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Exposure assessment of Portuguese population to multiple mycotoxins: The human biomonitoring approach

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ABSTRACT

Mycotoxins constitute a relevant group of food contaminants with several associated health outcomes such as estrogenic, immunotoxic, nephrotoxic and teratogenic effects. Although scarce data are available in Portugal, human biomonitoring studies have been globally developed to assess the exposure to mycotoxins at individual level. In order to overcome this lack of data, the present study concerned the analysis of mycotoxins in 24h urine and first-morning urine paired samples from 94 participants enrolled within the scope of the National Food, Nutrition, and Physical Activity Survey of the Portuguese General Population (2015–2016). Following a salt-assisted matrix extraction, urine samples were analysed by liquid chromatography–mass spectrometry for the simultaneous determination of 37 urinary mycotoxins' biomarkers and data obtained used to estimate the probable daily intake as well as the risk characterization applying the Hazard Quotient approach. Results revealed the exposure of Portuguese population to zearalenone, deoxynivalenol, ochratoxin A, alternariol, citrinin and fumonisin B_1 through the quantification in 24h urine and first-morning urine paired samples. Risk characterization data revealed a potential concern to some reported mycotoxins since the reference intake values were exceeded by some of the considered participants. Alternariol was identified for the first time in urine samples from a European country; however, risk characterization was not performed due to lack of reference intake value. These results confirmed mycotoxins as part of the human exposome of the Portuguese population reinforcing the need for further studies regarding the determinants of exposure.

1. Introduction

Over the last years, Human Biomonitoring (HBM) has been considered a powerful and promising tool to assess the exposure to contaminants from different origins, providing information of aggregated exposures from different routes (oral, dermal, inhalation) and contributing to the establishment of the total body burden of an individual (Zidek et al., 2017). HBM can be defined as a method for assessing the human exposure to chemical compounds or their effects through the systematic standardized measurement of the concentration of those compounds or its metabolites in human specimens (Choi et al., 2015; WHO, 2015). The choice of biomarkers is critical for the quality and pertinence of data to be obtained within a biomonitoring study. Biomarkers of exposure should be valid, feasible and relevant (IPCS/WHO, 2001, 1993). However, as referred by Choi et al. (2015), most of the biomarkers represent a compromise between these criteria. HBM studies allow determining the exposure at individual level and consequently the potential use of these data for risk assessment is of great importance. In the food safety area, exposure assessment is traditionally performed combining consumption and occurrence data. It is known that these methods rely on some assumptions and uncertainties, even when probabilistic approaches are used, and HBM studies may

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Abbreviations

deoxynivalenol DON
deepoxy-deoxynivalenol DOM-1
3-acetyl-deoxynivalenol 3-ADON
15-acetyl-deoxynivalenol 15-ADON
deoxynivalenol-3-glucoside DON-3G
deoxynivalenol-3-glucuronide DON-3-GlcA
deoxynivalenol-15-glucuronide DON-15-GlcA
zearalenone ZEN
zearalanone ZAN
alpha-zearalenol α-ZEL
beta-zearalenol β-ZEL
alpha-zearalanol α-ZAL
beta-zearalanol β-ZAL
alpha-zearalenol-glucuronide α-ZEL-GlcA
beta-zearalenol-glucuronide β-ZEL-GlcA
zearalenone-14-sulphate ZEN-14-Sulf
zearalenone-14-glucuronide ZEN-14-GlcA
ochratoxin A OTA
ochratoxin-alpha OTα
citrinin CIT
T-2 toxin T-2
T-2 triol toxin T-2 triol
T-2 tetraol toxin T-2 tetraol
HT-2 toxin HT-2
fumonisin B1 FB1
fumonisin B2 FB2
fumonisin B3 FB3
hydrolysed-fumonisin B1 HFB1
diacetoxyscirpenol DAS
roquefortine C ROQC
neosolaniol NEO
fusarenon-X FUS-X
sterigmatocystin STER
patulin PAT
alternariol monomethyl ether AME
alternariol AOH
nivalenol NIV
Hazard Quotient HQ
Probable Daily Intake PDI
Human Biomonitoring HBM

overcome some of these constraints (De Boevre et al., 2013; Assunção et al., 2016; de Nijs et al., 2016; Zidek et al., 2017).

