or other contaminants for which robust toxicity data are lacking, for several forms establishing relative potency factors (RPFs) for compounds of a substance group for which adequate toxicity data are not available. ZEN, T2 and HT2 and their modified forms have been used as examples.

2. Approach used for a combined assessment

The following approach was adopted to conduct a combined hazard assessment of mycotoxins and their modified forms in food. Several issues had to be considered to decide whether it is appropriate to set a combined health-based guidance value (HBGV) for a mycotoxin and its modified forms. Firstly, the modified forms of a certain mycotoxin were identified and described. Secondly, the occurrence of these modified forms in food commodities was verified, as a prerequisite for their relevance in dietary risk assessment. As a third step new studies on acute and chronic toxicity of the parent mycotoxin were reviewed in order to assess whether the existing HBGV needs re-evaluation. As a fourth step any information relevant for the assessment of the toxicity of the modified forms was evaluated. Both *in vitro* and in *vivo data* were considered, in particular information on the mode of action and whether this is similar or likely to be similar to that of the parent mycotoxin. The latter question is important when deciding on whether it would be appropriate to apply the principle of dose addition and establish a combined HBGV. In cases, where there were considerable differences in potency between the parent mycotoxin and various modified forms application of potency factors was considered appropriate and when using *in vitro* data possible differences in *in vivo* toxicokinetics were considered. This step-wise approach was applied to ZEN and T2/HT2 and is described in the present publication.

3. Biosynthesis of modified forms of ZEN and T2/HT2 toxin

3.1. Modified forms of ZEN

ZEN (CAS No. 17924-92-4, $C_{18}H_{22}O_5$, MW 318) is found in cereal crops such as maize, wheat, barley, sorghum and rye, primarily in the field, but it may also occur upon poor storage conditions (EFSA CONTAM Panel, 2011a). It is produced by various *Fusarium* species under moist and cool conditions, but in particular by *F. graminearum*, *F. culmorum, F.equiseti* and *F. verticillioides* (Sweeney et al., 1998; Doohan et al., 2003). ZEN has the ring system of a macrocyclic ß-resorcylic acid lactone (RAL). The reduction of the keto group to an alcoholic hydroxyl group yields the stereoisomeric compounds α-zearalenol (α-ZEL, CAS No. 36455-72-8, $C_{18}H_{24}O_5$, MW 320) and β-zearalenone (β-ZEL, CAS No. 71030-11-0, $C_{18}H_{24}O_5$, MW 320). Reduction of the olefinic double bond of ZEN leads to zearalanone (ZAN, CAS No. 5975-78-0, $C_{18}H_{24}O_5$, MW 320), and reduction of both the olefinic double bond and keto group to α-zearalanol (α-ZAL, CAS No. 26538-44-3, $C_{18}H_{26}O_5$, MW 322) and β-zearalanol (β-ZAL, CAS No. 42422-68-4, $C_{18}H_{26}O_5$, MW 322). The structures of ZEN and its major reduction products are depicted in Figure 1.

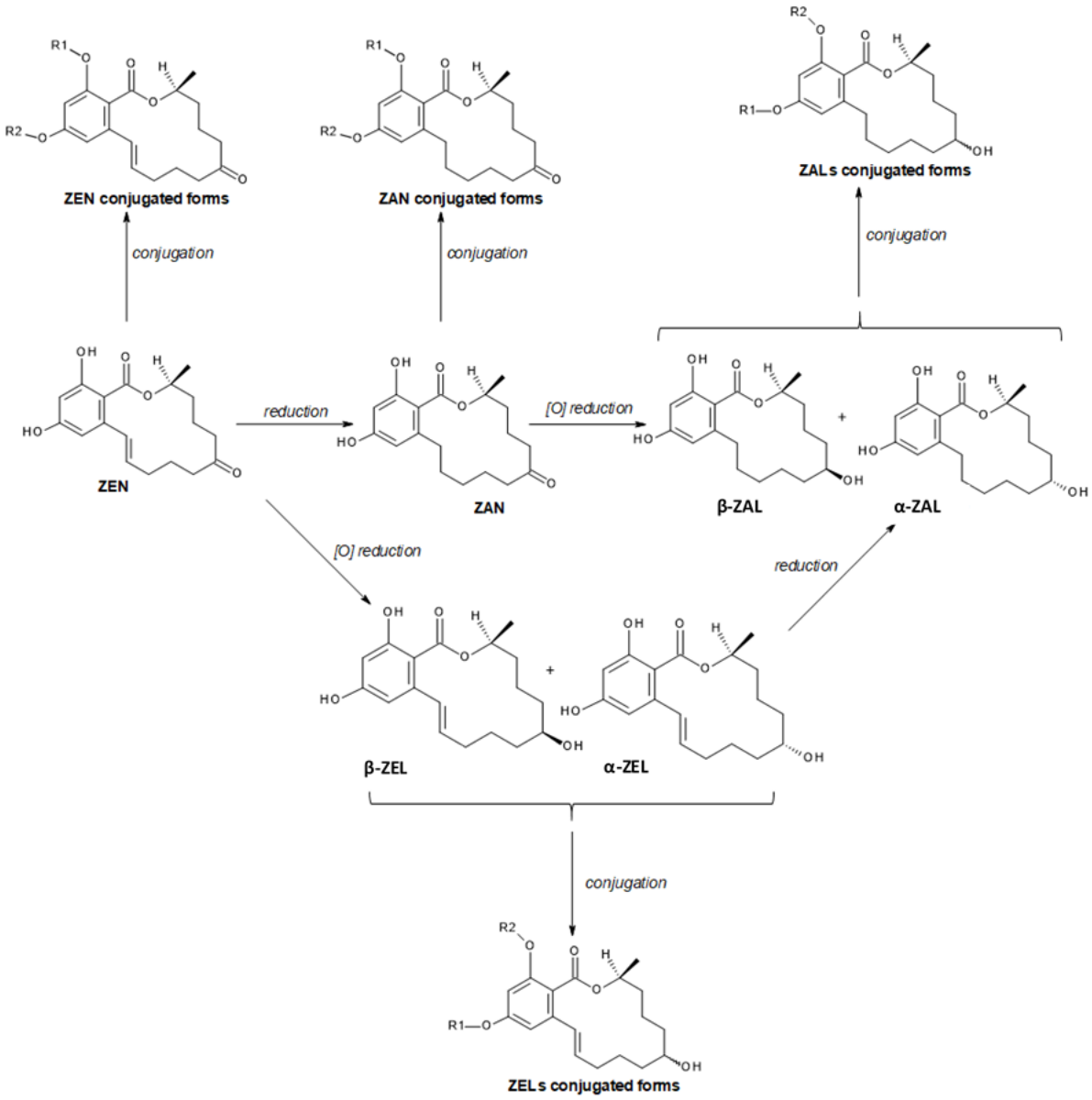

Figure 1: ZEN and its modified forms. R1 and R2 denote different conjugate groups.

α-ZAL, α-zearalanol; α-ZEL, α-zearalenol; β-ZAL, β-zearalanol; β-ZEL, β-zearalenol; ZAL, zearalanol; ZAN, zearalanone; ZEL, zearalenol; ZEN, zearalenone;

Next to the RALs depicted in Figure 1 and the cis-isomers of ZEN and ZELs, other RALs and related structures have been identified in cultures of *Fusarium* spp. or as mammalian phase I metabolites of ZEN and ZAL (e.g. 5-hydroxy-ZEN and 10-hydroxy-ZEN, ZEN-11, 12-oxide and its hydrolysis products, for review see Metzler et al., 2010). Their biological activity and relevance is not known and no conjugated forms of these RALs have yet been identified. Therefore, they are not considered further here. The phase I metabolites formed in the fungal, plant and/or mammalian organism are the initial compounds for phase II metabolism. In fungi and plants, the

major pathways of phase II metabolism of ZEN or its phase I metabolites are conjugation with hexose or sulfate (Berthiller et al., 2006; 2013). In plants, the hexose can be further modified via esterification with malonic acid or with another hexose. Due to its two hydroxyl groups at positions 14 and 16, ZEN can form two regioisomeric monoglucosides. Kamimura (1986) identified ZEN-14-O-ß-D-glucopyranoside (ZEN14βDGlcp, $C_{24}H_{32}O_{10}$, MW 480), and the regioisomeric ZEN-16-O-β-D-glucopyranoside (ZEN16βDGlcp, $C_{24}H_{32}O_{10}$, MW 480) has been characterised by Kovalsky Paris et al. (2014). The structure of ZEN14Sulf was confirmed by El-Sharkaway et al. (1991). Conjugation with glucuronic acid (GlcA), which leads to β-D-glucuronopyranosides, does not occur to any appreciable extent in plants and fungi, but is a major metabolic pathway in mammalian species. As currently no information is available on glucuronides of ZEN and its phase I metabolites in animal-derived foods, these modified forms are not considered here further.

