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Human exposure to mycotoxins and their masked forms through cereal-based foods in Belgium

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ABSTRACT

In the present study, a quantitative dietary exposure assessment of mycotoxins and their masked forms was conducted on a national representative sample of the Belgian population using the contamination data of cereal-based foods. Cereal-based food products (*n* = 174) were analysed for the occurrence of deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, zearalenone, α-zearalenol, β-zearalenol, T-2-toxin, HT-2-toxin, and their respective masked forms, including, deoxynivalenol-3-glucoside, zearalenone-4-glucoside, α-zearalenol-4-glucoside, β-zearalenol-4-glucoside and zearalenone-4-sulfate. Fiber-enriched bread, bran-enriched bread, breakfast cereals, popcorn and oatmeal were collected in Belgian supermarkets according to a structured sampling plan and analysed during the period from April 2010 till October 2011. The habitual intake of these food groups was estimated from a national representative food intake survey. According to a probabilistic exposure analysis, the mean (and P95) mycotoxin intake for the sum of the deoxynivalenol-equivalents, zearalenone-equivalents, and the sum of HT-2-and T-2-toxin for all cereal-based foods was 0.1162 (0.4047, P95), 0.0447 (0.1568, P95) and 0.0258 (0.0924, P95) μg kg^{-1} body weight day^{-1}, respectively. These values were below the tolerable daily intake (TDI) levels for deoxynivalenol, zearalenone and the sum of T-2 and HT-2 toxin (1.0, 0.25 and 0.1 μg kg^{-1} body weight day^{-1}, respectively). The absolute level exceeding the TDI for all cereal-based foods was calculated, and recorded 0.85%, 2.75% and 4,11% of the Belgian population, respectively.

KEYWORDS: mycotoxins, *Fusarium*, exposure assessment, probabilistic analysis, Monte Carlo simulation

ABBREVIATIONS

DON = deoxynivalenol

3ADON = 3-acetyl-deoxynivalenol

15ADON = 15-acetyl-deoxynivalenol

DON3G = deoxynivalenol-3-glucoside

β-ZEL = β-zearalenol

α-ZEL = α-zearalenol

ZEN = zearalenone

ZEN4G = zearalenone-4-glucoside

ZEN4S = zearalenone-4-sulfate

β-ZEL4G = β-zearalenol-4-glucoside

α-ZEL4G = α-zearalenol-4-glucoside

HT-2 = HT-2 toxin

T-2 = T-2 toxin

TDI = tolerable daily intake

DON-equivalents = DON, 3ADON, 15ADON and DON3G

ZEN-equivalents = ZEN, ZEN4G, ZEN4S, α-ZEL, β-ZEL, α-ZEL4G, β-ZEL4G

LOD = limit of detection

LOQ = limit of quantification

1. INTRODUCTION

Fusariotoxins are secondary metabolites produced by toxigenic micromycetes of the genus *Fusarium (F)* (Conkova et al., 2003). *Fusarium species* might endanger human health through the action of their toxic metabolites, mycotoxins (Bauer et al., 1980). Besides fumonisins, most dominant mycotoxin production includes trichothecenes and myco-estrogens. The clinical outcome is known as mycotoxicosis, which shows a variety of clinical symptoms where synergistic or additive effects between several mycotoxins can enhance the adverse health effects for the exposed organism (Grenier & Oswald, 2011).

Trichothecenes are a family of cyclic sesquiterpenoids and according to their functional groups, they are divided into four groups (A, B, C and D). Type A trichothecenes, T-2-toxin (T-2) and HT-2-toxin (HT-2), produced by *F. poae, F. langsethiae* and *F. sporotrichioides*, as well as deoxynivalenol (DON, type B), produced by *F. graminearum* and *F. culmorum*, are the most abundant trichothecenes (Richard et al., 2007). Type C-trichothecenes possess an additional epoxide nevertheless they are not produced by *Fusarium* species. Type D are also non-*Fusarium* mycotoxins and contain a macrocyclic ring; these airborne *Stachybotrys* mycotoxins include satratoxins, roridins and verrucarins and are prevalent in indoor environments. Type A and B are widely distributed in cereals as natural pollutants, whereas the macrocyclic trichothecenes rarely occur in food and feed.

Trichothecenes (type A and B) bind readily to eukaryotic ribosomes and are potent inhibitors of the translation process, inducers of apoptosis in lymphatic and haematopoietic tissues, and cause damage to cellular membranes (Ostry, 1998; Pestka & Smolinski, 2005). Acute exposure induces radiomimetic effects and gastro-intestinal manifestations such as diarrhoea, vomiting and melena, while chronic exposure reported effects as anorexia, reduced weight gain, retarded growth, nausea and degeneration of the immune, neural and reproductive systems.