Mycotoxins are natural toxins, secondary fungal metabolites, and one of the most relevant contaminants group in terms of chronic toxicity (Bennett and Klich, 2003). Mycotoxins contaminate food commodities in the different stages of field, storage and processing (Marin et al., 2013). They are distributed worldwide and responsible for acute and chronic health effects such as immunotoxic, teratogenic, mutagenic and endocrine disruptive effects, thus constituting a concern from the public health and economic perspectives (De Ruyck et al., 2015; Lorenz et al., 2019; Wu, 2014; Wu et al., 2014). Previous studies reported the occurrence of mycotoxins in food commodities across Europe, indicating that the European population is exposed through food consumption (Brera et al., 2011; De Boevre et al., 2013; Martins et al., 2018; Nathanail et al., 2015; Varga et al., 2013); however, the characteristics of exposure at individual level are not fully unraveled by the traditional assessment methods. Additionally, the exposure through inhalation from indoor and occupational environments should not be neglected (Polizzi et al., 2009; Viegas et al., 2018, 2019). In the last decade, the assessment of mycotoxin exposure in biomonitoring studies increased among research groups following the development of more powerful analytical techniques and allowing the identification of several mycotoxins' metabolites with potential use as biomarkers of exposure (Vidal et al., 2018b).

Mycotoxins comprise a group of compounds with a broad range of biochemical characteristics and subsequent differences in metabolism. Studies on the mycotoxins metabolism are mostly devoted to regulated mycotoxins, the ones presenting more concern from a public health perspective (Marín et al., 2018). Deoxynivalenol (DON) and zearalenone (ZEN) are metabolized through conjugation with glucuronic acid or sulfonation that are important pathways for detoxification (Vidal et al., 2018a; Warth et al., 2013). Ochratoxin A (OTA) presents hydroxylated forms as metabolites, and there is also evidence for the existence of glucuronide forms, but none has been identified so far (Muñoz et al., 2017; Vidal et al., 2018b). For citrinin (CIT), the identified urinary metabolite is dihydro-citrinone (Ali et al., 2015; Degen et al., 2018) that may be used in biomonitoring studies along with citrinin itself. Fumonisins B_1, B_2 and B_3 (FB_1, FB_2, FB_3) are characterized by low absorption, and excretion occurs mainly by faeces with less than 3% recovered in urine (Gambacorta et al., 2013; Souto et al., 2017; Turner et al., 2012). *Alternaria* toxins (alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA)) are emerging toxins and information about their metabolism is still scarce. Puntscher et al. (2019) and Hövelmann et al. (2016) developed some studies to gather data in excretion patterns and evidence of human exposure to these toxins. Besides the identification and quantification of parent toxins, there was evidence of the presence of urinary conjugated metabolites, namely alternariol monomethyl ether-3-sulphate (AME-3-S) (Puntscher et al., 2019). For other than these mycotoxins, some studies have also been developed, however, the knowledge on metabolites and biomarkers is still scarce (Marín et al., 2018).

To date in Portugal, human biomonitoring studies developed to assess the exposure of the Portuguese population to OTA, DON and FB_1 through the analysis of first-morning urine samples revealed an evidence of exposure to OTA and DON (Cunha and Fernandes, 2012; Duarte et al., 2009, 2012; Manique et al., 2008; Pena et al., 2006; Silva et al., 2010). To broaden the scope and to acquire more extensive data on exposure to mycotoxins and characterize the associated risk in Portugal, this study aims i) to present, for the first time, data on a human biomonitoring study developed to assess the exposure of the Portuguese population for 37 mycotoxins' urinary biomarkers determined in 24h urine and first-morning urine samples; ii) to characterize the risk associated to the exposure through the use of reverse dosimetry and hazard quotient approaches; iii) to analyze the differences of urinary mycotoxin' concentrations between the different type of urine samples used for the same participant.