3.2. Modified forms of T2 and HT2

T2 (CAS No. 21259-20-1, $C_{24}H_{34}O_9$, MW 466) and HT2 (CAS No. 26934-87-2, $C_{22}H_{32}O_8$, MW 424) are field contaminants in cereal grains like wheat, maize, oats, barley and rice (EFSA CONTAM Panel, 2011b). T2 and HT2 are trichothecenes, compounds sharing as a common structure a tetracyclic sesquiterpene with a spiro-epoxide group at C-12 and C13 and an olefinic double bond between C-9 and C-10. According to the substituents of the tetracyclic ring system, trichothecenes are grouped into different types (types A–D). T2 and HT2 belong to the type A trichothecenes, characterised by an esterified or free hydroxyl group at C-8, or an unsubstituted C-8. Together with type B trichothecenes (e.g. nivalenol or deoxynivalenol), which carry a keto group at C-8, they are the most important trichothecene mycotoxins in food and feed (for review see McCormick et al., 2011). The major pathways of biotransformation of T2/HT2 in fungi, plants and mammals are very similar and are depicted in Figure 3. The predominant pathway is the hydrolytic cleavage of one or more of the three ester groups of T2 (Wu et al., 2014). Reduction of the epoxide group at C-12 and C-13 to an olefinic double bond (deepoxidation) and hydroxylation at various positions of T2 and its hydrolytic metabolites, are other important pathways. Hydroxylation occurs predominantly at C-19, but also at C-20, C-8 and others. Deacetylation at C-4 leads to formation of HT2 and therefore, strictly spoken, HT-2 is a phase I metabolite of T2. As HT2 is always associated with T2 in contaminated food items, the deacetylation of T2 to HT2 appears to be very effective in the fungus. Therefore, HT2 is also considered as a parent compound by convention. In consequence, and for consistency reasons also in the present scientific review HT2 is considered

as a parent compound. Another hydrolytic metabolite of T2 is neosolaniol (NEO, CAS No. 36519-25-2, $C_{19}H_{26}O_8$, MW 382) which can be further hydrolysed to 15-acetyl-T2-tetraol ($C_{17}H_{24}O_7$, MW 340) and T2-tetraol (CAS No. 34114-99-3, $C_{15}H_{22}O_6$, 298). A major alternative pathway is hydroxylation of T2 to 19-HO-T2 ($C_{24}H_{34}O_{10}$, MW 482). Major hydrolysis products of HT2 are T2-triol (CAS No. 34114-98-2, $C_{20}H_{30}O_7$, MW 382) and in a further step T2-tetraol. De-epoxidation of HT2 yields de-epoxy- HT2 (DE-HT2, $C_{22}H_{32}O_7$, MW 408).

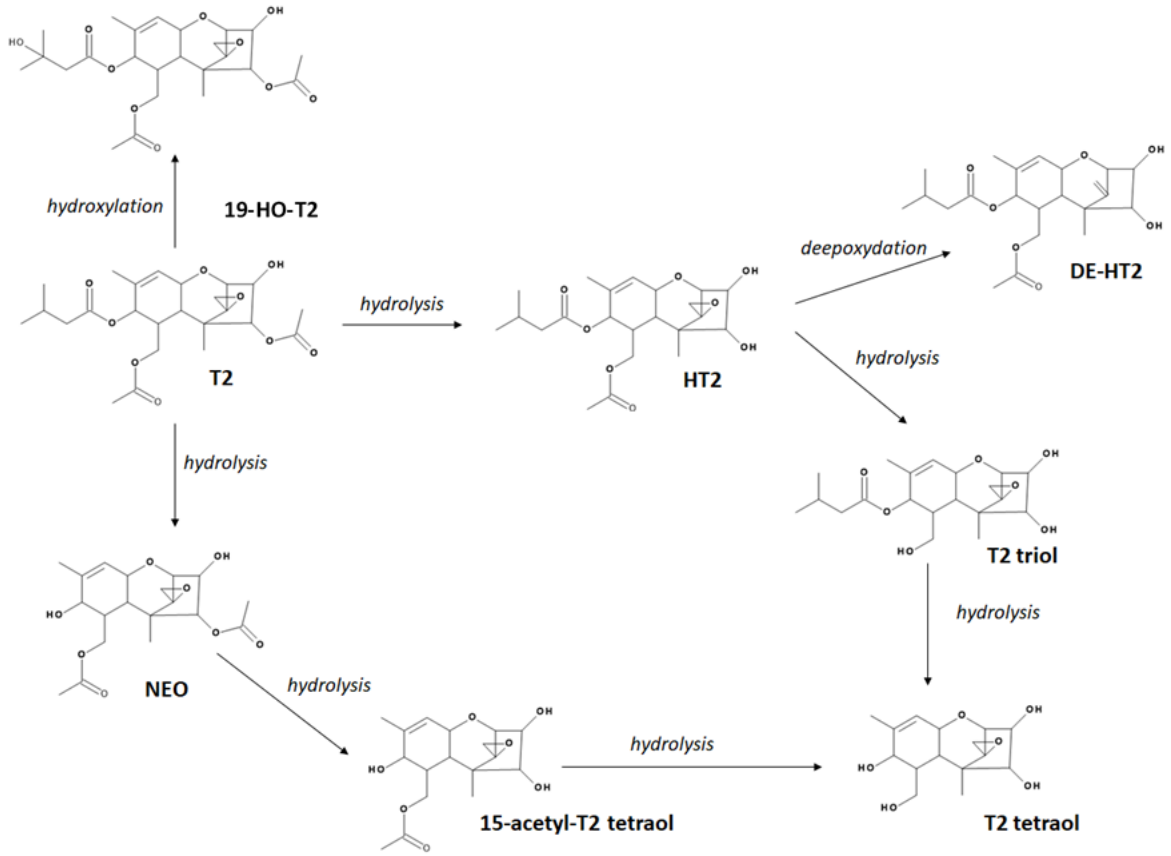

Figure 2: Major metabolic phase I pathways and metabolites of T2 and HT2

T2, T2-toxin; HT2, HT2-toxin; DE-HT2, de-epoxy-HT2; NEO, neosolaniol;

During phase II metabolism water-soluble conjugates are formed by covalent binding of parent compounds or phase I metabolites with highly polar molecules such as carbohydrates or sulfates. T2-3-Glc ($C_{30}H_{44}O_{14}$, MW 628) is a major conjugate of T2 and formed in fungi, yeast, and plants. Such glycosides can exist in two stereoisomeric forms (α and ß), which differ in the configuration of the glycosidic C-atom. Welsch and Humpf (2012) have isolated various glucuronides, namely T2-3-GlcA ($C_{30}H_{42}O_{15}$, MW 642), HT2-3-GlcA ($C_{28}H_{40}O_{14}$,

MW 600) and HT2-4-GlcA ($C_{28}H_{40}O_{14}$, MW 600) from incubations of T2 with pig liver microsomes and activated glucuronic acid (UDPGA). In addition to direct glucuronidation of T2, which has only one hydroxyl group, the microsomes catalysed the deacetylation of T2 to HT2 and the subsequent formation of two regioisomeric HT2 glucuronides.

4. Occurrence of modified forms of ZEN and T2/HT2

4.1. Occurrence of modified forms of ZEN

In fungi, α- and β-ZEL are formed from ZEN, but these can be further conjugated by either fungi and/or infected plants to α- and β-ZEL glucosides, that were co-occurring with ZEN and α- and β-ZEL in cereal-based products (De Boevre et al., 2013; Nathanail et al., 2015). In cereal based foods, the concentrations of α-ZEL and β-ZEL added another 40% (mean concentration 19 µg/kg) and 30% (mean concentration 14 µg/kg), respectively, to the amount of ZEN present (mean concentration 47 µg/kg). ZEN14Glc added another 50% (mean average concentration 23 µg/kg), α- and β-ZEL14Glc another 20% (mean average concentration 9 µg/kg and 10% (mean average concentration 5 µg/kg) each (De Boevre et al., 2013). ZEN14Sulf added an additional 20% (mean average concentration 10 µg/kg) to the amount of ZEN found (De Boevre et al., 2013). The highest concentration of α-ZEL found in breakfast cereals was 515 µg/kg (De Boevre et al., 2013). As compared with 450 µg/kg of ZEN it thereby added another 114% of modified ZEN to the concentration of the parent compound (De Boevre et al., 2013). The transfer of ZEN and its phase I and phase II metabolites to edible tissues in animals for food production is low (EFSA CONTAM Panel, 2011a; 2016). Overall, the data available show that modified forms contribute very significantly to the total ZEN (i.e. ZEN plus modified ZEN) concentrations in foodstuffs with adding up to a maximum of more than 100%.