Zearalenone (ZEN) is mainly produced by *F. graminearum, F. crookwellense, F. sporotrichioides* and *F. culmorum*, consequently co-occurrence with DON, T-2 and HT-2 was described (Pittet, 1998). ZEN, acting similarly to 17β-estradiol, causes strong estrogenic outcomes, alters consequently the reproductive tract and is associated with hyperestrogenism, although haematotoxic and genotoxic properties were also described (Minervini et al., 2005; Ostry, 1998; Turcotte et al., 2005). In humans, the occurrence of ZEN in plasma was associated with precocious puberty, endometrial adenocarcinomas and hyperplasia (Saenz de Rodriguez et al., 1985; Tomaszewski et al., 1998). The most abundant derivatives of ZEN are

α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL). Metabolisation of mycotoxins by plants can partly occur, and gives rise to the production of so called “masked mycotoxins" (Berthiller et al., 2009a). Three phases of chemical modifications of these xenobiotic compounds can be distinguished during the plant metabolism. The phase I process includes the reduction, oxidation or acetylation of the parent mycotoxin resulting in an activation of the derived molecule and a higher toxicity level (eg. α-ZEL). Phase II consists of the enzymatic transformation of these reactive groups such as conjugation, glucosidation and sulfatation (eg. deoxynivalenol-3-glucoside (DON3G)) leading to the formation of more hydrophilic compounds, facilitating the elimination of the masked mycotoxins and thus a decreased toxicity (Plasencia & Mirocha, 1991; Poppenberger et al., 2003; Vendl et al., 2009). Phase III comprises the compartmentalisation of the mycotoxins into the vacuole of the plant or binding to the cell wall (Berthiller et al., 2009a; Coleman et al., 1997; He et al., 2010; Zinedine et al., 2007). A potential risk for consumers is the possible hydrolysis of masked mycotoxins into their toxic parent forms during mammalian digestion (Grabley et al., 1992; Berthiller et al., 2011). The evaluation of masked mycotoxins is not (yet) available due to the lack of occurrence, bioavailability and toxicological data, however the Joint European Commission FAO/WHO Expert Committee (JECFA) considered DON3G and the acetylated forms 3ADON and 15ADON as an additional contributing factor of the total dietary exposure to DON (Codex, 2011; JECFA, 2010). Masked forms of zearalenone were not considered. Making these statements it encouraged industry and research to further investigate masked mycotoxins. Poppenberger *et al.* (2003) already proved that DON3G dramatically reduces the ability to inhibit protein synthesis of wheat ribosomes *in vitro*. Recently, Berthiller *et al.* (2011) showed the toxicological relevance of DON3G by demonstrating that several lactic acid bacteria can hydrolyse DON3G *in vitro* (Berthiller et al., 2011). However, Nagl *et al.* (2012) demonstrated that DON3G is partially bioavailable in rats. The majority of the administered DON3G was cleaved during digestion and subsequently excreted via faeces. Thus, DON3G present in food and feed seems to have a significantly lower toxic equivalency compared to DON. However, due to differences regarding anatomy and gut microbiota, bioavailability and metabolization may be species dependent and should be experimentally determined.

Concerning masked ZEN-forms Ayed *et al.* (2011) argued that ZEN and α-ZEL exhibited the same range of cytotoxicity and genotoxicity, and both were more cyto- and genotoxic than β-ZEL. Recently, a study was executed on the amount of these forms in rats (Versilovskis et al., 2012). After administration of ZEN4G, ZEN was found in the stomach suggesting that

hydrolysis is possible as was already shown for the acetylated DON-forms. Small amounts of ZEN4G were detectable in the small and large intestines suggesting that they were not fully hydrolysed. But, the large occurrence of α-ZEL (Videmann et al., 2012) and a sharp decrease of ZEN4G in the smal intestine proved hydrolysis (Versilovkis et al., 2012), as a consequence, the total human exposure and risk assessment to mycotoxins might be underestimated.

To be sure of the statements made for the masked forms of DON and ZEN, a full metabolism study should be carried out, preferably by incorporation of mycotoxins in the feed of reliable species.

The European Union (EU) has set maximum levels for certain mycotoxins as a risk management strategy, and to achieve a high level of public health protection (EC, 2006a). The Scientific Committee on Food has adopted opinions laying down a tolerable daily intake (TDI) for several toxins. It has established a TDI for DON of 1.00 μg kg^{-1} body weight (bw) day^{-1}, a provisional TDI of 0.25 μg kg^{-1} bw day^{-1} for ZEN and a combined provisional TDI of 0.06 μg kg^{-1} bw day^{-1} for the sum of T-2 and HT-2. However, the European Food Safety Authority has recently established a TDI of 0.10 μg kg^{-1} bw day^{-1} for the sum of T-2 and HT-2 (EFSA, 2011b; EFSA, 2011c).

