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Exploring the relationship between daily intake and urinary excretion of the mycotoxins T-2 and HT-2 toxin in humans

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ABSTRACT

T-2 toxin (T-2) and HT-2 toxin (HT-2) are mycotoxins that can contaminate food, especially cereals. Exposure to T-2 and HT-2 has mainly been estimated using dietary exposure assessment, however, human biomonitoring presents another valuable approach.

The relationship between daily intake and urinary excretion of T-2 and HT-2 over time in 40 Norwegian adults was modelled. T-2, HT-2 and T-2 triol were analysed in 24-h urine samples using LC-MS/MS. Dietary exposure of T-2 and HT-2 was calculated using 24-h weighed dietary records and concentration data in food derived from measured concentrations in raw food commodities. A statistical model was developed and fit to estimate the excreted fraction (f_{abs_excr}) and residence time parameters.

Without deconjugation prior to analysis, T-2, HT-2 or T-2 triol were not detected in the urine of the 40 adults. Applying a deconjugation step, total HT-2 (HT-2 and its glucuronides) was detected in almost all samples. Using the statistical model, the mean f_{abs_excr} was estimated to be 0.184, equivalent to 18.4 %. The estimated time in which 97.5 % of the ingested T-2 and HT-2 was excreted as total HT-2 was 14.3 h, and the elimination half-life was 4.0 h.

This study highlights the challenges involved in modelling the relationship between daily intake and urinary excretion of T-2 and HT-2 over time in humans. The findings indicate that approximately 20 % of the external exposure can be traced back in the urine within 24 h. However, additional research is required to support and strengthen these findings.

1. Introduction

T-2 toxin (T-2) and HT-2 toxin (HT-2) are mycotoxins belonging to the type A trichothecenes (Garvey et al., 2008), which are produced by various species of fungi, predominantly those belonging to the *Fusarium* genera (Cole et al., 2003; Cope, 2018). Globally, T-2 and HT-2 occurrence has been mainly reported in cereals (EFSA, 2017a; WHO, 2002). In Europe, oats and oat products are noted to be the most susceptible commodities to T-2 and HT-2 contamination (EFSA, 2011, 2017a; Meng-Reiterer et al., 2016; van der Fels-Klerx and Stratakou, 2010). Oats grown in Scandinavia have reportedly high concentrations, sometimes above established maximum levels (Fredlund et al., 2013; van der Fels-Klerx and Stratakou, 2010). Due to their relative heat and UV stability (Eriksen, 2003), T-2 and HT-2 can also persist in processed foods (EFSA, 2017a; Lancova et al., 2008; Pittet, 1998; van der Fels-Klerx and Stratakou, 2010).

T-2 can be metabolised in fungi, plants and mammals generating many metabolites. Such metabolites can occur together with T-2 as contaminants in food and feed. To date and to our knowledge, no publications are available studying the toxicokinetics of T-2 and its metabolites *in vivo* in humans, however, several experimental animal studies and *in vitro* studies are available (Broekaert et al., 2017; Corley et al., 1986; Gratz et al., 2017; Matsumoto et al., 1978; Pace, 1986; Pfeiffer et al., 1988; Robison et al., 1979; Sun et al., 2015; Weidner et al., 2012; Wu et al., 2011; Yang et al., 2013, 2017, 2020a).

Yang et al. (2013) investigated T-2 metabolism in rats, revealing

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distinct sex-based metabolic profiles. Following a single oral dose, urinary analysis identified 19 metabolites, with males predominantly excreting 3′-hydroxy-HT-2 (3′–OH–HT-2), de-epoxy-3′-hydroxy-HT-2 (de-epoxy-3′–OH–HT-2) and HT-2, while females primarily excreted HT-2, 3′–OH–HT-2 and 9′-hydroxy-T-2 (9′–OH–T-2). Further *in vitro* studies using rat liver microsomes and S9 fractions confirmed hydrolysis as the primary metabolic pathway, followed by hydroxylation. Building on these findings, Yang et al. (2017) explored T-2 metabolism in chickens, identifying 18 metabolites in faeces, bile, and plasma, 13 of which were previously unknown, including novel sulphate conjugates. Across multiple species, liver microsome incubations consistently identified HT-2 as the dominant metabolite, reinforcing its potential as a biomarker for T-2 exposure.

Based on the available animal data, it is assumed that in humans T-2 is to a large extent absorbed, and post absorption, the liver is the primary organ of metabolism yielding a broad range of metabolites. The estimated half-life of T-2 in human plasma is short, based on the plasma elimination phase half-life of 90 min in pigs (Corley et al., 1986). Moreover, based on animal data, it is estimated that T-2 and its metabolites are majorly eliminated via the urine within 24–48 h (Janik et al., 2021; Kuca et al., 2008; van den Brand and Mengelers, 2021). Limited conclusions can be drawn on the tissue sequestration potential of T-2 and its metabolites. A study found that T-2 was not detectable in all analysed tissues of orally exposed broiler chickens after 48 h, while HT-2 was detectable in all analysed tissues at 192 h post oral exposure (Yang et al., 2020a).

Repeated exposure to T-2 has been shown to induce a range of adverse effects in experimental animals and *in vitro* systems including immunotoxic, haematotoxic, neurotoxic, reprotoxic and cardiotoxic effects (Cimbalo et al., 2020; Dai et al., 2019, 2022; Janik et al., 2021; Li et al., 2011; Sato et al., 1978; Taroncher et al., 2021; Wu et al., 2020; Yang et al., 2020b; Zhang et al., 2020). According to the International Agency for Research on Cancer, T-2 does not appear to be carcinogenic to humans (Group 3) (IARC, 1993). Limited toxicological information is available for the metabolites of T-2 including HT-2. However, HT-2 has been shown to induce oxidative stress, DNA damage and mitochondrial dysfunction (Zhang et al., 2016, 2019). Modified forms (also referred to as masked or conjugated forms) of T-2 and HT-2, which have been reported in cereals (Broekaert et al., 2015; Bryła et al., 2018; Meng-Reiterer et al., 2016), can be deconjugated in the intestinal tract into the free form (Gratz et al., 2017), and therefore, should be considered in the exposure assessment.

Based on the immunotoxic and haematotoxic effects reported in rats, the European Food Safety Authority (EFSA) set a group-tolerable daily intake (TDI) of 0.02 μg/kg bw for the sum of T-2 and HT-2 in 2017 (EFSA, 2017b). In a dietary exposure assessment conducted by EFSA, potential concern associated with exposure to cumulative T-2 and HT-2 was indicated, particularly in younger populations (EFSA, 2017b). Similarly, in a dietary exposure assessment performed by the Norwegian Scientific Committee for Food Safety (VKM), the mean chronic exposure to cumulative T-2 and HT-2 was the highest in the youngest age groups, whereby the group-TDI was exceeded (VKM, 2013). Both EFSA and VKM used the sum of T-2 and HT-2 in their dietary exposure assessment and did not consider other metabolites of the mycotoxins due to lack of concentration data. Previously, EFSA stated that modified forms may add another 10 % to the T-2 and HT-2 concentration in food but that this should be confirmed in future research (EFSA, 2014).

Human biomonitoring (HBM) can be used to estimate internal exposure to chemicals in populations. It can be used as a tool to complement dietary exposure assessments or as an alternative approach (Louro et al., 2019). HBM involves the measurement of parent chemicals and/or their metabolites in biological samples. Urine is considered to be the optimal biological matrix to investigate exposure to T-2 and its metabolites based on the findings in experimental animal studies (Yang et al., 2013). HBM of T-2 and its metabolites in urine samples has been performed, both in European (De Ruyck et al., 2020; Gerding et al., 2014; Gratz et al., 2020; Heyndrickx et al., 2015; Ndaw et al., 2021a; Ndaw et al., 2021b; Rodríguez-Carrasco et al., 2014) and non-European populations (Fan et al., 2019; Niknejad et al., 2021; Warth et al., 2014). However, there are major discrepancies regarding the detected concentrations of T-2 and its metabolites in human urine, regardless of geographical location, suggesting that analytical techniques require refinement and harmonisation and/or that inter-individual differences exist. Additionally, HBM studies usually investigate T-2 and its major metabolite, HT-2, without considering other phase I and II metabolites, and thereby could underestimate exposure.