4.2. Occurrence of modified forms of T2 and HT2

NEO was detected in 13% of 169 coffee samples, concentrations ranging from 29 – 314 µg/kg whereas T2 and HT2 was reported in 29% and 9% of the samples, concentrations ranging from 2.4 - 12.2 µg/kg, respectively (García-Moraleja et al., 2015). Pereira et al. (2015) found NEO (87 µg/kg) and T2-tetraol (112 µg/kg) in 1 of 9 cereal containing baby food samples. In field barley, HT2-3-Glc was detected at concentrations of up to 163 µg/kg in 17 out of 18 samples whereas T2-3-Glc was found only in a few samples and at much lower concentrations (Lattanzio et al., 2015). In wheat up to 37% HT2-3-Glc relative to the amount of parent

compound was found, depending on the harvest season (Lattanzio et al., 2013). In barley, wheat and oats mono-glycosylated T2 and HT2 was detected and in barley also diglycosylated T2 and HT2 was identified (Veprikova et al., 2012). Nakagawa et al. (2013a, b) detected diglucosidic T2 conjugates and a phase I metabolite of NEO in maize. In a study with cereal grains and products thereof T2 and HT2 was detected in 67% of the total 30 samples while T2-3-Glc and HT2-3-Glc could not be verified (De Boevre et al., 2013). Nathanail et al. (2015) investigated occurrence of T2 and HT2 and some of their modified forms in oats, barley and wheat and found T2, HT2 and HT2-3-Glc in 21%, 35%, and 53% of the barley samples, the mean concentrations being 10.7 µg/kg, 20 µg/kg and 10.8 µg/kg, respectively. In oats T2, HT2 and HT2-3-Glc were found in 61.3%, 74.2%, and 58.1% of the samples, and mean values were 60.1 µg/kg, 159 µg/kg and 41.4 µg/kg, respectively. In wheat T2, HT2 and HT2-3-Glc were found in 46.7%, 63.3%, and 43.3% of the samples, and mean concentrations were 4.2 µg/kg, 9 µg/kg and 15 µg/kg, respectively. Concentrations were particularly high in oats, reaching maximum values of 548 µg/kg, 1830 µg/kg, and 300 µg/kg for T2, HT2, and HT2-3-Glc, respectively. In summary, the phase II metabolites of T2 and HT2 that according to the literature may occur in food and are therefore considered as of relevance for possible inclusion in the present assessment are T2-3-glucoside (T2-3-Glc), T2-3-diglucoside (T2-3-diGlc), T2-3-sulfate (T2-3-Sulf), T2-3-glucuronic acid (T2-3-GlcA), 3-acetyl-T2 (3-Ac-T2), 3-feruloyl-T2 (3-Fer-T2), HT2-3-glucoside (HT2-3-Glc), HT2-diglucoside (HT2-diGlc), HT2-glucuronic acid (HT2-GlcA), HT2-malonylglucoside (HT2-MalGlc), neosolaniol-glucoside (NEO-Glc) T2-triol glucoside (T2-triol-Glc) and T2-tetraol glucoside (T2-tetraol-Glc) (EFSA CONTAM Panel, 2017). No information about the occurrence of T2/HT2 or their modified forms in food of animal origin has been identified probably because they are rapidly excreted (at least partly) before any animal parts or products are consumed. This notion is supported by studies indicating that T2 and its metabolites are rapidly excreted mainly as glucuronides in urine and faeces (EFSA CONTAM Panel, 2011b; Welsch and Humpf, 2012). Overall, the data available show that modified forms contribute significantly to the total T2/HT2 concentration in plant derived foods adding up to 40% to the concentrations of T2/HT2.

5. Toxicity of ZEN and its modified forms

5.1. Toxicity of ZEN

In 2000, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2000) derived a no observed effect level (NOEL) of 40 µg/kg bw per day for ZEN based on oestrogenic effects (prolonged inter-

oestrous interval and maintenance of corpora lutea and increased progesterone concentrations in plasma) in a study where gilts received oral doses of 0, 40, 200 and 400 µg ZEN/kg bw per day, between day 5 and day 20 of the oestrus (Edwards et al., 1987). Based on this NOEL, a provisional maximum tolerable daily intake (PMTDI) for ZEN and its metabolites of 0.5 µg/kg bw per day was established using an uncertainty factor (UF) of 100. The Scientific Committee on Food (SCF) established a temporary tolerable daily intake (t-TDI) of 0.2 µg ZEN/kg bw per day (SCF, 2000) using the same NOEL, but applying an UF of 200 (taking into account next to interindividual and intraspecies variabilities also the deficiencies in the overall data base). Also EFSA, in their assessment of 2011 (EFSA CONTAM Panel, 2011a) identified oestrogenic activity as the critical adverse effect of ZEN and the pig as the most sensitive species. A NOEL of 10.4 µg/kg bw per day was identified based on results from a study, in which gilts received doses of 0.5, 3.0, 7.4, 10.4 and 17.6 µg ZEN/kg bw per day for 5 weeks and in which statistically significant increases in swollen and reddened vulvas and uteri weight were observed at the highest dose (Döll et al., 2003). A TDI of 0.25 µg/kg b.w. per day was derived applying an uncertainty factor of 40 (a reduced factor of 4 for interspecies differences because humans were considered not to be more sensitive than the female pig and a default factor 10 for intra-human variability). In none of these assessments was acute toxicity of ZEN observed and thus no ARfD was set. For the present scientific review the EFSA expert group reviewed all potentially relevant toxicity studies published since this last EFSA evaluation and overall, the new studies further corroborated oestrogenicity as critical effect of ZEN (see EFSA CONTAM Panel, 2016), but none of the studies reported adverse effects lower than the NOEL identified from the already previously evaluated study with female pigs (Döll et al., 2003) and therefore the TDI established in 2011 was retained for ZEN. No new studies became available suggesting acute effects of ZEN and thus no ARfD was set for ZEN.

5.2. Toxicity of modified forms of ZEN

A series of *in vivo* and *in vitro* studies investigating oestrogenicity related effects are available for ZEN phase I metabolites, but because of the design of these studies (i.e. the lack of dose-response information) they cannot be used for deriving no observed adverse effect levels/lowest observed adverse effect levels (NOAELs/LOAELs) or benchmark doses (BMDs) needed for setting HBGVs for the individual modified forms of ZEN. The majority of adverse effects observed with ZEN and its phase I metabolites are related to their endogenous steroidal sex hormone 17ß-estradiol (E2) like action that involves binding to oestrogen receptors (ERs) in oestrogen target organs. Consequently, the affinity and activation of ERs can be used as a direct measure of the oestrogenic potency of a compound (Enmark and Gustafsson, 1999). A landmark effect of

oestrogenic compounds is the induction of uterine growth in immature or ovariectomized female animals, which is assessed in the "uterotrophic assay" in which changes of uterus wet weights are used as endpoints in mice or rats (Thigpen et al., 1987). The *in vitro* tests reported were competitive binding assays with radiolabelled E2, the MCF-7 (Michigan Cancer Foundation–7) cell proliferation assay in which MCF-7 breast cancer cells are incubated with potentially oestrogenic compounds using E2 as reference compound, activation of reporter gene assays in which initiation of transcription of target genes is used to detect steroid hormone receptor (ant)agonistic compounds, the oestrogen receptor-alpha redistribution assay in which cellular activation and distribution of human oestrogen receptor-α in recombinant cells is measured in comparison with E2, the CALUX (chemical activated luciferase gene expression) assay in which light emission induced by the test compound in stably transfected cells is a measure of agonistic activity with E2. In principle, the activity of an oestrogenic agent can be modulated by metabolism both in *in vivo* and *in vitro* assays, but the impact of metabolism is, not or not well reflected in *in vitro* assays (Legler et al., 2002) . In order to compare the oestrogenic potency of the different phase I metabolites the available *in vitro* and *in vivo* studies were analysed and the oestrogenic potency relative to ZEN (which was set as 1) was determined on a molar basis. Table 1 lists phase I metabolites tested in such assays, the relative oestrogenic potencies of these metabolites, the assays used and the references to these studies.