Current legal limits and control strategies only focus on the parent mycotoxins. Cereal-based products are very important in the human diet and their quality and safety should be controlled during processing throughout the entire food chain (Yazar & Omurtag, 2008). Foodstuffs which are susceptible to trichothecene and ZEN contamination include wheat, maize, barley and cereal-based products such as breakfast cereals, bread and beer. Furthermore, in these matrices co-occurrence of masked and parent mycotoxins has been previously described (De Boevre et al., 2012a; Berthiller et al., 2009b; Desmarchelier & Seefelder, 2011; He et al., 2010; Kostelanska et al., 2009; Lancova et al., 2008; Vendl et al., 2010). To date however, no risk assessments were performed for masked mycotoxins. The objective of this study was to determine the occurrence of mycotoxins and their masked forms in cereal-based foodstuffs and to their estimate exposure for the Belgian population. Output of exposure was compared to the TDI of the parent mycotoxins.

2. MATERIALS AND METHODS

2.1 Reagents and chemicals

Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Disinfectol® (denaturated ethanol with 5% ether) was supplied from Chem-Lab NV (Zedelgem, Belgium). Methanol (LC-MS grade) was purchased from BioSolve BV (Valkenswaard, The Netherlands), while acetonitrile (Analar Normapur), n-hexane (Hipersolv Chromanorm) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) and tris (hydroxyl methyl) amino methane (Tris) were supplied by Merck (Darmstadt, Germany).

The mycotoxin solid calibration standards (1 mg) of DON, 3-acetyldeoxynivalenol (3ADON), 15-acetyldeoxynivalenol (15ADON), deepoxy-deoxynivalenol (DOM), zearalanone (ZAN), α-ZEL, β-ZEL, and HT-2 were obtained from Sigma Aldrich NV/SA (Bornem, Belgium). ZEN (5 mg) was supplied by Fermentek (Jerusalem, Israel). T-2 (1 mg, solid standard) and DON3G (50.2 ng μL^{-1}, in acetonitrile) were purchased from Biopure Referenzsubstanzen GmbH (Tulln, Austria). All mycotoxin solid standards were dissolved in methanol (1 mg mL^{-1}) and storable for a maximum of 1 year at -18 °C (Spanjer et al., 2008). The DON3G-solution was kept at 4 °C. ZEN4S, zearalenone-4-glucoside (ZEN4G), α-zearalenol-4-glucoside (α-ZEL4G) and β-zearalenol-4-glucoside (β-ZEL4G) were synthesized according to an in-house developed method based on the procedures of Grabley *et al*. (1992) and Schneweis *et al*. (2002).

The working solutions of DON, 3ADON, 15ADON, DOM, ZEN, α-ZEL, β-ZEL, ZAN, HT-2 and T-2 (10 ng μL^{-1}) were prepared in methanol and stored at -18 °C, while DON3G was dissolved in acetonitrile, stored at 4 °C and renewed monthly. The working solutions of the masked mycotoxins ZEN4G, α-ZEL4G, β-ZEL4G and ZEN4S (20 ng μL^{-1}) were prepared in methanol, stored at 4 °C and renewed monthly.

2.2 Collection of samples for (masked) mycotoxin analysis in foodstuffs

In the framework of a regular Belgian sampling program, a total of 174 cereal-based food products were collected between April 2010 and October 2011, including fiber-enriched bread (*n*=52), bran-enriched bread (*n*=36), breakfast cereals (*n*=62), popcorn (*n*=12) and oatmeal (*n*=13). The commercially available food products were purchased from different manufacturers in several supermarket chains with a good market share in Belgium.

Representative samples were obtained by collecting the cereal-based retail-products with different batch numbers every three months from the same manufacturers according to a structured sampling plan. Breakfast cereals, popcorn and oatmeal were stored at room temperature in the dark until analysis, and bread was concurrently analysed within 3 days to prevent moulding of the matrix.