Currently, both approaches, dietary exposure assessment and HBM, present uncertainty when estimating exposure to T-2 and its metabolites in humans. Combining data from both approaches is a comprehensive strategy to obtain a better understanding of real-life exposure and it can provide insights into the strengths and weaknesses of both exposure methods. Therefore, the aim of the current study is to explore the relationship between daily intake and excretion of T-2 and HT-2 over time in a group of adults from the EuroMix biomonitoring study.

2. Materials and methods

2.1. Study participants

Samples and data were obtained from a Norwegian biomonitoring study as part of the EU project “European Test and Risk Assessment Strategies for Mixtures” (EuroMix, 633172–2), which was funded by the Horizon 2020 programme. The EuroMix study was approved by the Regional Committee for Medical and Health Research Ethics (REK ID no. 2015/1868) and all the participants provided informed written consent. Participants were recruited among employees from governmental institutes and authorities, and universities in the counties of Oslo and Akershus in Norway between September 2016 and November 2017.

All participants (n = 145) provided weighed 24-h dietary records for two non-consecutive days and corresponding 24-h urine samples. Further detailed information related to the study design, sample collection, data collection, registration, and processing can be found in the publication by Husoy et al. (2019).

The target number of participants for the current research was 40 individuals. Regarding the selection of participants (see Fig. 1), firstly, a total of 80 individuals were pre-selected from the EuroMix participant pool (n = 145) based on their oat consumption. The data were retrieved from participants’ weighed 24-h dietary records. As previously mentioned, all participants had data and samples for two days and the day with (the highest) oat consumption was selected. Based on the dietary records, 72 individuals consumed oats on either or both days, thus those individuals were selected. Since our target number was 80 individuals, we selected an additional eight individuals based on their high consumption of unspecified cereals. The corresponding 24-h urine samples from those 80 individuals were analysed for total T-2, HT-2 and T-2 triol following a deconjugation step (where ‘total’ refers to the mycotoxins and their glucuronides). The summary statistics are displayed in Appendix A. Based on the results of the urinary analysis, the 40 participants with the highest total HT-2 concentrations were included in the study (to enhance the likelihood of analysing the mycotoxins in

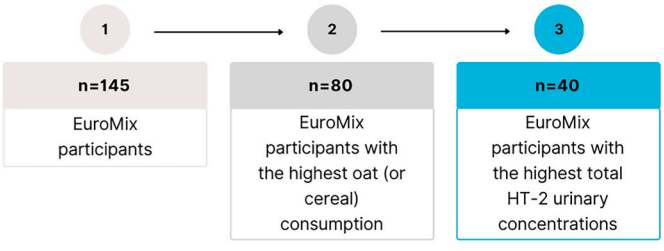

Fig. 1. Flowchart displaying the selection process for the 40 participants.

detectable concentrations). Total T-2 and T-2 triol were not detected in any sample. The final group consisted of 15 males and 25 females, with a mean age of 42 ± 11 and 39 ± 10 years, respectively. Male and female participants had a mean body weight of 78 ± 7 and 63 ± 7 kg, respectively.

2.2. Urinary analysis

2.2.1. Chemicals

Analytical standards of T-2 (100 μg/mL), HT-2 (100 μg/mL), T-2 triol (50 μg/mL), $^{13}C_{24}$ T-2 (25 μg/mL), $^{13}C_{22}$ HT-2 (25 μg/mL) and NEO (100 μg/mL) in acetonitrile were obtained from Romer Labs (Austria), while $^{13}C_{20}$ T-2 triol (10 μg/mL in acetonitrile) was obtained from Libios (France). Acetonitrile and methanol were obtained from Biosolve (the Netherlands). Ammonium formate, formic acid and potassium dihydrogen phosphate were purchased at Merck (the Netherlands). Potassium phosphate dibasic, β-glucuronidase from *Escherichia coli* type IX-A, HT-2 (1 mg), Strata X C18 columns and NaCl were purchased from Sigma-Aldrich (the Netherlands). Immunoaffinity columns (IAC) (EASI-EXTRACT® T-2 & HT-2) were purchased from R-Biopharm (the Netherlands), and water was purified by a Milli-Q purification system with a minimal resistance of 18.2 MΩ/cm.

2.2.2. LC-MS/MS system and parameters

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consisted of Waters Acquity UPLC equipped with an autosampler, two gradient pumps and a temperature control oven coupled to Waters Xevo TQS mass spectrometer with ESI interface. Analytical column Acquity UPLC HSS T3, 1.8 μm, 2.1 × 100 mm (Waters) was operated at 50 °C, and the analytes were separated using a gradient mode using water:methanol:formic acid (90:10:0.5) (v:v:v %, A) and methanol:formic acid (100:0.5) (v:v %, B), both with 5 mM ammonium formate as a mobile phase and a flow rate of 0.4 mL/min. The gradient started at 95 % A, held for 0.1 min, decreased to 5 % A at 9 min, was held for 1 min, and then increased to initial conditions of 95 % A at 10.1 min, held till 11.2 min. The instrument operated in positive ionisation mode, using MRM scan type, ion spray voltage of 4500 V, temperature 400 °C, curtain gas set at 40, and collision gas at medium. The *m/z* ions monitored, and other fragmentation settings are given in Appendix B.

2.2.3. Urine sample preparation and analysis

Prior to analysis, urine samples were stored at −80 °C and transported on dry ice. Since freezing slows down or can even halt chemical reactions and enzymatic activity in urine samples, we did not expect significant degradation of mycotoxins during storage and transport.

The samples were available as separate voids collected over a 24-h period. Two procedures were used for the analysis of urine samples from the 40 participants. Firstly, free T-2, HT-2 and T-2 triol were measured in pooled 24-h urine using LC-MS/MS. Prior to sample loading, 1.5 mL of urine was diluted with 2 mL of potassium phosphate buffer 75 mM at pH 6.8.

Secondly, following a deconjugation step, total T-2, HT-2 and T-2 triol were measured using LC-MS/MS. For this analysis, urine samples were pooled into three collection time intervals, morning (6:00–12:00 h), afternoon (12:00–18:00 h) and evening (18:00–08:20 h). Urine voids were pooled based on the exploratory nature of this study. To note, the evening time intervals was set at 18:00–08:20 h as opposed to 18:00–06:00 h since one participant did not fall into the 24-h time window. This participant provided their first urine void at approximately 08:00 h in the morning and last urine void at 08:20 h the following morning. In total, 117 samples were subject to analysis since participants had a morning, afternoon and evening sample. To note, three samples were missing (two morning and one afternoon). The deconjugation step was performed by adding 180 μL of β-glucuronidase solution from *Escherichia coli* (25 kU/mL) to 1.5 mL of urine diluted with 2 mL of potassium phosphate buffer 75 mM pH 6.8. Internal standards were added, and the reaction was maintained for at least 16 h in a water bath overnight at 37 °C. Samples were cooled down to room temperature prior to sample loading.

Analytes were extracted on an IAC dedicated to T-2 and HT-2, which exerted cross reactivity towards T-2 triol. NEO was not retained on the IAC and thus, could not be analysed. After draining the remaining liquid, the total sample was loaded onto the column and let pass by gravity. Columns were rinsed with 2x3 mL water and excess water was removed by passing air through the column. Analytes were eluted with 3 mL of methanol and evaporated under a nitrogen stream at 55 °C. Dry residues were reconstituted in 100 μL of methanol/water (50/50, v/v) and analysed by LC-MS/MS. The recovery, repeatability and limit of detection (LOD)/limit of quantification (LOQ) values for T-2, HT-2 and T-2 triol in urine are given in Table 1.