Table 1: Relative oestrogenic potency of modified forms of ZEN relative to ZEN calculated from the results of comparative *in vitro* assays

Assay/Endpoint	**Compound (molar potency relative to ZEN[a])**	**Reference**
Binding to calf uterine cytosolic oestrogen receptor	cis-ZEN (2)	Kiang et al. (1978)
	ß-ZEL (0.31); α-ZAL(12); ß-ZAL (2.4)	Arts and van den Berg (1989)
Binding to rat uterine cytosolic oestrogen receptor	α-ZAL (9); ß-ZAL (0.3)	Katzenellenbogen (1979)
	α-ZEL (10); ß-ZEL (0.05); ZAN (0.5); α-ZAL (7) ß-ZAL (0.15)	Branham et al. (2002)
Binding to recombinant human oestrogen receptor	ß-ZEL (0.14)	Miksicek (1994)
	α-ZAL (11[b]; 4[c])	Takemura et al. (2007)
Oestrogen receptor-alpha redistribution	ß-ZEL (19); ß-ZEL (0.09); α-ZAL (21); ß-ZAL (5.4);	Ehrlich et al. (2015)
MCF-7 cell proliferation	ß-ZEL (0.05); α-ZAL (56);	Welshons et al. (1990)
	α-ZEL (92); α-ZAL (18); ß-ZEL (0.44); ZAN (2.5); ß-ZAL (3.5); cis-ZEN (0.73)	Shier et al. (2001)
	α-ZEL (2200); ß-ZEL (0.6); α-ZAL (18); ß-ZAL (12);	Minervini et al. (2005)
	α-ZEL (63); ß-ZEL (0.45); ZAN (3.1); α-ZAL (27); ß-ZAL (1.7);	Molina-Molina et al. (2014)
	α-ZEL (563); ß-ZEL (0.06); ZAN (13); α-ZAL (14); ß-ZAL (0.5); cis-ZEN (1.5); cis-α-ZEL(36); cis-ß-ZEL (0.62)	Drzymala et al. (2015)
Activation of reporter gene	α-ZEL (33); ß-ZEL (0.25); α-ZAL (5); ß-ZAL (1.8);	Coldham et al. (1997)
	α-ZEL (73); ß-ZEL (0.41);	Frizzell et al. (2011)

Assay/Endpoint	Compound (molar potency relative to ZEN[a])	Reference
	α-ZEL (11); ß-ZEL (0.06); α-ZAL (4); ß-ZAL (0.5);	Stypula-Trebas et al. (2015)
	α-ZAL (10);	Jefferson et al. (2002)
CALUX assay	α-ZEL (51); ß-ZEL (0.2); α-ZAL (26); ß-ZAL (1.7);	Ehrlich et al. (2015)
AP assay in human endometrial adenocarcinoma cells	α-ZEL (15);	Vejdovszky et al. (2017)

[a] Oestrogenicity of the different compounds is expressed on a molar basis relative to ZEN, the activity of which is defined as 1 in each assay

[b] Binding to oestrogen receptor-alpha

[c] Binding to oestrogen receptor-beta

AP, alkaline phosphatase; α-ZAL, α-zearalanol; α-ZEL, α-zearalenol; ß-ZAL, ß-zearalanol; ß-ZEL, ß-zearalenol; CALUX assay, chemical activated luciferase gene expression assay; cis-α-ZEL, cis-α-zearalenol; cis-ß-ZEL, cis-β-zearalenol; cis-ZEN, cis-zearalenone; MCF-7, human breast adenoma cell line; ZAN, zearalanone; ZEN, zearalenone;

Table 2: Relative oestrogenic potency of modified forms of ZEN (zearalenone) relative to ZEN calculated from the results of comparative *in vivo* assays

Assay/Endpoint	Compound (Molar potency relative to ZEN[a])	Reference
Uterotrophic activity in immature rats	α-ZAL (3[b]); ß-ZAL (0.3[b]);	Katzenellenbogen et al. (1979)
	α-ZAL (4.4[c]); α-ZEL (62[c]); ß-ZAL (1.9[c]), ß-ZEL (0.18[c]); ZAN (1.6[c])	Everett et al. (1987)
Uterotrophic activity in immature mice	α-ZAL (1[b]);	Jefferson et al. (2002)
	α-ZAL (2[d])	Ueno and Tashiro (1981)
Uterotrophic activity in ovariectomised mice	α-ZAL (4[b]);	Takemura et al. (2007)
	cis-ZEN (0.8[e]); cis-α-ZEL (8[d]); cis-ß-ZEL (0.8[e])	Peters (1972)

[a] Oestrogenicity of the different compounds is expressed on a molar basis relative to ZEN, the activity of which is defined as 1 in each assay

[b] subcutaneous administration

[c] oral administration

[d] intraperitoneal application

[e] added to feed

α-ZAL, α-zearalanol; α-ZEL, α-zearalenol; ß-ZAL, ß-zearalanol; ß-ZEL, ß-zearalenol; cis-α-ZEL, cis-α-zearalenol; cis-ß-ZEL, cis-β-zearalenol; cis-ZEN, cis-zearalenone; ZAN, zearalanone; ZEN, zearalenone;

The results listed in Tables 1and 2 show that α-ZEL has a higher oestrogenic activity than ZEN, suggesting that conversion of ZEN to α-ZEL constitutes a metabolic activation. This is particularly obvious in *in vitro* assays that reflect better the intrinsic oestrogenic potencies than *in vivo* assays, because the compound is not metabolized in the *in vitro* assays. In contrast, the epimeric ß-ZEL exhibits consistently less oestrogenicity than ZEN. Although not to the same extent, a similar pattern is seen for the α- and ß-stereoisomers of ZAL, as α-ZAL is more oestrogenic than ß-ZAL and ZAN. ß-ZAL and ZAN have about the same potency as ZEN. Data on the oestrogenicity of cis-ZEN are limited but suggest that isomerization of the olefinic double bond in ZEN and the ZEL epimers has no significant effect on oestrogenic potency. Notably, α -ZAL showed similar potency when given s.c. or orally, whereas ß-ZAL was clearly more potent after oral than after s.c. administration (Katzenellenbogen et al., 1979; Everett et al., 1987). A possible explanation for the higher potency of ß-ZAL after oral uptake may be its faster metabolic conversion to ZAN and α-ZAL in the liver as compared to *s.c.* application.

According to the *in vitro* and *in vivo* data presented in Tables 1 and 2, the oestrogenic potency of the different ZEN congeners can be ranked as follows: α-ZEL > α-ZAL ≈ cis-α-ZEL > ZEN ≈ ZAN ≈ cis-ZEN ≈ ß-ZAL ≈ cis-ß-ZEL > ß-ZEL. For ZEN conjugates there is almost no information with regard to their oestrogenicity. When the oestrogenic activity of ZEN14GlcA, α-ZEL14GlcA, α-ZEL7GlcA, ß-ZEL14GlcA and ß-ZEL16GlcA was investigated in an oestrogen-responsive reporter gene assay it was found that, overall, the oestrogenic activity of these glucuronides amounted only to 0.01% and 1% of E2 and ZEN, respectively (Frizzell et al., 2015). The authors attributed this weak oestrogenic activity exclusively to trace amounts of free ZEN and ZELs probably arising from the cleavage of the conjugates and present in the incubation medium. Hence, it can be assumed that the phase II conjugates as such were devoid of oestrogenic activity. All three conjugate groups are esters and therefore prone to hydrolysis and release of their aglycone. In mammals, conjugates of xenobiotic compounds may be hydrolysed in the gastrointestinal tract both by enzymes of the intestinal epithelium and the gut microflora (EFSA CONTAM Panel, 2014; Binder et al., 2017; Catteuw et al., 2019). This has been demonstrated repeatedly for the glucuronides excreted in bile, resulting in enterohepatic circulation of the aglycone and consequently delayed excretion. Like glucuronides also sulphates may be enzymatically hydrolysed, but since they are more susceptible to acid hydrolysis than glucosides (Roy, 1982) they are probably hydrolysed, at least in part, already in the stomach. In summary, orally ingested glucuronides, sulphates and glucosides of ZEN or its phase I metabolites would be prone to gastro-intestinal hydrolysis with release of their respective aglycones as has been demonstrated recently in studies with pigs (Binder et al., 2017; Catteuw et al., 2019). In the absence of data on the extent of this hydrolysis in humans, the expert group concluded that it is appropriate to assume complete release of the aglycones as a reasonable worst case.