2. Material and methods

2.1. Participants

During the years 2015–2016, the National Food, Nutrition, and Physical Activity Survey of the Portuguese General Population aimed to collect nationwide data on dietary habits (foods, nutrients, dietary supplements, food safety and insecurity), physical activity (sedentary behaviours, sports and active choices in daily living) and their relation with health determinants in Portugal (Lopes et al., 2018). A convenience sample of 95 participants was selected from adult population. One participant was excluded due to problems in biological samples transportation. A convenience sample of 94 adult participants (aged 48.4 ± 15.2 years) was considered including females (n = 46, 48.9%) and males (n = 48, 51.1%). From each participant 24h urine and first-morning urine paired samples were collected following a standardized protocol (Lopes et al., 2017). For these sampling, the following

conditions were considered as exclusion criteria: taking diuretics, pregnancy, lactating, having diabetes or kidney disease, haemophilia or any condition requiring supplemental oxygen, donating blood or plasma during or < 4 weeks before the study, following prescribed dietary therapy and/or having had a urinary tract infection within 1 month of commencing the study.

Ethical approval was obtained from the National Commission for Data Protection (Authorization nº 4940/2015) and the Ethical Committee of the Institute of Public Health of the University of Porto (Decision nº CE 16053). All participants provided their written informed consent according to the Ethical Principles for Medical Research involving human subjects expressed in the Declaration of Helsinki and the national legislation. Data collection was performed under pseudo-anonymization, and all documents with identification data were treated, and stored in a different dataset (Lopes et al., 2018).

2.2. Chemicals and reagents

Mycotoxin standards nivalenol (NIV), DON, deepoxy-deoxynivalenol (DOM1), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), deoxynivalenol-3-glucoside (DON-3G), CIT, OTA, ochratoxin alpha (OTα), T-2 toxin (T-2), T-2 triol toxin (T-2 triol), T-2 tetraol toxin (T-2 tetraol), HT-2 toxin (HT-2), FB_1, FB_2, FB_3, hydrolysed fumonisin B_1 (HFB_1), ZEN, zearalanone (ZAN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), diacetoxyscirpenol (DAS), roquefortin C (ROQC), neosolaniol (NEO), fusarenon-X (FUS-X), sterigmatocystin (STER), patulin (PAT), AME, AOH and isotope-labelled internal standards ($^{13}C_{15}$) deoxynivalenol ($^{13}C_{15}$ DON), isotope-labelled ($^{13}C_{15}$) zearalenone ($^{13}C_{15}$ ZEN), isotope-labelled ($^{13}C_{15}$) fumonisin B_1 ($^{13}C_{15}$ FB_1) and isotope-labelled ($^{13}C_{15}$) OTA ($^{13}C_{15}$ OTA) were purchased from Sigma Aldrich (Bornem, Belgium). Mycotoxins biomarkers standards (deoxynivalenol-

Table 1
Optimized MS and MS/MS parameters for biomarkers of exposure to mycotoxins (n = 37) and internal standards (n = 4).