2.3. Dietary exposure assessment

Dietary exposure estimates for T-2 and HT-2 per meal were calculated using participants' food consumption data (weighed 24-h dietary records) and concentration data of T-2 and HT-2 in food. The exposure estimates for T-2 and HT-2 per meal were modelled separately. For the presentation of summary statistics in Section 3.2, the exposure estimates were summed on a molar basis to derive cumulative exposure (expressed in terms of T-2 equivalents). Molecular weights of 466.5 and 424.5 g/mol were used for T-2 and HT-2, respectively.

Probabilistic dietary exposure estimates were performed by Monte Carlo simulations with 1000 iterations using the software R (version 3.6.2).

Exposure estimates for T-2 and HT-2 were calculated for each meal consumed by the 40 participants over a 24-h period. In the EuroMix biomonitoring study, the time point of each meal was registered in the 24-h weighed dietary records (Husoy et al., 2019). Therefore, exposure estimates could be grouped by morning (06:00–12:00 h), afternoon (12:00–18:00 h) and evening (18:00–08:20 h) time intervals.

Concentration data of T-2 and HT-2 in raw food commodities (e.g., oats, barely, wheat and rye) and foods (e.g., bread, pasta and biscuits) were gathered from public literature. Concentration data of T-2 and/or HT-2, in Europe, from a total of 23 publications, were used (Babič et al., 2021; Bertuzzi et al., 2014; Błajet-Kosicka et al., 2014; Bogdanova et al., 2018; De Boevre et al., 2012, 2013; Fredlund et al., 2013; Gambacorta et al., 2018; Grajewski et al., 2019; Ibáñez-Vea et al., 2012; Juan et al., 2013; Kosicki et al., 2020; Kovač et al., 2022; Kuzdraliński et al., 2013; Pleadin et al., 2013; Rasmussen et al., 2012; Rodriguez-Carrasco et al., 2012, 2015; Rubert et al., 2013; Tolosa et al., 2021; Topi et al., 2020; van der Fels-Klerx et al., 2012). The majority of extracted concentration data were aggregated data (e.g., percentage positive samples, mean, median, range, etc.). Where several aggregated datapoints were available from a publication, the data were modelled so that all datapoints were considered. To this end, for each publication, for each raw food commodity or food, concentration data for T-2 and/or HT-2 were sampled from a log-normal distribution defined by the aggregated data equal to the number of analyses presented. The resulting modelled concentration data were compiled for each raw food commodity or food. The R software (version 4.3.0) was used to model the concentration data (see link to R code in Appendix C). Regarding the treatment of left-censored concentration data, a middle-bound scenario was used,

Table 1
Recovery, repeatability and LOD/LOQ values of T-2, HT-2 and T-2 triol in urine.

Mycotoxin	Mean recovery[a] (%)	Repeatability (%)	LOD/LOQ (ng/mL)
T-2	97.0	4.0	0.013/0.050
HT-2	106.0	9.0	0.020/0.050
T-2 triol	102.8	10.3	0.100/0.300

LOD: limit of detection; LOQ: limit of quantification.
[a] Recovery following spiking at the LOQ value for each mycotoxin.

where values below the LOD were set to half the LOD value and values below the LOQ were set to half the LOQ value.

The concentration data for T-2 and HT-2 per raw food commodity or food were then used to calculate the exposure estimates of T-2 and HT-2. The foods consumed by the participants did not match the foods with T-2 and HT-2 concentration data. Using the concentration data for the raw food commodities, the concentrations in various foods that were consumed by the participants were estimated based on recipes and declarations of contents. The food recipes were taken from the official Norwegian web page called "Matvaretabellen", where recipes representative for the most common food items in the Norwegian diet are included (Mattilsynet, 2024). These estimations were made for the following foods: (1) bakery (including bread, polar bread, hamburger bread, crispbread, buns and rolls, wheat tortilla, rye flour and wheat flour); (2) oatbread; (3) cereals; and (4) oat milk.

For the exposure estimates per meal, the T-2 or HT-2 concentration in each food was multiplied by the amount of the consumed food as reported in participants' weighed 24-h dietary records. To note, when setting a concentration for T-2 or HT-2 in a food, the concentrations were sampled by Monte Carlo simulations for the number of analyses for the original raw food commodity. Overall, the exposure estimates per meal were calculated as follows:

$$D = \sum_{food=1}^{food} \left(C_{food} \times x_{amount}\right)$$

where:

D = dietary exposure estimate for T-2 or HT-2 in a given meal (ng);

C = concentration of T-2 or HT-2 in a given food (ng/g);

x = amount of a given food eaten in a specific meal or snack (g).

The R software (version 4.3.0) was used to calculate the exposure estimates (see link to R code and data files in Appendix C).

2.4. Statistical model

A statistical model was developed to describe the relationship between daily intake and excretion of T-2 and HT-2 over time. The model can be interpreted as a compartmental model with transition rates. The input data for the model included urinary excreted amounts (i.e., urinary concentration multiplied by volume) and dietary exposure estimates. No imputation method was used when input data were not available. We defined our model in terms of nanomoles.

The parameters estimated by the model were the excreted fraction (f_{abs_excr}) and the residence time. The f_{abs_excr} should be interpreted as a lumped parameter since it combines the absorbed proportion of T-2 and HT-2 into the body, the proportion of T-2 that is metabolised into HT-2, and the excretion of the mycotoxins via the urine. The residence time can be defined as the time duration between intake and excretion. This time duration was assumed to be right-skewed, and following previous work done by van den Brand et al. (van den Brand et al., 2021), we assumed a log-normal distribution. The residence time was parametrised by the mean and standard deviation. As a result, the mean residence time corresponds to 63.2 % elimination from the body.

The parameters of the statistical model were estimated by fitting the model to the input data of all individuals over time. Since the urinary excreted amounts depend on the parameters of the model in a non-linear way, a non-linear regression method was applied. The regression function describes the relation between the dietary exposure estimates being the independent variables and the urinary excreted amounts being the dependent variables.

Explorative regression analyses of the data showed that the urinary excreted amounts best fit a normal distribution. Explorative analyses also showed that the ratios between dietary exposure estimates and urinary excreted amounts were different between participants, indicating population heterogeneity. Therefore, this heterogeneity was addressed by assuming the f_{abs_excr} to be random. Moreover, a logistic transformation was applied to ensure that the f_{abs_excr} was between a value of 0 and 1. Since there was no statistical or physiological reason to define the residence time as a random parameter, it was assumed to be fixed. The input data did not allow for differentiating between participants age and sex, so no co-variates were included in the model.

The final statistical model was a non-linear mixed effects (nlme) model (Fig. 2), as this type of models can be advantageous when dealing with complex data and individual variability. In addition, the excreted amounts depend on the model parameters in a non-linear way and both fixed effects (residence time parameters) and random effects (f_{abs_excr} parameter) are included. The data of both males and females were grouped together (n = 40) in the model, similar to the statistical model previously developed for deoxynivalenol by van den Brand et al. (van den Brand et al., 2021).

The input data, urinary excreted amounts and dietary exposure estimates, reflect a daily setting meaning that values can be zero, although unlikely, and there are multiple intake time intervals during a 24-h time window. The urinary excreted amount in the first morning void relates to a previous and unknown dietary exposure outside the 24-h time window. In addition, the last evening dietary exposure may result in an unknown urinary excreted amount excreted after the last urine void of the 24-h time window. These unknown values are so-called missing values. To address this, the missing values were interpreted as non-zero missing values and imputed according to the following procedure. For the previous and unknown evening dietary exposure outside the 24-h time window, it was assumed to be equal to the evening dietary exposure within the 24-h time window. For the unknown urinary excreted amount excreted after the last urine void of the 24-h time window, it was assumed that the last dietary exposure would not result in a measurable urinary excreted amount after the 24 h time window. Therefore, two time intervals were included after stopping the experiment (after the time interval of 24 h): (1) 24–48 h, where excretion from the last dietary exposure could occur but values were not measured and thus missing (but not zero); and (2) 48–72 h, where the urinary excreted amount must be zero.