2.3 Determination of (masked) mycotoxin concentrations in foodstuffs

Bread, popcorn, oatmeal and breakfast cereals were pulverized with the Moulinette 320-grinder (Moulinex, Barcelona, Spain). A cleaning and decontamination routine of the equipment was performed using water and disinfectol® after each milling practice. The sample preparation was executed according to De Boevre *et al.* (De Boevre et al., 2012b). Briefly, the ground material (2.5 g) was extracted with 10 mL acetonitrile/water/acetic acid (79/20/1, v/v/v), and combined with a simultaneous hexane defatting (5 mL) using the Agitator decanter overheadshaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 60 minutes. The upper layer (hexane) was removed after centrifugation (3,000 g, 15 min). Then, the aqueous layer was filtered and evaporated to dryness (N_2, 40 °C). Finally, the residue was redissolved in 100 µL injection solvent, consisting of methanol/water (50/50, v/v) and 10 mM ammonium acetate, adjusted to pH 3 with glacial acetic acid. The chromatographic conditions and method were described in detail in De Boevre *et al.* (De Boevre et al., 2012b). The developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was successfully validated based on the Commission Regulation (EC) N° 401/2006 of 23 February 2006 (EC, 2006b). Matrix matched calibration plots were applied for the determination of the mycotoxins. DOM and ZAN, structural analogues of type B-trichothecenes and ZEN, respectively, were used as internal standards in the multi-mycotoxin analysis. Five blank samples of each matrix were spiked at five concentration levels of 25, 50, 100, 200 and 400 µg kg^{-1} for DON, 3ADON, 15ADON and DON3G, and 2.5, 10, 20, 40, 80 µg $kg^{-1}$for ZEN, α-ZEL, β-ZEL, ZEN4G, α-ZEL4G, β-ZEL4G, ZEN4S, HT-2 and T-2. In case the obtained results were out of the range of the calibration curve, the sample was re-analysed in order to fit in the range of a newly constructed calibration plot. Every run contained a standard control mix, five samples of the calibration curve and a maximum of 20 samples.

Evaluating the linearity, the homogeneity of variance was checked before applying the linear model. The linearity was determined graphically using a scatter plot. The apparent recovery for each mycotoxin was determined by dividing the observed value by the spiked level. The obtained values, between 70% and 108%, were in conformity with the ranges set in legislation

(EC, 2006b). The limits of detection (LODs) for maize, wheat, oats, breakfast cereals and bread varied in the following ranges [5-12 µg kg^{-1}], [5-11 µg kg^{-1}], [5-12 µg kg^{-1}], [7-12 µg kg^{-1}] and [8-13 µg kg^{-1}], respectively. Limits of quantification (LOQs) for maize, wheat, oats, breakfast cereals and bread were in the following ranges [10-24 µg kg^{-1}], [10-22 µg kg^{-1}], [10-24 µg kg^{-1}], [14-24 µg kg^{-1}] and [16-26 µg kg^{-1}], respectively, which guaranteed quantification at a low ppb-level. A precision study was performed by determining the repeatability (intraday precision) and the reproducibility (interday precision) at the five concentration levels, and was fulfilled in accordance with the described criteria. The criteria for the parent mycotoxins were also implemented for their masked forms. The precision was calculated in terms of the relative standard deviation (RSD): the obtained RSDs for repeatability were within 11-21%, 7-26%, 5-28%, 5-27%, 8-24% for maize, wheat, oats, breakfast cereals and bread, respectively, and the obtained RSDs for reproducibility were within 12-28%, 9-22%, 5-38%, 6-23%, 5-27%, respectively. The specificity was tested by the analysis of 20 blank samples of the different matrices. The results of the performance characteristics of the LC-MS/MS method were in good agreement with the criteria mentioned in the Commission Regulation (EC) No 401/2006 (EC, 2006b), and were described in detail in De Boevre *et al.* (De Boevre et al., 2012b).

2.4 Consumption data

The food consumption data were obtained from the Belgian National Food Consumption Survey of 2004. The survey is a representative sample of food consumption data of 3,083 participants living in Belgium at the age of 15 years or older (De Vriese et al., 2005). The consumption data of foodstuffs analysed for their mycotoxin content were extracted from the survey using the food category and food name data (IBM SPSS 19). The usual intake of each of the food groups was estimated from the 2 day 24h recalls by correcting for the intra person variability (Harttig et al., 2011; Haubrock et al., 2011; German Institute of Human Nutrition Postdam-Rehbrücke (DIfe), 2011). All subjects were considered habitual consumers of the cereal products. The usual food intake was determined from the total data set including the 'zero intakes' (when a certain food type is not consumed by an interviewed individual) with the Multiple Source Method program (German Institute of Human Nutrition Postdam-Rehbrücke (DIfe), 2011). The usual food intake was expressed as µg kg^{-1} bw day^{-1} using the self-reported body weight (bw) data collected during the survey.

2.5 Exposure assessment

Three different scenarios were included for the mycotoxin dietary exposure assessment in relation to the data treatment of the non-detects (< LOD): lower, medium and upper bound. Non-detects were considered as zero, 1/2 LOD and LOD for lower, medium and upper bound, respectively. For the exposure assessment, the foods were grouped in five categories: fiber-enriched bread, bran-enriched bread, breakfast cereals, popcorn and oatmeal. Also, a sixth category "all cereal based foods", was included and contained the total of the five categories.