For phase I metabolites of ZEN, including also metabolites that have so far not been detected in food or feed, the expert group assigned relative potencies factors (RPF) for oestrogenicity with ZEN as the comparator. This would allow including the metabolites in a group HBGV with ZEN. These RPFs were based on the relative oestrogenic potencies determined in *in vivo* uterotrophic assays (see Table 2) because only oral *in vivo* assays reflect the entire oestrogenic response including both toxicokinetics and the cellular processes of estrogen action, whereas *in vitro* assays consider only parts of it such as the binding of a compound to estrogen receptors. In cases where several results from uterotrophic assays were available those yielding the highest relative potency was used applying a conservative approach. The *in vitro* results are presented in Table 1 for the reason that they support, albeit rather in a qualitative than quantitative manner, the relative potencies derived from the *in vivo* uterotrophic assays. No *in vivo* uterotrophic assays with phase II metabolites of ZEN or ZEN phase I

metabolites have been identified and as indicated above, phase II metabolites were assigned the same RPF as their respective aglycones. Because no information was identified on the transfer of glucuronides of ZEN and its phase I metabolites to animal derived foods these were not considered in Tables 1 and 2.

The potency factors relative to ZEN (which was set as 1) derived from *in vivo* uterotrophic assays (presented in Table 2) are summarised in Table 3.

Table 3: Relative potencies factors (RPFs) for the different phase I and phase II metabolites of ZEN as derived from *in vivo* uterotrophic assays

Relative potency factor	Compounds
0.2	β-ZEL; β-ZELGlcs; β-ZELSulfs
1.0	ZEN; ZENGlcs; ZENSulfs; cis-ZEN; cis-ZENGlcs; cis-ZENSulfs; cis-β-ZEL; cis-β-ZELGlcs; cis-β-ZELSulfs;
1.5	ZAN; ZANGlcs; ZANSulfs;
2.0	β-ZAL; β-ZALGlcs; β-ZALSulfs
4.0	α-ZAL; α -ZALGlcs, α -ZALSulfs
8.0	cis-α-ZEL; cis-α-ZELGlcs; cis-α-ZELSulfs
60	α-ZEL; α-ZELGlcs; α-ZELSulfs

α-ZAL, α-zearalanol; α-ZALGlcs, α-ZAL-glucosides; α-ZALSulfs, α-ZAL-sulfates; α-ZEL, α-zearalenol; α-ZELGlcs, α-ZEL-glucosides; α-ZELSulfs, α-ZEL-sulfates; β-ZAL, β-zearalanol, β-ZALGlcs, β-ZAL-glucosides; β-ZALSulfs, β-ZAL-sulfates; ß-ZEL, ß-zearalenol; β-ZELGlcs, β-ZEL-glucosides; β-ZELSulfs, β-ZEL-sulfates; cis-α-ZEL, cis-α-zearalenol; cis-α-ZELGlcs, cis-α-ZEL-glucosides; cis-α-ZELSulfs, cis-α-ZEL-sulfates; cis-β-ZEL, cis-β-zearalenol; cis-β-ZELGlcs, cis-β-ZEL-glucosides, cis-β-ZELSulfs; cis-β-ZEL-sulfates; cis-ZEN, cis-zearalenone; cis-ZENGlc, cis-ZEN-glucosides; cis-ZENSulfs, cis-ZEN-sulfates; ZAN, zearalanone; ZANGlcs, ZAN-glucosides; ZANSulfs, ZAN-sulfates; ZEN, zearalenone; ZENGlcs, ZEN-glucosides; ZENSulfs, ZEN-sulfates;

Thee RPFs as listed in Table 3 can be applied for dietary risk assessment assuming that the oestrogenic effects of ZEN and its modified forms are additive. Assuming dose-addition as principle of combined action is supported by present knowledge, albeit in principle synergistic or antagonistic effects cannot be excluded. The RPFS are

based on data for phase I metabolites and are expressed on a molar basis. As the molecular weights (MWs) of phase I metabolites are about the same as the MW of ZEN, it has a negligible impact when using a weight scale instead of a molar scale. On the contrary, the MWs of phase II metabolites are much higher. Hence, when applying RPFs in a dietary risk assessment to the phase II metabolites listed in Table 3, the quantities of the phase II metabolites need to be expressed on a molar scale. A finding of particular importance when applying the RPF approach is that the relative potency of α-ZEL is 60 times higher than that of ZEN. This finding has a major impact on the risk assessment of ZEN and exemplifies the importance of considering the differential toxicity of modified mycotoxins for a comprehensive risk assessment.

5.3. Toxicity of T2 and H2 and its modified forms

5.3.1. Toxicity of T2 and HT2

In 2001, the JECFA identified a LOEL of 29 µg T2/kg bw per day based on changes in white and red blood cells observed in a 3-week study with pigs (Rafai et al., 1995) and established a provisional maximum tolerable daily intake (PMTDI) of 60 ng/kg bw per day by adding an UF of 500 (considering next to a standard factor for intra-species and intra-individual variability also the absence of a NOEL and deficiencies in the database, in particular the lack of long-term studies). Since the effects might be attributable also to HT2 (which is rapidly formed from T2) the Committee concluded that the PMTDI is applicable for T2 and HT2 alone or in combination (FAO/WHO, 2001). In the same year, the SCF derived a TDI of 60 ng/kg bw for the sum of T2 and HT2 that was based on identical considerations (SCF, 2001). In their assessment of 2011, EFSA concluded that T2 inhibits the synthesis of proteins, DNA and ribonucleic acid (RNA), causes apoptosis, necrosis and lipid peroxidation, and confirmed that immunological and haematological effects are the critical endpoints of T2 toxicity (EFSA, 2011a). Using observations of reduction in antibody response to a specific antigen in pigs in a 3-weeks study in which pigs received doses of 0, 29, 62, 105 and 129 µg T2/kg bw per day (Rafai et al., 1995) for a BMD analysis, a $BMDL_{05}$ (95% lower confidence limit of the BMD_{05}, the dose associated with a 5% change in response relative to the control group) of 10 µg T2/kg bw per day was derived. By applying a standard UF of 100, a TDI of 0.1 µg/kg bw was established, applicable to T2, HT2 and the sum of T2/HT2. For the present scientific review the EFSA expert group reviewed all new studies published since the last EFSA assessment (2011) and concluded that a reduction of total leukocyte count observed in a new 90-day study in which rats were orally dosed with 0, 45, 68 and 90 µg T2/kg bw per day (Rahman et al., 2014) was the most

appropriate adverse effect for setting a TDI for T2. Following respective EFSA guidance (EFSA Scientific Committee, 2017) a $BMDL_{10}$ (95% lower confidence limit of the BMD_{10}, the dose associated with a 10% change in response relative to the control group) of 3.33 µg T2/kg bw per day was calculated. By applying an UF 200, (taking into account next to the standard factors for inter- and intra-species variability also a factor of 2 for derivation of a chronic HBGV from a subchronic study, see EFSA, 2012) we established a revised TDI of 0.02 (rounded from 0.017) µg/kg bw for T2, which is also applicable for HT2 and the sum of T2/HT2.

In new studies, anorectic effects in mice and mink upon short term exposure to T2 and HT2 were observed (Wu et al., 2014; 2016). The expert group identified emetic events (retching, vomiting) in mink upon oral exposure to single doses of 0.0, 0.005, 0.25 and 0.5 mg/kg bw to both T2 and HT2 as appropriate for setting an ARfD. Following EFSA guidance (EFSA Scientific Committee, 2017) a benchmark dose analysis was carried out combining the data sets for T2 and HT2 resulting in a $BMDL_{10}$ of 2.97 µg T2 or HT2/kg bw which by adding an UF of 10 resulted in an ARfD of 0.3 (rounded from 0.297) µg/kg bw that is applicable for T2, HT2 or the sum of T2 and HT2. A factor for inter-species variability was omitted as humans are not expected to be more sensitive than mink with regard to emesis as shown by experiments with emetine (Gordon, 1985; Zhang et al., 2006; Percie du Sert et al., 2012)..

5.3.2. Acute *in vivo* toxicity of T2/HT2 in comparison with their phase I metabolites

Whereas there is a relative abundance of *in vivo* acute toxicity studies on T2, there were only a few *in vivo* studies investigating the toxicity of phase I metabolites of T2 and HT2. These studies compared specific acute effects upon exposure to single doses of T2/HT2 and at least one phase I metabolite. The results are briefly summarized in Table 4 together with the relative molar potencies calculated for the specific endpoints.