We applied the nlme-procedure in the software R (version 4.3.0) to fit the model to the data (see link to R code and data files in Appendix D).

Several assumptions that were made when constructing the model were validated using four sensitivity analyses outlined in Appendix D. The following assumptions were tested: (1) the excreted amounts in urine were assumed to be normally distributed; (2) the missing evening intake prior to the 24-h time window was assumed to be equal to the evening intake within the 24-h time window; (3) the last intake amount was assumed to not result in a measurable excreted amount (zero) after more than 24 h; (4) fixed intake amounts were assumed. The achieved QQ-plots of the residuals were compared to determine the best model fit. To note, the QQ-plot should (approximately) be a straight line, if the

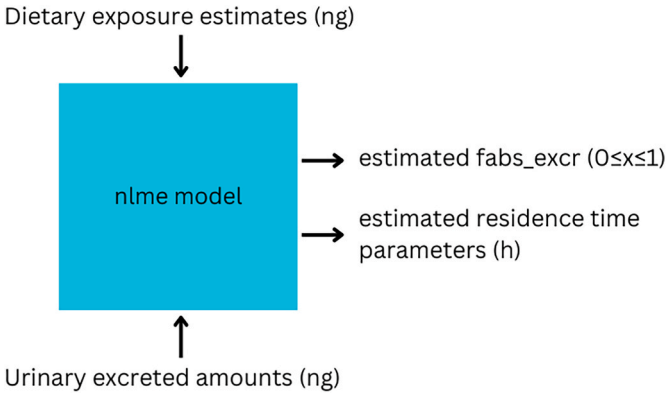

Fig. 2. Simple visualisation of the statistical model used to calculate the f_{abs_excr} and the residence time parameters. Abbreviations: f_{abs_excr}: excreted fraction; h: hour; nlme: non-linear mixed effects.

resulting regression model residuals are indeed normally distributed. In addition, the Akaike Information Criterium (AIC) was applied (Appendix D). The AIC-value quantifies the model fit, taking into account the number of parameters included in the model (Burnham and Anderson, 2002). In general, the favourable statistical model to describe the residence time of the cumulative dietary exposure to T-2 and HT-2 is the one with the lowest AIC value.

3. Results

3.1. Urinary excretion data

Twenty-four-hour urine samples from 40 participants were analysed for free T-2, HT-2 and T-2 triol using LC-MS/MS without applying a prior deconjugation step. Free T-2, HT-2 and T-2 triol were not detected in any sample.

Following a deconjugation step, participants' urine samples were analysed for total T-2, HT-2 and T-2 triol using LC-MS/MS, where 'total' refers to the mycotoxins and their glucuronides. For this analysis, urine samples were pooled into three collection time intervals, morning (06:00–12:00 h), afternoon (12:00–18:00 h) and evening (18:00–08:20 h). Total T-2 was not detected in any sample. Total T-2 triol was detected above the LOD in an afternoon and evening sample of one participant, but both concentrations were below the LOQ. To note, T-2 triol and its corresponding internal standard exhibited instability throughout the sample preparation process, but the validation criteria were met since the response of the analyte was corrected by the response of the internal standard. Total HT-2 was analysed above the LOQ in almost all samples (≥97 %) (Table 2).

The summary statistics of total HT-2 concentrations are presented in Table 2. The mean total HT-2 concentration excreted over the 24-h period was 0.676 ng/mL. For morning, afternoon and evening collection time intervals, the mean total HT-2 concentrations were 0.238, 0.249 and 0.208 ng/mL, respectively. The mean total HT-2 concentrations were higher in male participants compared to female participants (Appendix E).

Participants' urinary volumes are also provided in Table 2. The mean 24-h urinary volume across all participants was 2057 mL (range: 1025–3505 mL). For morning, afternoon and evening collection time intervals, the mean urinary volumes were 595, 559 and 933 mL, respectively. The mean urinary volumes were similar between male and female participants, however, this was not the case when accounted for body weight, with males having lower urinary volumes per kg bw than females (Appendix E).

The mean amount (i.e., urinary concentrations multiplied by volumes) of total HT-2 excreted over the 24-h period was 440 ng (Table 2). For morning, afternoon and evening collection time intervals, the mean amounts of total HT-2 were 148, 116 and 186 ng, respectively. Participants' individual amounts were used as input data for the statistical model. When stratified by sex, mean amounts of HT-2 were higher in males compared to females (see Appendix E).

3.2. Dietary exposure estimates

For the same 40 participants, the dietary intake of T-2 and HT-2 over a 24-h period was estimated using food consumption data (weighed 24-h dietary records) and modelled concentration data of T-2 and HT-2 in food (see Section 2.3). The summary statistics for cumulative T-2 and HT-2 exposure estimates are given in Table 3. The exposure estimates for T-2 and HT-2 were modelled separately and then summed on a molar basis to derive cumulative exposure. The mean daily exposure estimate for cumulative T-2 and HT-2 was 8907 ng. For morning, afternoon and evening time intervals, the mean exposure estimates for cumulative T-2 and HT-2 were 6298, 2657 and 2772 ng, respectively. Noticeably, the highest mean exposure estimate was calculated for the morning time interval, followed by the evening and then the afternoon time interval. Participants' individual exposure estimates were used as input data for the statistical model.

3.3. Statistical model

A statistical model was developed to describe the relationship between daily cumulative intake of T-2 and HT-2 and excretion of HT-2 over time in the human body using urinary concentrations and dietary exposure estimates as input data. The data were defined in terms of nanomoles for the model. Input data from a total of 32 participants were used to estimate the parameters in the statistical model, where eight participants were excluded due to missing urinary excreted amounts.

Table 3
Summary statistics for cumulative T-2 and HT-2 exposure estimates, where 'cumulative' refers to the sum of T-2 and HT-2 on a molar basis in 40 participants.

	Morning (06:00–12:00 h)	Afternoon (12:00–18:00 h)	Evening (18:00–06:00 h)	Entire day (24 h)
Mean[a] ± SD (ng)	6298 ± 12353	2657 ± 6958	2772 ± 10645	8907 ± 14662
Median (ng)	2939	883	672	5094
Range (ng)	23.6–537850	4.23–404872	6.24–740583	90.8–741588

[a] Summation based on molecular weights of 466.5 and 424.5 g/mol for T-2 and HT-2, respectively.

Table 2
Summary statistics of total HT-2 in urine, where 'total' refers to HT-2 and its glucuronides in 40 participants, where each participant had a morning, afternoon and evening urine sample.

	Morning (06:00–12:00 h)	Afternoon (12:00–18:00 h)	Evening (18:00–08:20 h)[a]	Entire day (24 h)
No. samples[b]	38	39	40	117
% >LOD/LOQ	100/95	97/95	100/100	99/97
Mean ± SD[c]				
ng/mL	0.238 ± 0.161	0.249 ± 0.215	0.208 ± 0.129	0.218 ± 0.131
ng[d]	148 ± 136	116 ± 106	186 ± 126	440 ± 303
Median (ng/mL)	0.183	0.178	0.158	0.184
Range (ng/mL)	0.021–0.667	0.010–0.980	0.040–0.473	0.078–0.476
Urine volume (mL)	595 ± 329	559 ± 313	933 ± 279	2057 ± 618
Urine volume (mL/kg bw)	8.82 ± 4.86	8.21 ± 4.40	13.8 ± 4.39	30.4 ± 9.23

LOD: limit of detection; LOQ: limit of quantification.
[a] The evening time interval was set at 18:00–08:20 h because 08:20 h was the time of the last participants urine void within the 24-h time window.
[b] Three samples were missing (two morning and one afternoon).
[c] One afternoon sample was analysed below the LOD and in for the calculation of sum parameters this concentration was set to half the value of the LOD.
[d] The amount (ng) of total HT-2 was calculated by multiplying the concentration (ng/mL) by urinary volume (mL).