2.6 Deterministic exposure assessment

A deterministic analysis was performed, in a first attempt to assess the dietary exposure of the parent and masked mycotoxins. Calculations were executed using Microsoft Excel 2007. The estimated intakes of the parent and masked mycotoxins were determined by multiplying the mean mycotoxin concentrations by the mean, maximum or P95-percentiles of the consumption data. To minimize the risk to the consumer, the mean mycotoxin concentration was considered as fixed, while the consumption data were variable (mean, maximum, P95) (Vromman et al., 2010). The three scenarios (lower, medium and upper) were determined with regard to the data treatment. Deterministic estimations of exposures assume that all individuals consume the cereal-based foods at the same period of time and at a same level. In addition, the parent mycotoxins and their masked forms are considered to be continuously present at an average level. As these might cause an overestimation, an additional probabilistic analysis was performed in order to allow a more detailed exposure assessment.

2.7 Probabilistic exposure assessment

Calculations were executed using the software @Risk® for Microsoft Excel version 5.5 (Palisade Corporation, USA). Best fit distributions were formed to the lower, medium and upper bound scenario of all mycotoxin concentrations in the six different food categories and to the respective consumption data. The type of distribution selected as best fitted for the upper bound was applied upon the other two scenarios (lower-medium). Best fit was based on chi-square statistics. Also, the probability/probability plots (P/P) and the quantile/quantile plots (Q/Q), resulting from the cumulative distributions, were a parameter if the cumulative distributions corresponded to the theoretical cumulative distributions. First order Monte Carlo simulations were performed considering 10,000 iterations. The estimated intake of the parent and masked mycotoxins (mean, standard deviation, maximum and percentiles) was determined separately per food category. The total intake (all cereal based foods) was calculated by considering all concentration data of the food categories and the consumption

data according to the estimates from the 2 day 24h recalls by correcting for the intra person variability. Hence, the sum of the means of the subcategories intake is not necessarily equal to the mean intake of the overall category.

3. RESULTS AND DISCUSSION

3.1 Determination of (masked) mycotoxins in foodstuffs

A total of 174 cereal-based samples were analysed for the occurrence of DON, 3ADON, 15ADON, DON3G, T-2, HT-2, ZEN, α-ZEL, β-ZEL, α-ZEL4G, β-ZEL4G, ZEN4G and ZEN4S. Five food categories were considered: fiber-enriched bread, bran-enriched bread, breakfast cereals, popcorn and oatmeal. The difference between fiber- and bran-enriched bread was made according to the labelling of the product. The definition was based on the percentage of fiber/bran per weight depending on the processing of the grains. **Table 1** shows the mean concentration, standard deviation and maximum concentration (μg kg^{-1}) of the mycotoxins and their masked forms found in each food category. The analysed foods were contaminated with an average of 3 to 8 different mycotoxins (median = 5) including 1 to 3 masked forms (median = 1). These masked forms comprized DON3G, ZEN4G, ZEN4S, α-ZEL4G and β-ZEL4G. The percentage of mycotoxins occurring in the samples was calculated by the amount of samples (> LOD) divided by the total amount of samples analysed.

DON is a common contaminant in many cereal-based matrices. A total of 85% and 44% of the fiber and bran-enriched bread samples were contaminated with DON with low maximum values considering the EU-maximum level of 500 μg kg^{-1}. Breakfast cereals (58%) however showed higher concentrations regarding the EU-maximum limit of 500 μg kg^{-1}. While DON is a major contaminant in the wheat based matrices, only 38% of oatmeal samples were contaminated. The acetylated forms, 3ADON and 15ADON, were present in 40% and 38% of the fiber-enriched bread samples, respectively. Bran-enriched bread samples were contaminated with 39% 3ADON and 11% 15ADON (**Table 1**). The acetylated forms appeared in 55% and 48% of the breakfast cereal samples, respectively. 3ADON and 15ADON occurred in 100% of the popcorn samples, and 62% and 77% of the oatmeal samples. DON3G occurred in half of the fibre-enriched bread samples (52 %). Also, 50% of the breakfast cereal samples were contaminated with DON3G. More DON3G was observed in popcorn (92%) and oatmeal (77%).

A relative contamination level of 63% (T-2) and 69% (HT-2) of the fiber-enriched samples was noticed, while in bran-enriched bread 14% (T-2) and 22% (HT-2) of the samples was contaminated. Concerning the breakfast cereals, a contamination level of 61% and 65%, respectively was obtained. The T-2 and HT-2-contamination was rather low in popcorn as

only 25% and 17% of the samples were contaminated, in contrast to the higher incidence in oatmeal (69%, T-2; 69%, HT-2).