Analyte	Retention time (min)	Q1 (mz^{-1})	CV (v)	Q2 (mz^{-1})	CE	Dwell	ESI-source
					(v)	(msec)	
DON	3.24	297.0	40	231.0/249.0[a]	9/9	0.130	+
DOM-1	4.28	281.1	40	215.1/233.1[a]	9/9	0.011	+
DON-3G	3.02	476.1	15	249.0/297.0[a]	18/12	0.110	+
3-ADON	5.26	339.0	15	213.1/231.1[a]	9/15	0.080	+
15-ADON	5.26	339.0	15	137.1[a]/261.1	9/15	0.080	+
DON-3-GlcA	2.65	471.0	60	113.0/193.0[a]	30/24	0.080	–
DON-15-GlcA	2.78	471.0	60	113.0/193.0[a]	30/24	0.080	–
$^{13}C_{15}$ DON	3.24	311.9	30	103.4/245.2[a]	10/10	0.110	+
ZEN	9.67	319.0	27	283.1[a]/301.1	19/20	0.023	+
ZAN	9.52	321.0	40	189.1/303.3[a]	12/18	0.023	+
α-ZEL	9.33	321.2	30	175.1/177.0	22/17	0.023	+
β-ZEL	8.05	321.5	30	177.2[a]/189.1	15/20	0.023	+
α-ZEL-GlcA	6.01	481.3	50	293.2[a]/301.2	30/34	0.023	–
β-ZEL-GlcA	5.6	481.1	26	275.1[a]/319.1	34/14	0.023	–
α-ZAL	8.99	323.2	30	123.0/305.1[a]	23/23	0.080	+
β-ZAL	8.12	323.2	30	189.1/305.1[a]	23/23	0.080	–
ZEN-14-GlcA	7.12	498.4	16	283.2/319.1[a]	28/14	0.023	+
ZEN-14-Sulf	6.25	397.0	32	175.0/317.0[a]	34/22	0.023	–
$^{13}C_{15}$ ZEN	9.67	337.3	40	199.1[a]/214.9	22/22	0.023	+
OTA	9.37	403.9	40	238.9[a]/358.0	22/12	0.023	+
OTα	5.82	257.0	30	221.1[a]/239.1	20/10	0.023	+
$^{13}C_{15}$ OTA	9.37	424.1	30	250.2/360.1[a]	23/17	0.023	+
CIT	7.02	281.0	60	205.0/249.0[a]	25/15	0.029	–
FB_1	8.31	722.2	40	334.2[a]/352.1	36/32	0.023	+
FB_2	10.49	706.1	70	336.2/354.2[a]	36/30	0.230	+
FB_3	9.38	706.2	70	354.3[a]/530.2	30/28	0.023	+
HFB_1	8.01	406.4	20	254.3/334.3[a]	20/20	0.023	+
$^{13}C_{15}$ FB1	8.31	756.4	40	356.2/374.2[a]	36/32	0.023	+
AOH	8.24	258.9	40	185.1[a]/213.1	30/26	0.023	+
AME	10.29	272.9	57	199.3/258.2[a]	30/26	0.023	+
NIV	2.09	313	35	175.0/177.0[a]	21/16	0.321	+
FUS-X	5.27	355	16	137.1/247.1[a]	21/12	0.080	+
DAS	7.01	384.1	35	247.1/307.1[a]	12/9	0.029	+
ROQC	8.17	390.1	25	193.0[a]/322.0	24/24	0.003	+
NEO	5.44	400	30	215.0[a]/305.0	12/9	0.080	+
T-2	8.80	484.32	40	215.2[a]/305.2	18/12	0.023	+
HT-2	7.99	447	40	285.0/345.0[a]	12/12	0.058	+
T-2 triol	7.19	405.2	30	125.2/303.1[a]	14/30	0.029	+
T-2 tetraol	6.95	299.15	30	173.2/175.2[a]	18/15	0.230	+
PAT	2.21	152.94	40	81.0/108.8[a]	14/10	0.321	–
STER	10.28	325.0	40	281.0[a]/310.0	24/30	0.023	+

DON = Deoxynivalenol; DOM-1 = deepoxy-deoxynivalenol; 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; DON-3-GlcA = deoxynivalenol-3-glucuronide; DON-15-GlcA = deoxynivalenol-15-glucuronide; DON-$^{13}C_{15}$ = isotope-labelled ($^{13}C_{15}$) deoxynivalenol; ZEN = zearalenone; ZAN = zearalanone; α-ZEL = alpha-zearalenol; β-ZEL = beta-zearalenol; α-ZAL = alpha-zearalanol; β-ZAL = beta-zearalanol; α-ZEL-GlcA = alpha-zearalenol-glucuronide; β-ZEL-GlcA = beta-zearalenol-glucuronide; ZEN-14-Sulf = zearalenone-14-sulphate; ZEN-14-GlcA = zearalenone-14-glucuronide; ZEN-$^{13}C_{15}$ = isotope-labelled ($^{13}C_{15}$) zearalenone; OTA = ochratoxin A; OTα = ochratoxin-alpha; OTA-$^{13}C_{15}$ = isotope-labelled ($^{13}C_{15}$) ochratoxin A; CIT = citrinin; T-2 = T-2 toxin; T-2 triol = T-2 triol toxin; T-2 tetraol = T-2 tetraol toxin; HT-2 = HT-2 toxin; FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; FB_3 = fumonisin B_3; HFB_1 = hydrolysed-fumonisin B_1; FB1–$^{13}C_{15}$ = isotope-labelled ($^{13}C_{15}$) fumonisin B_1; DAS = Diacetoxyscirpenol; ROQC = roquefortine C; NEO = neosolaniol; FUS-X = Fusarenon-X; STER = Sterigmatoscystin; PAT = Patulin; AME = Alternariol monomethyl ether; AOH = Alternariol, NIV = Nivalenol.
[a] Quantifier transition.