A non-linear mixed effects model was used to estimate the f_{abs_excr} and residence time parameters (Table 4). The population mean f_{abs_excr} was estimated to be 0.184, equivalent to 18.4 %. The mean residence time was estimated to be 4.9 h. The median, which is equivalent to the elimination half-life of the cumulative T-2 and HT-2 exposure, and the 97.5th percentile residence times were estimated to be 4.0 and 14.3 h, respectively. Following our assumption of a log-normal distribution, the mean residence time was indeed larger than the median value.

4. Discussion

In this study, the relationship between daily intake and excretion of T-2 and HT-2 over time in humans was explored using a statistical model. Total T-2, HT-2 and T-2 triol were measured in participants' 24-h urine samples, pooled by morning, afternoon and evening collection time intervals, where 'total' refers to the mycotoxins and their glucuronides. For the same time intervals, the dietary exposure estimates for T-2 and HT-2 were calculated. Using the urinary concentrations and dietary exposure estimates as input data, a statistical model was developed and optimised.

The urinary analysis revealed the importance of a deconjugation step before sample analysis since none of the mycotoxins were detected without enzymatic treatment. When a deconjugation step was applied, total HT-2, a well-known hydrolysis metabolite of T-2, was detected in almost all urine samples (≥99 %), while little or no total T-2 or T-2 triol were detected. This finding demonstrates that most of the total HT-2 present in the urine samples was in the conjugated form.

For the deconjugation step, β-glucuronidase from *Escherichia coli* was applied in the current study, which has also been applied in previous studies (Gratz et al., 2020; Ndaw et al., 2021a,b). This enzyme does not allow for the deconjugation of sulphate metabolites which can occur alongside glucuronide metabolites (Yang et al., 2017). Alternatively, β-glucuronidase/arylsulfatase derived from *Helix pomatia* can be used to deconjugate glucuronides and sulphates. However, previous research has shown that T-2 was unstable when incubated for 24 h with enzymes derived from *Helix pomatia* but stable when incubated with enzymes derived from bovine liver and *Escherichia coli* (Welsch and Humpf, 2012). Regarding HT-2, it exhibited stability in the presence of all three types of enzymes (Welsch and Humpf, 2012). Based on these findings, β-glucuronidase/arylsulfatase derived from *Helix pomatia* was not applied in the current study, and consequently, no conclusions can be drawn on the possible presence of sulphate metabolites in urine. In the current study, no analytical standards were available for T-2 and HT-2 glucuronides. Therefore, the performance of the β-glucuronidase from *Escherichia coli* applied could not be tested.

A non-linear mixed effects model was developed to estimate the f_{abs_excr} and residence time parameters. In the model the following assumptions were made: (1) the excreted amounts in urine were assumed to be normally distributed; (2) the missing evening intake prior to the 24-h time window was assumed to be equal to the evening intake within the 24-h time window; (3) the last intake amount was assumed to not result in a measurable excreted amount (zero) after more than 24 h; (4) fixed intake amounts were assumed. Four sensitivity analyses were performed to test the assumptions made on the model fit and in addition, the AIC was applied (Appendix D). The results demonstrated that the statistical model parameters were most sensitive to one of the sensitivity analyses in particular, whereby the excreted amounts in urine were assumed to be log-normally distributed as opposed to normally distributed (main analysis). This affected both the calculated f_{abs_excr} and residence time, however, the error terms were not normally distributed, and it was concluded that the model fit was not valid. In addition, the derived AIC value was incomparable to the other AIC values, notably, far higher.

Excreted concentrations of chemicals including mycotoxins in urine are typically log-normally distributed rather than normally distributed often due to biological variation. However, we see that our data better fit a normal distribution. This is likely caused by bias in our participant selection process since we selected a limited number of 40 individuals from the EuroMix participant group with the highest intake and excretion of the mycotoxins. This was done in an attempt to better predict the relationship between intake and excretion of the mycotoxins. However, by selecting individuals with actual (high) exposure and avoiding individuals with zero exposure, we reduced the variability among participants. In future research, if additional concentration data of the mycotoxins in a larger and more diverse participant group would become available, our model could be further tested and validated. The other sensitivity analyses had little or no impact on the statistical model fit, highlighting the robustness of the assumptions made.

Applying the statistical model, the population mean f_{abs_excr} was estimated to be 0.184, equivalent to 18.4 %. This finding indicates that approximately 20 % of the external exposure can be traced back in the urine within 24 h. This raises the question of the fate of the remaining approximate 80 %. This could hypothetically imply that T-2 and HT-2 have limited absorption and following ingestion the majority may be excreted via the faeces. This could be explored by means of an intervention study, whereby, blood, urine and faecal samples are collected following a known ingested dose of T-2 or HT-2. Such a controlled toxicokinetic study would provide valuable insight into the true absorption potential of the mycotoxins in humans.

The excreted fraction estimated in the current study is similar to that presented in research reported at the World Mycotoxin Forum 14th Conference (WMF, 2023). A healthy female took an oral dose of T-2 (at the TDI level), and two days later, she took an oral dose of HT-2 (at the TDI level). After each dose, her urine was collected. Following the T-2 dose, within 24 h, 20 % of the ingested dose was excreted as total HT-2 in urine (where 'total' refers to the mycotoxins and their glucuronides) (excretion percentage expressed as T-2 equivalents). No total T-2 or T-2 triol were detected. Following the HT-2 dose, within 24 h, 17 % of the ingested dose could be traced back to total HT-2 in urine. No total T-2 or T-2 triol were detected. This research provides insights into the toxicokinetics of T-2 and HT-2 following ingestion, however, it was conducted in only volunteer, therefore, additional research with a larger (and more diverse) sample size is required.

On the other hand, the estimated f_{abs_excr} could be impacted by the assumptions made in the current study. Firstly, in our model, we assumed that all dietary intake of cumulative T-2 and HT-2 was excreted as total HT-2 in urine, since total T-2 and total T-2 triol were not or rarely detected. In future research, we recommend that other metabolites should be explored in urine since experimental animal and *in vitro* studies have demonstrated a broad metabolic profile of T-2 (Janik et al., 2021; Kuca et al., 2008; Yang et al., 2013, 2017). Other hydrolysis metabolites that could be present in human urine and are of interest for

Table 4
The estimated f_{abs_excr} and residence time parameters of cumulative T-2 and HT-2 exposure, after fitting the data of 32 participants in the statistical model.

Parameter	Value
f_{abs_excr}	
Mean	0.184
Relative uncertainty (SE)[a]	0.024
Relative heterogeneity (SD)[b]	0.134
Residence time	
Mean	4.9 h
Median (half-life)	4.0 h
95 % CI	1.1–14.3 h

f_{abs_excr}: excreted fraction; SD: standard deviation; SE: standard error.

[a] SE describes the standard error of the calculated population mean value, and thus measures the uncertainty, defined on an original scale.

[b] SD describes the standard deviation of the random effect, and thus measures the heterogeneity, defined on an original scale.

future research include NEO, T-2-tetraol and 15-acetyl-T-2-tetraol. NEO and T-2-tetraol can also be present in conjugated forms, therefore, a deconjugation step prior to analysis is advised. Hydroxylation metabolites could also be of interest for future research. In a HBM study conducted by Narvaez et al. (2021), 3′–OH–T-2 was tentatively identified in 99.7 % of urine samples from Italian adults. Furthermore, de-epoxidation metabolites could also be investigated including de-epoxy-3′-hydroxy-HT-2 and de-epoxy-3′-hydroxy-T-2-triol. In addition, the presence of sulphate metabolites could be of interest. Overall, accurate and quantitative measurement of the aforementioned metabolites is required. Therefore, further method development, refinement and validation with (internal) standards is needed which may bring us closer to accurately detecting and quantifying the complete metabolic profile of T-2 and HT-2 following ingestion.