Fiber-enriched bread samples (44%) were contaminated with ZEN of which 7 of these samples did not meet the EU-maximum limit of 50 µg kg^{-1}. ZEN occurred in the bran-enriched samples (39%) and in more than half of the breakfast cereal samples (52%), where a maximum was observed, which is 9 times the EU-maximum limit. An incidence of ZEN (58%) was noticed in popcorn while the highest incidence of ZEN (62%) was observed for oatmeal. In fiber-enriched bread and bran-enriched bread α-ZEL was observed in 31% and 8% of the samples, while approximately the same abundancies for β-ZEL were noticed in 23% and 17% of the bread samples. Regarding the breakfast cereals, α-ZEL and β-ZEL were present in 52% and 42% of the samples with concerningly high maxima. Only two samples of popcorn were contaminated with α-ZEL and β-ZEL, in contrast to the oatmeal samples (38% and 31%). ZEN4G, ZEN4S, α-ZEL4G and β-ZEL4G occurred in 29%, 8%, 10% and 19% of the fiber-enriched bread samples. In the bran-enriched bread samples ZEN4S occurred in 6% of the samples, the glucosylated forms in 6% (ZEN4G), 3% (α-ZEL4G) and 6% (β-ZEL4G). Concerning the breakfast samples, α-ZEL4G, β-ZEL4G and ZEN4S occurred in the same incidence (26%, 29% and 27%), while ZEN4G was observed in 40% of the samples. None of the popcorn samples contained α-ZEL4G nor ZEN4G; all other masked forms also occurred in very small amounts (8%). Only two oatmeal samples were contaminated with ZEN4S and β-ZEL4G; ZEN4G appeared in 38% of the samples (**Table 1**). A more detailed interpretation is described in De Boevre *et al.*(De Boevre et al., 2012a).

3.2 Consumption data

A sample of 3,083 persons provided data about their eating habits on cereal-based foods. Approximately 30% consume the foodstuffs investigated on the interview days. Oatmeal and popcorn were consumed by few persons (**Table 1**).

3.3 Deterministic exposure assessment

In **Table 2** the estimated intakes (µg kg^{-1} bw day^{-1}) by the Belgian adult population for the different food categories are presented for the lower, medium and upper bound, respectively.

The total mean intake for the upper bound (worst case) scenario for the consumption of all cereal-based food for the mycotoxins DON, 3ADON, 15ADON, DON3G is 0.040, 0.024, 0.016, 0.025 µg kg^{-1} bw day^{-1}, respectively. Insofar as no TDI's for 3ADON, 15ADON and DON3G are described, the TDI of the parent DON was used. These values are approximately

25 to 63 times lower than the TDI set for DON (1.00 μg kg^{-1} bw day^{-1}). However, extrapolating this value to DON-derivatives is notably a worst case scenario, assuming that these toxins are equally toxic as their parent or that total hydrolysis occurs. The sum of the DON-equivalents was incorporated, which is the sum of DON, 3ADON, 15ADON and DON3G, and compared to the DON TDI. A mean intake for fiber-enriched bread, bran-enriched bread, breakfast cereals, popcorn, oatmeal and all cereal based foods of 0.125, 0.077, 0.050, 0.019, 0.106 and 0.104 μg kg^{-1} bw day^{-1} was obtained, which is 8 to 53 times lower than the DON TDI-value.

High consumers of fiber-enriched bread exceeded the TDI of 0.10 μg kg^{-1} bw day^{-1} for the sum of HT-2 and T-2 (P95, 0.128 μg kg^{-1} bw day^{-1}; max, 0.227 μg kg^{-1} bw day^{-1}). Also, consumers, who daily consume the maximum of all cereal based foods, were exposed to a potential risk to HT-2 and T-2 (max intake, 0.146 μg kg^{-1} bw day^{-1}).

The highest exposure of the investigated mycotoxins is attributed to the myco-estrogens. High consumers of breakfast cereals and all cereal foods nearly exceeded the TDI of 0.25 μg kg^{-1} bw day^{-1} for ZEN (0.201 μg kg^{-1} bw day^{-1} and 0.219 μg kg^{-1} bw day^{-1}intake, respectively). The sum of the ZEN-equivalents was designated as the sum of ZEN, α-ZEL, β-ZEL, ZEN4G, ZEN4S, α-ZEL4G, and β-ZEL4G. Fiber-enriched bread, bran-enriched bread and breakfast cereals are responsible for a high intake of these toxins with a maximal exceeding factor of 1.8, 1.1 and 2.3 times the ZEN TDI. The maximum consumption of all cereal based foods included a 0.594 μg kg^{-1} bw day^{-1} intake which is of high concern. Popcorn and oatmeal were consumed without exceedment of TDI, possibly due to lower consumption.

An extra calculation was investigated concerning the som of the mean (and P95) intakes of the five different cereal based food matrices. The mean DON-exposure for consuming bread, breakfast cereals, popcorn and oatmeal was 0.146 (0.319, P95) μg kg^{-1} bw day^{-1}, which does not imply any risk. For the sum of T-2 and HT-2 an exceedment of the TDI was observed for mean and P95 intake, 0.101 and 0.220 μg kg^{-1} bw day^{-1}, respectively. Concerning ZEN, consumers were not exposed to any risk for the mean intake (0.119 μg kg^{-1} bw day^{-1}, nevertheless the TDI was achieved for P95 (0.250 μg kg^{-1} bw day^{-1}).