Table 4: *In vivo* acute toxicity studies comparing the toxicity of T2 with HT2 and their phase I metabolites

Endpoint	Effective molar dose (molar potency relative to T2[a])	Reference
Minimum emetic subcutaneous dose in ducklings	T2: 0.21 µmol/kg bw (1); HT2: 0.24 µmol/kg bw (0.88); NEO: 0.26 µmol/kg bw (0.81)	Ueno et al. (1974)
Oral LD_{50} in broiler chicks	T2: 10.7 µmol/kg bw (1); HT2: 17.0 µmol/kg bw (0.63) NEO: 65.1 µmol /kg bw (0.16); T2-triol: 79.0 µmol/kg bw (0.14); T2-tetraol: 113 µmol/kg bw (0.094)	Chi et al. (1978)
Intraperitoneal LD_{50} in	T2: 11.2 µmol/kg bw (1); HT2: 21.2 µmol/kg bw (0.53) NEO: 38.0 µmol/kg bw (0.29); T2-triol: 283 µmol/kg bw (0.04)	Ueno et al.

mice		(1986)
Induction of apoptosis in murine thymus cells (% DNA fragmentation above negative control (2%)/ dose of 1.56 mg toxin/kg bw	T2 (3.35 µmol/kg bw), 22% (1) ; HT2 (3.68 µmol/kg bw), 3% (0.14) 19-HO-T2[c] (3.24 µmol/kg bw), 24% (1.1) ; 19-HO-HT2[c] (3.55 µmol/kg bw), 4% (0.2) ; NEO (4.05 µmol/kg bw), 0.5% (0.023) ; T2-tetraol (5.23 µmol/kg bw), 0%	Islam et al. (1998)

[a] For the derivation of relative molar potencies the doses have been converted from weight to moles and the effective molar dose has been set as 1 for T2

[b] Only 3 doses tested

[c] In the original paper (Islam et al., 1998), these compounds were designated as 3'-HO-T2 and 3'-HO-HT2 due to a different numbering system

LD_{50}, median lethal dose; HT2, HT2-toxin; NEO, neosolaniol; T2, T2-toxin;

From the results described in Table 4 it can be seen that the potency to induce vomiting in ducklings upon acute exposure is identical for T2, HT2 and NEO, as the minimal effective dose is 0.1 mg/kg bw for all three compounds (Ueno et al., 1974). Potential for lethality of the different phase I metabolites decreased in the same order in broilers and mice and was T2 > HT2 > NEO > T2-triol > T2-tetraol (the last phase I metabolite only tested in broilers) (Chi et al., 1978; Ueno et al., 1986). The ranking of potency regarding induction of apoptosis was T2 ≈ 19-HO-T2 > HT2 ≈ 19-HO-HT2 > NEO ≈ T2-tetraol ≈ negative control (Islam et al., 1998). T2 and HT2 induce anorexia/emesis in different species, but the mode of action has not being fully elucidated. It has been hypothesised that satiety hormones and pro-inflammatory cytokines mediate these effects (Gaigé et al., 2014). T2 upregulates messenger RNA (mRNA) levels of interleukin (IL)-1 beta, IL-6 and tumour necrosis factor (TNF)-alpha in rodents (Agrawal et al., 2012; Gaigé et al., 2014), and. these proinflammatory cytokines induce sickness behavior, including anorexia (Kelley et al., 2003). In mink, T2- and HT2-induced emesis was paralleled by increased plasma concentrations of pancreatic peptide YY3-36 (PYY3–36) and 5-hydroxytryptamine (5-HT) (Wu et al., 2016), two hormones which are known to be involved in emesis (Wu et al., 2013; Lebrun et al., 2015. With regard to phase I metabolites, NEO was equipotent with T2 and HT2 when tested for vomiting in ducklings (Ueno et al., 1974, see Table 4). Therefore, the CONTAM Panel decided that NEO could be included with a RPF of 1 together with T2 and HT2 in a group ARfD that is based on this endpoint. None of the other phase I metabolites have been tested with regard to emetic response. The other data

on comparative toxicity of T2 and HT2 with its metabolites presented in Table 4 cannot be related to emesis and therefore not be used to assess relative emetic potency of metabolites. Consequently, due to the lack of appropriate data, other phase I metabolites cannot be included in a group ARfD. Since phase II metabolites of T2, HT2 and NEO are assumed to be hydrolysed to their parent compounds (aglycones) after ingestion, they were included in a group ARfD with the same potency as their parent compounds.

5.3.3. *In vitro* toxicity of T2 , HT2 in comparison with their phase I metabolites

Whereas only four acute *in vivo* studies have been identified, there is a series of *in vitro* studies where toxicity of T2/HT2 and their phase I metabolites were comparatively investigated. Like for the *in vivo* studies the results obtained in the *in vitro* studies allowed calculations of relative toxic potencies for HT2, and phase I metabolites of T2 and HT2 relative to T2 (the toxic potency of which was set as 1 for each test and endpoint). In Table 5 these studies and their results are briefly summarised and their relative molar potencies are listed. Only metabolites that are naturally occurring have been included.

Table 5*: In vitro* toxicity studies with T2 and HT2 and their phase I metabolites in various cell systems

Endpoint	Effective concentration (molar potency relative to T2[a])	Reference
Inhibition of mitogen-induced blastogenesis in primary human lymphocytes; 72 h exposure; EC_{50}	T2: 3.2 nM (1) ; HT2: 8.3 nM (0.39) T2-triol: 390 nM (0.008); T2-tetraol: 500 nM (0.006);19-HO-T2: 8.3 nM (0.39); 19-HO-HT2: 115 nM (0.028)	Forsell et al. (1985)
Inhibition of protein synthesis (^{3}H-leucin incorporation) in kidney epithelial cell line (Vero cells, African green monkey); 1 h exposure; EC_{50}	T2: 24 nM (1); T2-tetraol: 22,800 nM (0.0011)	Ehrlich and Daigle (1985)
Inhibition of protein synthesis (^{3}H-leucin incorporation) in mouse erythroleukemia cell line; 1 h exposure; EC_{50}	T2: 6 nM (1); T2-tetraol: 6,400 nM (0.00094)	Ehrlich and Daigle (1985)
Inhibition of protein synthesis (^{3}H-leucin incorporation) in kidney epithelial cell line (Vero cells, African green monkey); 1.5 h exposure; EC_{50}	T2: 14 nM (1); HT2: 65 nM (0.22); NEO: 273 nM (0.051); T2-triol: 1120 nM (0.013); T2-tetraol: 5856 nM (0.0024)	Thompson and Wannemacher (1986)
Inhibition of protein synthesis (^{3}H-leucin incorporation) in primary rat spleenocytes; 1.5 h exposure; EC_{50}	T2: 6 nM (1); HT2: 10 nM (0.60); NEO: 127 nM (0.047); T2-triol: 151 nM (0.040); T2-tetraol: 7832 nM (0.0008)	Thompson and Wannemacher (1986)
Inhibition of protein synthesis (^{3}H-leucin incorporation) in rabbit reticulocytes; 1.5 h exposure; EC_{50}	T2: 21.5 nM (1); HT2: 70.8 nM (0.30); NEO: 654 nM (0.033); T2-triol: 5240 nM (0.004); T2-tetraol: 8390 nM (0.002)	Ueno et al. (1986)
Cell viability (trypan blue exclusion) in human acute promyelocytic leukemia cell line (HL 60); concentrations needed to kill 100% of the cells in 5 days	T2: 17 nM (1); HT2: 19 nM (0.89); NEO: 340 nM (0.050); T2-triol: 262 nM (0.065); T2-tetraol: 470 nM (0.036)	Samara et al. (1987)
Cell viability; 6 days exposure of adult human liver cell line (Chang liver); EC_{50}	T2: 5 nM (1); HT2: 10 nM (0.50); NEO: 200 nM (0.025); T2-triol: 105 nM (0.048); T2-tetraol: 800 nM (0.0062)	von Milczewski (1987)
Cell viability in human epidermoid carcinoma cell line (HEp-2); 6 days exposure; EC_{50}	T2: 5 nM (1); HT2: 10 nM (0.5); NEO: 180 nM (0.028); T2-triol: 86 nM (0.058) T2-tetraol: 670 nM (0.0075);	von Milczewski (1987)
Cell viability in human heart cell line (clone GHc7); 6	T2: 9 nM (1); HT2: 12 nM (0.75); NEO: 210 nM (0.043); T2-triol: 76 nM (0.12);	von Milczewski (1987)