Secondly, in our model, we also assumed that almost complete excretion of the intake amount occurs within a 24-h time window, but potential excretion could occur after this timepoint, for instance through enterohepatic recirculation and/or tissue sequestration leading to delayed bodily clearance. Using the statistical model, the estimated time in which 97.5 % of the ingested T-2 and HT-2 was excreted as total HT-2 was 14.3 h, supporting our assumption that most of the excretion occurs within 24 h. This assumption is supported by animal data which indicate rapid plasma elimination and a bodily clearance within 24–48 h (Corley et al., 1986; Janik et al., 2021; Kuca et al., 2008; van den Brand and Mengelers, 2021; Yang et al., 2017; Yang et al., 2013). In future research, a longer excretion window (e.g., 48 or 72 h) should be explored.

There are also several uncertainties associated with the dietary exposure estimates calculated in the current study which should be considered, particularly concerning the concentration data in food. This uncertainty can be seen back in the large standard deviation of the mean cumulative T-2 and HT-2 exposure estimates (Table 3, Section 3.2). For the dietary exposure estimates, concentration data of T-2 and HT-2 in foods and raw food commodities in Europe were gathered from 23 publications with varied characteristics and limited data points (mainly aggregated data available). In future research, a larger concentration dataset with favorably individual-level concentration data of T-2, HT-2 and additional metabolites could be used to better estimate dietary exposure. In addition to this uncertainty, no foods eaten by the participants matched the foods with concentration data. Therefore, we modelled the concentration of the mycotoxins in foods based on concentrations in raw food commodities, adding additional uncertainty which we did not quantify. The collection of food samples (per meal, snack or drink, not pooled over 24 h) from participants would have been a more favourable approach whereby the concentrations of mycotoxins in the foods consumed could have been measured. In addition, concentration data for T-2 and HT-2 were used, not considering modified forms due to lack of data. Since modified forms can occur alongside T-2 and HT-2 in food, our exposure estimates could be underestimated. Previously, EFSA stated that conjugated forms may add another 10 % to the concentration of T-2 and HT-2, however, this value was not applied in our dietary exposure assessment due to too much associated

Table 5
Summary of results from other HBM studies investigating the presence of T-2, HT-2 and other metabolites in urine of adults.

Country	Study population	Type of urine	Mycotoxin	Positive %	Analytical technique	Enzymatic treatment	Method sensitivity (ng/mL)	Reference
European								
Norway	General population (n = 40)	24-h	T-2[a] HT-2[a] T-2 triol[a]	0 99 2	LC-MS/MS	β-glucuronidase	LOD = 0.013 LOD = 0.020 LOD = 0.100	Current study
Italy	General population (n = 72)	Morning	T-2 HT-2	19 28	UHPLC-Q-Orbitrap-HRMS	No	LOQ = 0.200 LOQ = 0.400	Narvaez et al. (2021)
Belgium, Czech Republic, France, Netherlands, Norway	General population (n = 188)	24-h	T-2 HT-2 T-2 triol T-2 tetraol NEO	22 6 1 4 6	UPLC-MS/MS	No	LOD = 0.008 LOD = 0.022 LOD = 0.086 LOD = 0.944 LOD = 0.018	De Ruyck et al. (2020)
Germany	General population (n = 101)	Spot	T-2 HT-2 HT-2-4-GlcA	1 0 0	LC-MS/MS	No	LOD = 0.025 LOD = 0.200 LOD = 0.025	Gerding et al. (2014)
Belgium	General population (n = 239)	Morning	T-2 HT-2	0 0	LC-MS/MS	No	NR	Heyndrickx et al. (2015)
Spain	General population (n = 22)	Spot	T-2 HT-2 NEO	0 14 0	GC-MS/MS	No	LOD = 0.500 LOD = 1.000 LOD = 0.250	Rodríguez-Carrasco et al. (2014)
France	Working males (n = 18)	Spot	T-2 HT-2	4 4	LC-HR-MS/MS	No	LOQ = 1.000 LOQ = 0.500	Ndaw et al. (2021b)
France	Working males (n = 3)	Spot	T-2[a] HT-2[a]	0 0	LC-HR-MS/MS	β-glucuronidase	LOQ = 0.500 LOQ = 1.000	Ndaw et al. (2021a)
Non-European								
China	General population (n = 260)	Morning	T-2	2	UHPLC-MS/MS	No	LOD = 0.050	Fan et al. (2019)
Thailand	General population (n = 60)	Morning	T-2 HT-2	0 0	LC-MS/MS	No	LOD = 1.000 LOD = 12.000	Warth et al. (2014)
iran	General population (n = 10)	Morning	T-2 HT-2 NEO	0 10 40	GC-MS/MS	No	LOD = 0.500 LOD = 1.000 LOD = 0.250	Niknejad et al. (2021)

GC-MS/MS: gas chromatography-tandem mass spectrometry; HBM: human biomonitoring; HT-2-GlcA: HT-2-4-glucuronide; LC-HR-MS/MS: liquid chromatography-high-resolution mass spectrometry; LC-MS/MS: liquid chromatography-tandem mass spectrometry; LOD: limit of detection; LOQ: limit of quantification; NR: not reported; UHPLC-MS/MS: ultra-high performance liquid chromatography-tandem mass spectrometry.

[a] Measured as total T-2, HT-2 or T-2 triol (mycotoxins and their glucuronides) since a deconjugation was applied before analysis.

uncertainty (EFSA, 2014). Overall, the occurrence of modified forms of the mycotoxins should be explored in future research.

To add to the complexity of this research, varied results have been reported in other HBM studies investigating the presence of T-2, HT-2 and other metabolites in urine. An overview of the results from European and non-European HBM studies in adults is provided in Table 5. Large differences in the detection frequency of the mycotoxins between studies are apparent. In the current study, the detection frequency of HT-2 was substantially higher compared to the other studies which analysed HT-2 in urine (De Ruyck et al., 2020; Gerding et al., 2014; Heyndrickx et al., 2015; Narvaez et al., 2021; Ndaw et al., 2021a; Ndaw et al., 2021b; Niknejad et al., 2021; Rodríguez-Carrasco et al., 2014; Warth et al., 2014). In the current study, T-2 was not detected in any urine sample from the 40 participants, even after a deconjugation step was applied. Alternatively, T-2, as free T-2, since no deconjugation step was applied, was detected in urine samples in several other studies (range of detection: 1–22 %) (De Ruyck et al., 2020; Fan et al., 2019; Gerding et al., 2014; Narvaez et al., 2021; Ndaw et al., 2021b), demonstrating that T-2 can indeed be excreted unchanged in the urine. The conflicting results found between the current study and the other HBM studies could be attributed to differences in sample preparation techniques (e.g., deconjugation step applied or not applied, different clean-up procedures), analytical methods (e.g., LC-MS/MS or HRMS) and method sensitivity (e.g., LOD and LOQ values). Overall, further method development and harmonisation is required so that the results generated from different studies can be compared. The conflicting results between studies could also suggest inter-individual differences in metabolic capacity. On the other hand, the detection frequency of T-2 triol in the current study was in line with that found in one other study (De Ruyck et al., 2020).