3.4 Probabilistic exposure assessment

In probabilistic analysis every possible value that each variable can have and the weight of each possible scenario for the probability of its occurrence is taken into consideration (Vose, 1996), therefore allowing a more accurate (masked) mycotoxin intake estimation. Best fit distributions were formed for respectively all mycotoxin concentrations in the six different

categories and all consumption data. In case constructing a distribution was not possible due to insufficient numbers of observed data for parent or masked mycotoxins, the uniform distribution analysis method was applied (**Table 1 and 3**). The more data available, the more the theoretical cumulative distributions approximated the observed cumulative distributions. The best fit distributions determined for the upper bound scenario of parent and masked mycotoxin concentrations in the food categories and food intakes, further applied for the probabilistic calculations are listed in **Table 3**. **Table 4 (a, b, c, d and e)** represents the probabilistic estimates of the parent and masked mycotoxin intake (mean, standard deviation, maximum, percentiles) (µg kg^{-1} bw day^{-1}) by the Belgian population for the upper bound (worst case scenario) from the different food categories.

Table 4a showed for DON that 0.02%, 0.03% and 0.01% of the Belgian population exceeds the TDI of 1.00 µg kg^{-1} bw day^{-1} for fiber-enriched bread, popcorn and all cereal based foods. The share of DON intake to its equivalents was determined by dividing the excess DON to the excess of the DON-equivalents. The part of share of DON to the estimated intake of the DON-equivalents **(Table 4b)** for fiber-enriched bread, popcorn and all cereal based foods, was 2%, 4% and 1%, respectively. These results assumed that the intake was mainly attributed to the DON-derivatives, 3ADON, 15ADON and DON3G. The sum of the DON-equivalents showed that high consumption of fiber-enriched bread, popcorn and all cereal based foods enhances the exposure. As the percentage of population which exceeded the TDI is difficult to interpret, the risk was expressed on the absolute Belgian population (10.839.905, (Statbel, 2012)). 124,659; 73,711 and 92,139 persons were exposed daily to threshold exceeding DON-equivalents for fiber-enriched bread, popcorn and all cereal-based foods, respectively. However, based on the lack of toxicity data for the DON-derivatives no conclusions regarding the possible risk can be drawn.

The mean and P 99.5 intakes for breakfast cereals, oatmeal and popcorn for the sum of T-2 and HT-2 were 0.0119, 0.0060, 0.0032, 0.0630, 0.0541 and 0.0334 µg kg^{-1} bw day^{-1}, respectively. These values were below the provisional TDI of 0.10 µg kg^{-1} bw day^{-1} **(Table 4c)**. Though, fiber-enriched bread, bran-enriched bread and all cereal based foods had T-2 and HT-2 intakes (P 99.5) of 0.7052, 0.1335 and 0.1950 µg kg^{-1} bw day^{-1}, respectively, potentially indicating that the concentrations found in the analysed foods cause on daily scale a high exposure to the Belgian population (1.799.424; 204,874; 445,520 persons exceeding the TDI, respectively).

Data in **Table 4d** showed a high ZEN-exposure with P 99.5 of 0.4491, 0.6130, and 0.9662 µg kg^{-1} bw day^{-1} for high consumers of fiber-enriched bread, bran-enriched bread, breakfast

cereals and all cereal based foods, respectively. These values corresponded to an excess of 629,798, 888,872 and 187,530, respectively of the Belgian population which is of high concern. The attribution of ZEN to the ZEN-equivalents was investigated by dividing the excess of ZEN to the overplus of its equivalents **(Table 4e).** The share of ZEN for fiber-enriched bread, bran-enriched bread, breakfast cereals and all cereal based foods was 39%, 55% and 63%, respectively, which was high in contrast to the attribution of DON to its DON-equivalents. Concerning the sum of the ZEN-equivalents, only oatmeal can be consumed without any risk due to low consumption (mean: 0.0150 µg kg^{-1} bw day^{-1}, P 99.5: 0.1081 µg kg^{-1} bw day^{-1}). The other five foodstuff groups had P 99.5 intakes of 0.9180, 0.4696, 0.7892, 0.1178 (max 0.2442), and 0.3603 µg kg^{-1} bw day^{-1} for fiber and bran-enriched bread, breakfast cereals, popcorn and all cereal based foods which corresponds with a population of 2,168 to 1.616.131 consumers. The latter high value is especially disturbing since these adults consume breakfast cereals on a daily basis and thus are exposed to a corresponding amount of ZEN and equivalents.