3-glucuronide (DON-3-GlcA), deoxynivalenol-15-glucuronide (DON-15-GlcA), α-zearalenol-glucuronide (α-ZEL-GlcA), β-zearalenol-glucuronide (β-ZEL-GlcA), zearalenone-14-sulphate (ZEN-14-Sulf), zearalenone-14-glucuronide (ZEN-14-GlcA)) were kindly supplied by Dr. Huybrechts (Sciensano, Tervuren, Belgium). Mycotoxin solid standards were dissolved in methanol or acetonitrile according to the respective certificate of analysis, and were stored at −18 °C. The working solutions were prepared in methanol, and stored at −18 °C. Ultrapure water was produced on a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol (MeOH) (LC-MS grade), acetonitrile, formic acid (99%) and acetic acid (99.5%) were purchased from BioSolve (Valkenswaard, The Netherlands). Magnesium sulphate anhydrous (99.3%) and sodium chloride (> 99.9%) were supplied by VWR Chemicals (Radnor, Pennsylvania, US).

2.3. Sample preparation

A QuEChERS-based procedure (Quick, Easy, Cheap, Effective, Rugged, Safe) was used for sample preparation. The method is according to Vidal et al. (2018a). Briefly, the urines were allowed to acclimatise to room temperature, and 2 mL of urine sample were spiked with 10 µL of internal standards, and mixed with 18 mL of acetonitrile/water/formic acid (52/45/3, v/v/v) in a 50 mL centrifuge tube. Thereafter, 4 g of magnesium sulphate and 1 g of sodium chloride were added into the extraction tube and the mixture was vigorously hand and vortex shaken. Samples were shaken for 30 min on a rotary shaker. After centrifugation (4000 *g*, 6 min), 5 mL of the organic layer were taken, and evaporated to dryness under a gentle nitrogen air at 40 °C. The residue obtained was dissolved in 0.5 mL of injection solvent (H_2O/MeOH, 85/15, v/v), filtered (0.22 µm, PVDF, Durapore®, Cork, Ireland) and transferred to a UPLC vial upon LC-MS/MS analysis.

2.4. Instrumental analysis

Sample analysis was performed in a Waters® Acquity UPLC system coupled to a Quattro XEVO TQS mass spectrometer (Waters®, Manchester, UK). The software used for data acquisition and processing was MassLynx™ version 4.1 and QuanLynx® version 4.1 (Waters®, Manchester, UK). Separation of analytes was carried out by a HSS T-3 column (2.1 × 100 mm, 1.8 µm) (Waters®, Manchester, UK). Two different mobile phases were used, mobile phase A (water/methanol/acetic acid; 94/5/1, v/v/v) and mobile phase B (methanol/water/acetic acid; 97/2/1, v/v/v), both buffered with 5 mM ammonium acetate, that were set at a flow rate of 0.3 mL/min. The total run time was 18 min. Five µL of sample was injected into the UPLC system (Vidal et al., 2018a). The gradient started at 95% A, decreasing to 35% A until 7 min, followed by a decrease to 25% A until 11 min. Mobile phase B increased to 99% until 13 min, kept in the same conditions until 14.10 min, and immediately changed to initial conditions (95% mobile phase A) and allowed to equilibrate until 18 min. The ESI-source was operated both in negative and positive mode (ESI^- and ESI^+), and parameters were optimized and programmed for all measurements as follows: source and desolvation temperatures were 150 °C and 200 °C, respectively; the capillary voltage was 30 kV and nitrogen was applied as spray gas. The argon collision gas pressure was 9×10^{-6} bar, the cone gas and desolvation gas flow were 150 and 550 L/h, respectively. A number of two characteristic Multiple Reactions Monitoring (MRM) transitions with a specific dwell time per analyte were monitored to ensure accurate identification (Table 1).