5. Conclusions

This study is the first of its kind to explore the relationship between daily intake and urinary excretion of T-2 and HT-2 over time in humans using a statistical model, and it highlights the challenges involved. The urinary analysis revealed the importance of a deconjugation step prior to analysis since none of the mycotoxins were detected without enzymatic treatment. Total HT-2 (HT-2 and its glucuronides) was detected in almost all urine samples, while little or no total T-2 and total T-2 triol were detected. Using the individual urinary excreted amounts and dietary exposure estimates as input data, the statistical model was developed, optimised and applied. The population mean f_{abs_excr} was estimated to be 0.184, equivalent to 18.4 %. The estimated time in which 97.5 % of the ingested T-2 and HT-2 was excreted as total HT-2 was 14.3 h, and the elimination half-life was 4.0 h. The findings indicate that approximately 20 % of the external exposure can be traced back in the urine within 24 h, suggesting that the mycotoxins may have limited absorption or that the assumptions made in the current research play an influential role and require further refinement. Therefore, further research is needed to fully understand the relationship between daily intake and excretion of T-2 and HT-2 so that the associated health risks can be better assessed.

CRediT authorship contribution statement

Hannah P. McKeon: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Rudolf Hoogenveen:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Marta M. Sopel:** Writing – review & editing, Investigation. **Marloes A.A. Schepens:** Writing – review & editing, Project administration, Methodology, Conceptualization. **Marcel J.B. Mengelers:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Annick D. van den Brand:** Writing – review & editing, Conceptualization. **Judith A. de Heer:** Writing – review & editing, Investigation. **Anne Lise Brantsæter:** Writing – review & editing, Methodology. **Maria Kalyva:** Writing – review & editing, Methodology. **Trine Husøy:** Writing – review & editing, Resources, Methodology, Investigation, Formal analysis.

Institutional review board statement

The EuroMix biomonitoring study was approved by the Regional Committee for Medical and Health Research Ethics (REK ID no. 2015/1868) and conducted according to the guidelines of the Declaration of Helsinki.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hannah McKeon reports financial support was provided by Food and Consumer Product Safety Authority. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A

Table A1
Summary statistics for urinary concentrations (ng/mL) of total T-2, HT-2 and T-2 triol in 24-h urine samples of pre-selected individuals (n = 80). Since a deconjugation step was performed prior to sample analysis, 'total' refers to the mycotoxins and their glucuronides.

	T-2	HT-2	T-2 triol
No. samples	80	80	80
LOD/LOQ	0.013/0.05	0.02/0.1	0.1/0.3

(*continued on next page*)

Table A1 (*continued*)

	T-2	HT-2	T-2 triol
% >LOD/LOQ	0/0	93/44	1/0
Mean ± SD[1]	–	0.137 ± 0.132	–
Median[1]	–	0.086	–
Range	–	<LOD-0.510	–

LOD: limit of detection; LOQ: limit of quantification.

[1] Six samples were analysed below the LOD and for the sum parameters the concentrations were set to half the value of the LOD.

Appendix B

Table B1
MS/MS fragmentation settings.

Q1 (m/z)	Q3 (m/z)	Rt (min)	Analyte	Cone voltage	CE (V)
483.4	185.1	7.1	**T-2 (qn)**	25	18
484.3	215.2	7.1	**T-2 (ql)**	25	20
508.3	198.2	7.1	**$^{13}C_{24}$ T-2**	25	18
442.17	215.13	6.6	**HT-2 (qn)**	25	11
442.17	263.15	6.6	**HT-2 (ql)**	25	13
464.1	278.2	6.6	**$^{13}C_{22}$ HT-2**	25	13
400.3	215.2	5.9	**T-2 triol (qn)**	25	15
400.3	281.2	5.9	**T-2 triol (ql)**	25	11
420.3	229.2	5.9	**$^{13}C_{20}$ T-2 triol**	25	15
400.2	185.2	3.05	**NEO**	25	20
400.2	215.2	3.05	**NEO**	25	15

Appendix C

The R code and data files used to model the concentration data of the mycotoxins in raw food commodities and food are available on GitHub (https://github.com/TrineHusoy/Exposure_T2_HT2.git).

The R code and data files used to calculate the dietary exposure estimates are available on GitHub (https://github.com/TrineHusoy/Exposure_T2_HT2.git). Individual food consumption data from participants in the EuroMix study cannot be openly shared for data protection reasons. Therefore, a simulated food consumption dataset was created to test the R code, whereby the original data have been stochastically rearranged (including the ID numbers).

Appendix D

The R code and data files used to fit the statistical model are available on GitHub (https://github.com/TrineHusoy/Exposure_T2_HT2.git).

Four sensitivity analyses were performed in order to validate the statistical model, and check the consequences of changing certain assumptions. The respective R code and data files are available on GitHub.

The main statistical model was developed using the following assumptions: (1) the excretion amounts in urine were assumed to be normally distributed; (2) the missing evening intake prior to the 24-h time window was assumed to be equal to the evening intake within the 24-h time window; (3) the last intake amount was assumed to not result in a measurable excreted amount (zero) after more than 48 h; (4) fixed intake amounts were assumed. The QQ-plot of the residuals achieved from the main analysis can be seen in Figure D1. To note, the QQ-plot should show (approximately) a straight line, if the resulting regression model residuals are indeed normally distributed.

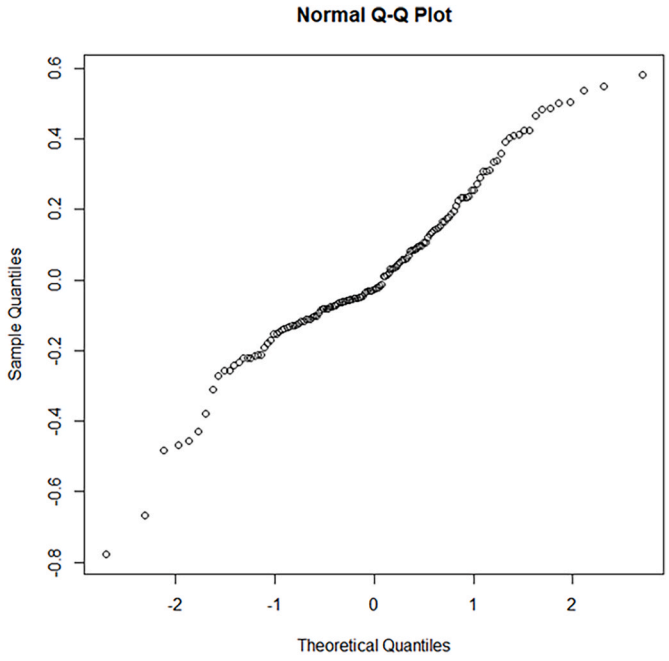

Fig. D1. The QQ-plot of the residuals from the main analysis.

All four assumptions were tested using sensitivity analyses and an overview of the results are displayed in Table D1.

In the main model, we assumed that the excretion amounts in urine were normally distributed. In this sensitivity analysis, we assumed a log-normal distribution which affected both the calculated f_{abs_excr} and residence time, however, the error terms were not normally distributed, and it was concluded that the model fit was not valid (see Figure D2).

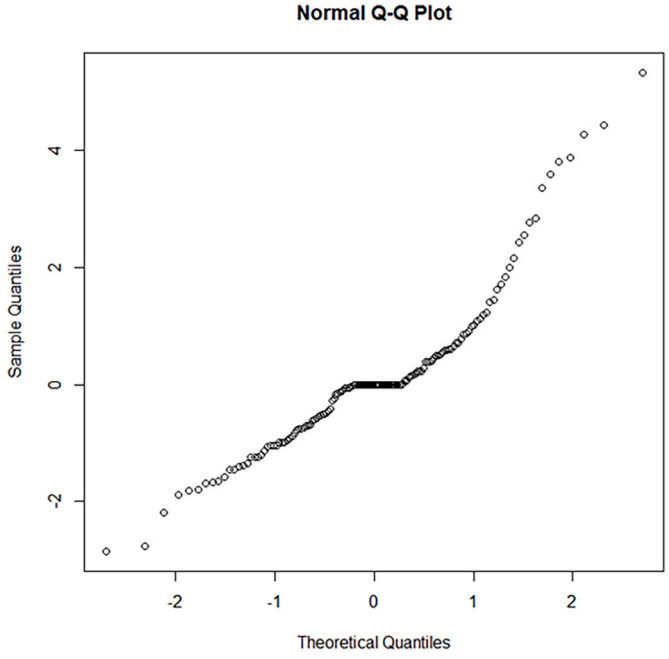

Fig. D2. The QQ-plot of the residuals from sensitivity analysis 1, whereby a log-normal distribution was applied for the excretion amounts in urine as opposed to a normal distribution as used in the main analysis.