Endpoint	Effective concentration (molar potency relative to T2[a])	Reference
days exposure; EC_{50}	T2-tetraol: 470 nM (0.019)	
Cell viability in pig kidney cell line (clone Amc6sc8); 6 days exposure; EC_{50}	T2: 10 nM (1); HT2: 11.3 nM (0.88); NEO: 3-6 nM (3.33-1.67)[b]; T2-triol: 39 nM (0.26); T2-tetraol: 540 nM (0.019)	von Milczewski (1987)
Cell viability in mouse lymphoma cell line (L5178); 3 daysexposure; EC_{50}	T2: 3.9 nM (1); HT2: 7.1 nM (0.55); NEO: 105 nM (0.037) ; T2-triol: 260 nM (0.015) T2-tetraol: 340 nM (0.012)	Anderson et al. (1989)
Cell viability in human fibroblast cell line (GM5757); 48 h exposure; EC_{50}	T2: 122 nM (1); HT2: 383 nM (0.32); NEO: 342 nM (0.36); T2-tetraol: 6540 nM (0.019)	Kim et al. (1991)
Cell viability in human liver cell line (HepG2); 48 h exposure; neutral red uptake; EC_{50}	T2: 21 nM (1); HT2: 37 nM (0.57); T2-triol: 1040 nM (0.020); T2-tetraol: 1490 nM (0.014)	Babich and Borenfreund, (1991)
Cytotoxicity in baby hamster kidney cell line (BHK-21 cells); 24 h exposure; approximate LC_{100}	T2: 10.7 nM (1); HT2: 240 nM (0.045); NEO: 26.2 nM (0.41); T2-tetraol: 33,500 nM (0.0003); 19-HO-T2: 10.3 nM (1.03)	Senter et al. (1991)
Cell viability Primary human renal proximal tubule epithelial cells; 48 h exposure; CCK-8 assay; EC_{50}	T2: 200 nM (1); HT2: 800 nM (0.25); NEO: 3000 nM (0.067); T2-triol: 14600 nM (0.014); T2-tetraol: 25100 nM (0.008)	Königs et al. (2009)
Cell viability in primary human lung fibroblasts; 48 h exposure; CCK-8 assay; EC_{50}	T2: 500 nM (1); HT2: 700 nM (0.71); NEO: 2000 nM (0.25); T2-triol: 10600 nM (0.047); T2-tetraol: 8300 nM (0.060)	Königs et al. (2009)
Cell viability in lung fibroblast cell line from male Chinese hamster (V79); 48 h exposure; neutral red uptake; EC_{50}	T2: 3 nM (1); HT2: 14 nM (0.21)	Behm et al. (2012)

[a] The doses reported in the table have been recalculated from weight to molar units and relative molar potency has been set as 1 for T2.

CCK-8, Cell Counting Kit-8, using reduction of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt; EC_{50}, half maximal effective concentration; HT2, HT2-toxin; LC_{100}, absolute lethal concentration; NEO, neosolaniol; T2, T2-toxin;

Summarising *in vitro* tests for T2/HT2 metabolites, either cell toxicity, cell viability or inhibition of protein synthesis were investigated. T2 consistently had the highest potency for each endpoint in each study, followed by HT2 being almost equipotent. The other hydrolytic T2 metabolites NEO and T2-triol were less potent with T2-tetraol exerting the lowest toxicity. Oxidation of the side chain of T2 to 19-HO-T2 did not change its toxicity. For the other metabolites no clear potency pattern could be established. Notably, the relative potencies observed *in vitro* appeared similar to those identified in the in acute *in vivo* studies. The toxic modes of action of T2, and to a lesser extent of HT2 (which is a readily formed transformation product of T2), are relatively well investigated. T2 is a protein synthesis inhibitor binding to the 60S subunit of the ribosome, interfering with the elongation step of protein synthesis and specifically with the peptide bond formation (Garreau de Loubresse et al., 2014). Ribosome binding by T2 activates mitogen-activated protein kinase (MAPK) via a mechanism known as the "ribotoxic stress response" (Yang et al., 2000; Wan et al., 2015). MAPKs are important transducers of downstream signalling events related to immune response and apoptosis. DNA and RNA synthesis are also inhibited by T2 and it has been suggested that this could be secondary to the inhibition of protein synthesis (Rakkestad et al., 2010). Inhibition of DNA synthesis by T2 has been reported at concentrations generally exceeding those that cause inhibition of protein synthesis (EFSA CONTAM Panel, 2011b). T2 causes apoptosis *in vitro* in various cell types and *in vivo* in lymphoid organs, intestinal crypt, brain, skin and fetal tissues (EFSA CONTAM Panel, 2011b). Apoptosis of hematopoietic progenitors by T2 blocks the renewal of blood cells in the bone marrow. As the lifetime of leukocytes and platelets (approximately 10 days in humans) is much shorter than the life time of red blood cells (approximately 120 days in humans) decreases in leukocyte and thrombocyte counts in circulating blood cells with increasing the risk of septicemia and haemorrhage are early signs of toxicity. As can be seen in Table 5, phase I metabolites of T2 and HT2 elicit similar effects in mechanistic *in vitro* studies as T2 and HT2. Therefore, it can be assumed that these metabolites would cause effects of the similar nature as those of T2 in repeated dose tests. As *in vitro* cell systems (presented in Table 5) usually have a limited capacity for detoxification, results would in general overestimate toxicity of T2 in comparison with those expected in the *in vivo* situation.

In the absence of *in vivo* data that would preferentially be used to calculate relative potencies we used *in vitro* data establishing RPFs and furthermore chose the highest potency to avoid underestimation of relative toxicity. Only phase I metabolites that have been examined either *in vivo* or *in vitro* have been considered for establishing relative potencies as listed in Table 6. Because it can reasonably assumed that phase II metabolites of T2 and HT2 (i.e. T2-3-Glc, T2-3-diGlc, T2-3-Sulf, T2-3-GlcA, 3-Ac-T2, 3-Fer-T2, HT2-3-Glc, HT2-diGlc,

HT2-GlcA, HT2-MalGlc, NEO-Glc, T2-triol-Glc and T2-tetraol-Glc) might be completely hydrolysed to their parent compounds (aglycons) after ingestion, they were included in a group TDI with the same potency as their parent compounds assuming dose additivity in the absence of information although antagonistic or synergistic effects cannot be excluded in principle. The RPFs have been calculated for each compound in each study on a molar basis (see Tables 4 and 5). They have been rounded up to half orders of magnitude i.e. 1, 0.3 or 0.1 to avoid spurious accuracy and retaining a conservative approach.

Table 6: Relative potency factors (RPFs) for chronic effects of modified forms of T2

Relative potency factor (RPF)a	Compound
0.1	T2-triol, T2-triol-Glc, T2-tetraol
0.3	19-HO-HT2, NEO, NEO-Glc, T2-tetraol-Glc
1	T2, T2-3-Glc, T2-3-diGlc, T2-3-Sulf, T2-3-GlcA, 3-Ac-T2, 3-Fer-T2, 19-HO-T2, HT2, HT2-3-Glc, HT2-diGlc, HT2-GlcA, HT2-MalGlc

[a]RPFs have been rounded up to half an order of magnitude i.e. to either 1, 0.3 or 0.1;

T2, T2-toxin; HT2, HT2-toxin; 3-Ac-T2, 3-acetyl-T2; 3-Fer-T2, 3-feruloyl-T2; HT2-diGlc, HT2-diglucoside; HT2-3-Glc, HT2-3-glucoside; HT2-GlcA, HT2-glucuronic acid; HT2-MalGlc, HT2-malonylglucoside; NEO, neosolaniol; NEO-Glc, neosolaniol-glucoside, T2-3-diGlc, T2-3-diglucoside; T2-3-Glc, T2-3-glucoside; T2-3-GlcA, T2-3-glucuronic acid; T2-3-Sulf, T2-3-sulfate; T2-tetraol-Glc, T2-tetraol glucoside; T2-triol-Glc, T2-triol-glucoside;

6. Discussion and conclusions

The question of the relevance of modified mycotoxins regarding both their occurrence in food and their toxicity has received increased attention by the scientific community and also by European regulators over the last decade. This is also reflected in the increased number of publications dealing with this relatively new scientific field. Previous reviews have highlighted various aspects of modified mycotoxins including their relevance, addressing in particular modified Fusarium toxins (see for example Dall'Asta and Battilani, 2016; Payros et al., 2016; Righetti et al., 2016; Gratz, 2017; Bryla et al., 2018; Freire and Sant'Ana, 2018). In a recent review, Lorenz et al. (2019) identified the lack of standardised and sensitive analytical methods, insufficient occurrence and toxicity data and co-exposure as major data gaps in the field of assessment of modified mycotoxins. The authors also suggest use of biomarkers as a useful tool to complement existing exposure assessments of

(modified) mycotoxins, a conclusion resulting also from a recent biomarker study with ZEN and its modified forms in Germany (Ali and Degen, 2018).