In **Figure 1, 2 and 3** relative contributions of the sum of the DON-equivalents, T-2 and HT-2, and the ZEN-equivalents are indicated for the six different food categories. The values (%) show the percentages of the examined population which exceeds the TDI. It is clearly proven that the consumption of bread that has been highly enriched with fibres and all cereal based food causes a risk impact as their shares in the graphs are the most prevalent.

3.5 Uncertainty related to exposure assessments

There are always uncertainties associated with exposure assessments which need to be considered for the interpretation of results. A possible overestimation of the subgroup ‘all cereal based foods’ is acknowledged inasmuch high consumers of bread might not be at the same time frame high consumers of breakfast cereals. Current software however, does not allow to incorporate these relationships in the exposure assessment estimations. The hypothesis that masked mycotoxins during ingestion are totally converted to their parents is assumed. The most recent food consumption data of the Belgian population were used in this study. Although the data originate from 2004, our findings are of public health importance to date in Belgium. Although eating habits might change over time, the changes in the consumption of cereal based food are limited (Kearney, 2010). Furthermore, this study applied a dietary assessment characterized by inherent under or over-reporting of the consumption data. Despite this, the 24 hour recall is the recommended method to estimate food intake in large food consumption surveys in Europe (de Boer et al., 2011).

Mycotoxin (DON, ZEN, T-2 and HT-2) dietary estimations have been performed by several EU-countries (SCOOP 2003, 2003; JECFA, 2010; Reddy et al., 2010; Bail et al., 2005; Binder et al.,2007; Harcz et al., 2007; Maragos, 2011). From 2002 to 2005, Harcz et al. investigated the exposure for DON and ZEN in Belgium, whereas similar results were obtained (Harcz et al., 2007).

According to SCOOP (2003) the DON-levels for adults were below the TDI, which can be confirmed in the observed results (**Table 4**). Mean estimates of chronic dietary exposure to the sum of T-2 and HT- based on the available occurrence data were in a range of 0.0034 µg kg^{-1} bw day^{-1} to 0.018 µg kg^{-1} bw day^{-1}, with P95 ranged from 0.0072 µg kg^{-1} bw day^{-1} to 0.039 µg kg^{-1} bw day^{-1} (EFSA, 2011b). Also, our study proved the relevance of T-2 and HT-2 in cereal-based foods. The EFSA report concluded that in the adult population the mean dietary exposure to ZEN across survey studies ranged from 0.0024 µg kg^{-1} bw day^{-1} to 0.029 µg kg^{-1} bw day^{-1}, whereas the P95 ranged from 0.0047 µg kg^{-1} bw day^{-1} to 0.054 µg kg^{-1} bw day^{-1} (EFSA, 2011c). These results were not in accordance with the high values obtained in this study (mean 0.1199, P95 0.4094 µg kg^{-1} bw day^{-1}). The results obtained were transferred to the regional risk management authority for further monitoring of the data. The largest contribution was breakfast cereals which was also observed in the EFSA-report.

To date however, no risk assessments were performed for masked mycotoxins. The multiple exposure assessments in other countries indicated that small children were exposed to high levels of the mycotoxins investigated in contrast to the adult population (Serrano et al., 2012; Gauchi et al., 2002). The average intakes for regions such as the Mediterranean area estimate that the exposure is generally below the levels deemed as tolerable. However, certain regions and definitely developing countries will encounter problems (e.g. climate, high humidity) for certain mycotoxins, particularly in subpopulations where the contamination of cereal-based food is extraordinary (Kearney, 2010). The discrepancies between studies could be explained by the use of different methodologies, quantification strategies and food consumption surveys.

4. CONCLUSION

To our knowledge, this is the first study assessing the exposure of the Belgian population to DON, 3ADON, 15ADON, ZEN, α-ZEL, β-ZEL, T-2, HT-2 and their masked forms. The population is expected to be exposed to moderate levels of several mycotoxins. Although the majority of the Belgian population does not exceed the TDI of 1.0, 0.2 and 0.1 μg kg^{-1} bw day^{-1} DON, ZEN and the sum of T-2 and HT-2, respectively, there is a large subpopulation, exceeding those safety values. A reduction of (masked) mycotoxin concentrations in cereal-derived foodstuffs is a prerequisite to reach the safety levels. In view of the ZEN exposure reported in our study, a call for an overhaul of legislation focusing on ZEN is necessary. Edwards (Edwards, 2011) reported already the risk of ZEN in European wheat; our study indubitably confirms his statements. Official instances are focused on the levels of the individual mycotoxins that exceed the maximum limits. From a scientific point of view however, also the mycotoxin derivatives and masked forms should be taken into consideration. Nevertheless, the evaluation of masked mycotoxins should be treated with caution due to the lack of occurrence, bioavailability and toxicological data. Based on the results of the risk assessment a systematic monitoring of mycotoxins and their masked forms remain necessary to gain more concentration data and specific toxicokinetic data for the masked forms.

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