In the main model, we assumed that the missing evening intake prior to the 24-h time window was equal to the evening intake within the 24-h time window. In this sensitivity analysis, we assumed the evening intake prior to the 24-h time window to be zero. Only minor changes were shown in relation to the model parameters (Table D1) and therefore, we concluded that the model was not sensitive to this assumption (see Figure D3).

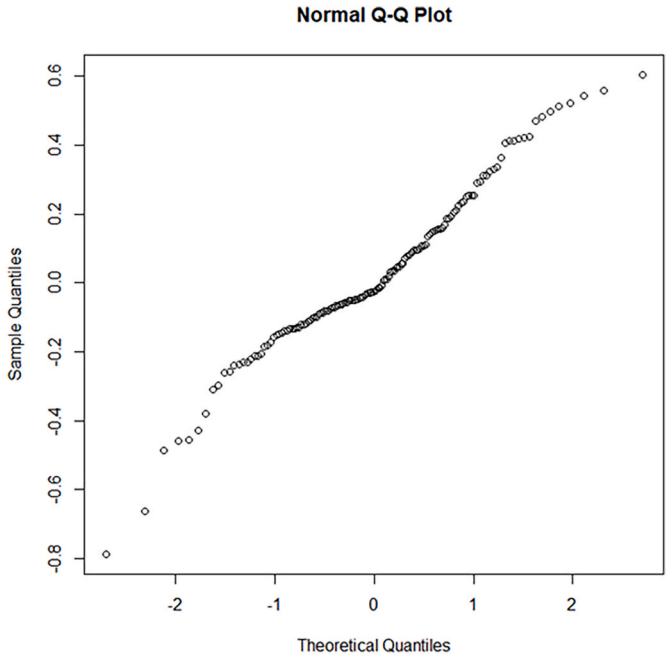

Fig. D3. The QQ-plot of the residuals from sensitivity analysis 2, whereby we assumed that the evening intake prior to the 24-h time window to be zero as opposed to assuming that the evening intake prior to the 24-h time window was equal to the evening intake within the 24-h time window as used in the main analysis.

In the main model, we assumed that the last intake amount would not result in a measurable excreted amount after more than 48 h. In this sensitivity analysis, instead of applying time-intervals of 24–48 h and 48–72 h, we applied time-intervals of 24–44 h and 44–64 h. Only minor changes were shown with the model fit being slightly worse (Figure D4). Overall, we concluded that the model was not sensitive to this assumption.

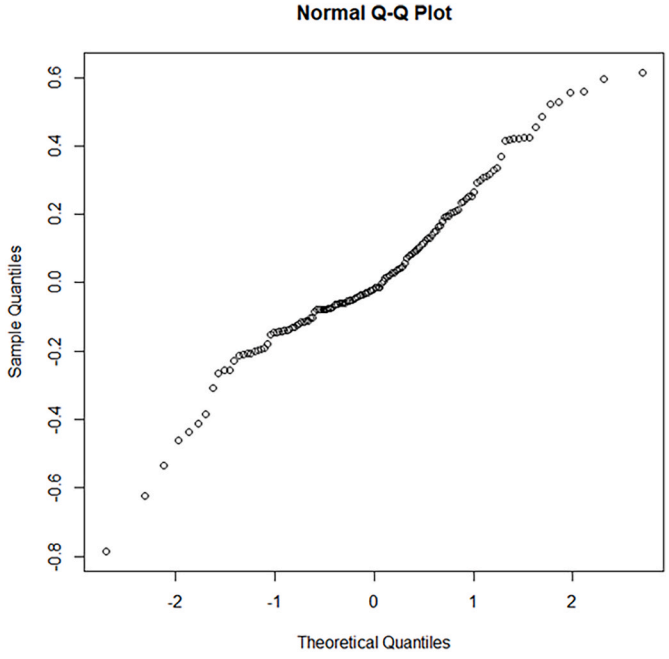

Fig. D4. The QQ-plot of the residuals from sensitivity analysis 3, whereby we applied time-intervals of 24–44 h and 44–64 h instead of applying time-intervals of 24–48 h and 48–72 h as used in the main analysis.

In the main model, we assumed fixed intake amounts. In this sensitivity analysis, random instead of fixed intake amount values were applied using Monte Carlo simulations. Overall, we concluded that the model was not sensitive to this assumption. The Monte Carlo sensitivity analysis produces a QQ-plot for each run, therefore, all of those plots are not presented.

Table D1
Overview of the results from all four sensitivity analyses along with the results of the main analysis.

Analysis	AIC	Population mean	Mean	Median	95 % CI
		f_{abs_excr}	Residence time		

(*continued on next page*)

Table D1 (*continued*)

Analysis	AIC	Population mean	Mean	Median	95 % CI
		f_{abs_excr}	Residence time		
Main	−16.6	0.184	4.9	4.0	1.1–14.3
SA 1	506.5	0.231	5.3	4.7	1.8–12.5
SA 2	10.3	0.181	4.8	3.9	1.1–13.4
SA 3	−8.6	0.181	4.6	3.6	1.0–13.7
SA 4	35.6	0.172	5.7	4.7	5.1–6.2

AIC: akaike information criterium; CI: confidence interval; f_{abs_excr}; excreted fraction; SA: sensitivity analysis.

Appendix E

Table E1
Summary statistics of total HT-2 in urine in male (n = 15) and female participants (n = 25), where 'total' refers to HT-2 and its glucuronides.

	Morning (06:00–12:00 h)	Afternoon (12:00–18:00 h)	Evening (18:00–08:20 h)[a]	Entire day (24 h)
No. samples[b]				
M	14	14	15	43
F	24	25	25	74
% >LOD/LOQ				
M	100/100	100/100	100/100	100/100
F	100/92	96/92	100/100	99/95
Mean ± SD				
ng/mL				
M	0.331 ± 0.191	0.301 ± 0.203	0.269 ± 0.140	0.282 ± 0.136
F	0.183 ± 0.113	0.220 ± 0.220	0.171 ± 0.109	0.180 ± 0.113
ng[c]				
M	210 ± 173	152 ± 112	264 ± 156	603 ± 370
F	108 ± 95.2	95.2 ± 99.4	139 ± 75.7	342 ± 206
Median (ng/mL)				
M	0.276	0.290	0.316	0.260
F	0.163	0.141	0.144	0.134
Range (ng/mL)				
M	0.088–0.667	0.063–0.784	0.068–0.451	0.090–0.476
F	0.021–0.425	0.010–0.980	0.040–0.473	0.078–0.456
Urine volume (mL)				
M	607 ± 274	576 ± 323	995 ± 274	2100 ± 547
F	588 ± 362	549 ± 314	895 ± 281	2032 ± 667
Urine volume (mL/kg bw)				
M	7.26 ± 3.77	6.90 ± 4.44	12.86 ± 3.61	27.02 ± 6.89
F	9.40 ± 5.52	8.67 ± 4.54	14.42 ± 4.78	32.48 ± 9.95

LOD: limit of detection; LOQ: limit of quantification.
[a] The evening time interval was set at 18:00–08:20 h because 08:20 h was the time of the last participants urine void within the 24-h time window.
[b] One afternoon sample was analysed below the LOD and in for the calculation of sum parameters this concentration was set to half the value of the LOD.
[c] The amount of total HT-2 was calculated by multiplying the concentration (ng/mL) by urinary volume (mL).

Data availability

Data will be made available on request.

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