The present scientific review uses as a starting point parts of the evaluations carried out by an EFSA expert group for recently finalised EFSA opinions (EFSA, 2016; 2017). Such opinions are usually drafted to provide hazard and exposure assessments in order to support risk management decisions in the EU. In contrast to this, the present scientific review attempted to go beyond such a scope, namely to develop and test a methodology for hazard assessment that could be applied to other groups of structurally related mycotoxins or other contaminants for which toxicity data are lacking or insufficient, using ZEN and T2 and HT2 and their modified forms as an example. We chose ZEN and T2/HT2 and their modified forms as examples because the latter have been identified, based on their toxic properties and their occurrence in food, as relevant for dietary risk assessment. In terms of mass the modified forms of these mycotoxins contribute very considerably to the overall mycotoxin burden since modified forms of T2/HT2 can add another 40% in addition to the amount of parent compound present in certain food commodities. The corresponding figure for modified ZEN is even higher, i.e. around 100%. Modified mycotoxins, even though they may likely represent a food safety problem, are currently not considered in the EU legislative framework on food safety and in consequence are not considered in routine analyses. Thus, despite their potentially high relevance with regard to co-occurrence with their parent compounds there are very limited data on their occurrence in food, also owing to the fact that for modified forms standards and calibrants are usually not readily available. Both ZEN and T2/HT2 are well characterised toxicologically, also in repeated dose studies allowing dose response relationships to be established. Both mycotoxins exert pronounced toxicity (oestrogen-mediated adverse effects on reproduction in the case of ZEN and emesis and haemato-/immunotoxicity in the case of T2/HT2) at relatively low doses. However, no dose-response data are available for their modified forms. The assessment approach provided in the present scientific review, namely the establishment of reference points for the parent compounds, the evaluation of their applicability for their modified forms based on considerations of similarity and mechanistic data justifying application of the principle dose addition, allows for the first time a realistic joint estimation of the hazard of these compounds. Based on the sheer number of ZEN and T2/H2 metabolites a full toxicological characterisation of all these compounds appears unrealistic for the foreseeable future. The combination of mechanistic information available for all the metabolites with robust toxicological evidence for the parent compounds enables risk assessors to include the individual modified forms in risk assessments reflecting realistically the entire toxicity burden. The present evaluation has also made obvious that phase I or phase II

metabolism can either reduce the toxicity of ZEN and T2/HT2, leave it unchanged or even yield compounds with a much higher toxic potency. This is illustrated best with the case of α-ZEL, for which a 60-fold higher toxic potency in comparison with the parent compound ZEN could be identified following this novel approach. The present scientific review furthermore demonstrates that non-inclusion of modified forms in HBGVs (as is currently the case) would lead to an underestimation of the risks from modified mycotoxins based both on the considerable concentrations at which they occur in food and their toxic potential. Likewise can it be seen that assumption of equal toxicity for all metabolites either under- or overestimates the toxicity of individual modified forms. Thus, application of potency factors appears to be the most appropriate tool unless data becomes available overruling the set potency factors. As already indicated above, we conclude that the approach applied here for the two selected mycotoxins could also be applied for other groups of mycotoxins or other groups of structurally related compounds for which robust data are only available for one or a few compounds, but mechanistic information is available for all compounds (e.g. deoxynivalenol and its modified forms). Finally, it needs to be stressed that the present approach, namely using surrogate information to compensate for appropriate toxicity data is obviously associated with considerable uncertainties,,and using such an approach is more likely to overestimate than underestimate the risk. Any outcome with regard to toxic potency and assumptions regarding dose additivity should be overruled once appropriate toxicity data become available for any of the modified mycotoxins or combinations of the mycotoxins included in the analyses. The same goes for the assumptions of complete cleavage of phase II metabolites of ZEN and T2/HT2 to their parent compounds upon ingestions and that toxicity of parent compound and modified forms is additive. Therefore, more data on toxicokinetics and appropriate toxicity data for modified mycotoxins are needed and the dose additivity assumption needs to be tested.

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Conflict of interest

The authors have no conflict of interest.

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Figure

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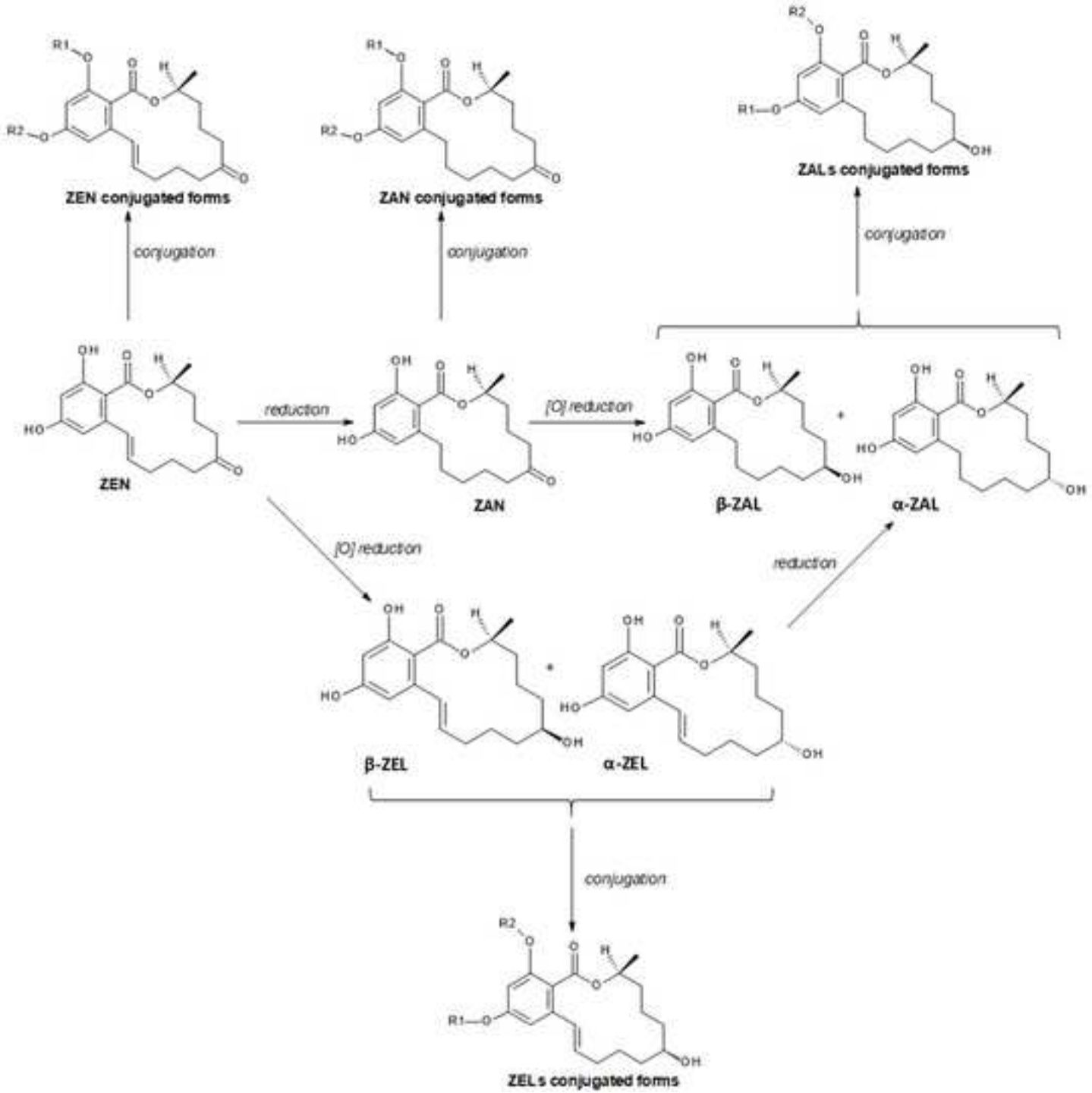

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