2.5. Validation experiments

An in-house validation was conducted following EU Commission Decision 2002/657/EC (European Commission, 2002). The parameters investigated included limit of detection (LOD), limit of quantification (LOQ), intra and inter-day precision expressed as relative standard deviation (RSD), apparent recovery and linearity. Validation assays were performed in triplicate during four different working days. Matrix-matched calibration plots were constructed and internal standards were used in the multi-mycotoxin analysis. Evaluating the linearity, the homogeneity of variance was checked before fitting the linear model. The linearity was interpreted graphically using a scatter plot. Limit of detection (LOD) was calculated as three times the standard error of the intercept, divided by the slope of the standard curve; the limit of quantification (LOQ) was similar, differing by six times the standard error. The calculated LOD and LOQ were verified by the signal-to-noise ratio (s/n), which should be more than 3 and 10, respectively according to the IUPAC guidelines (Todd, 1995).

2.6. Probable daily intake and risk characterization

In order to assess the risk associated to the determined exposure levels, a probable daily intake (PDI) of mycotoxins was estimated, assuming that consumption of contaminated food constituted the main source of exposure. PDI for each mycotoxin was determined through reverse dosimetry calculation in order to convert the urinary mycotoxin concentrations into intake levels, expressed as µg/kg bw/day. The deterministic method of intake mass balance was applied, considering the concentration of biomarker in urine (µg/L), the urinary volume produced in 24h (L), the body weight (kg), the excretion rate for each mycotoxin (%) and the mass balance between the parent compound and the metabolite (de Nijs et al., 2016; Horvat et al., 2017; Steckling et al., 2018) (Supplementary file S1). All the data regarding the participants (body weight, urinary biomarkers concentration and urinary volume in 24h) were considered at individual level.

Regarding excretion rates, available data was used to estimate PDI: 64.0% (Vidal et al., 2018a) and 72.3% (Turner et al., 2010) for DON; 0.075% (Van Der Westhuizen et al., 2011) and 0.5% (Riley et al., 2012) for FB_1; 9.6% for ZEN (Warth et al., 2013), 2.5% for OTA (Studer-Rohr et al., 2000), 14.75% for CIT (Degen et al., 2018), and 8.3% for AOH (Puntscher et al., 2019).

Risk characterization was performed comparing the estimated PDI for each mycotoxin with the correspondent health-based guidance values, when available, to determine the respective Hazard Quotient (HQ). When the HQ was < 1, the exposure was considered to be within safe limits (Assunção et al., 2018, 2015; EFSA, 2013). The estimated PDI was compared with the Tolerable Daily Intake (TDI) for DON (group TDI of DON, 3-ADON, 15-ADON and DON-3G; 1.0 µg/kg bw/day) (EFSA, 2017), ZEN (group TDI for ZEN and metabolites; 0.25 µg/kg bw/day) (EFSA, 2016), FB_1 (group TDI for FB_1, FB_2, FB_3 and FB_4; 1.0 µg/kg bw/day) (EFSA, 2018) and for CIT (0.2 µg/kg bw/day) (EFSA, 2012a), and the Provisional Tolerable Weekly Intake (PTWI) for OTA (0.120 µg/kg bw/week) (EFSA, 2006).

2.7. Statistical analysis

The results of the biomarkers of exposure were presented as volume-weighted concentrations (µg/L), creatinine adjusted concentrations (µg biomarker/g crea) and as daily excretion for determinations in 24h urine (µg biomarker/day). Samples were considered positive for exposure to mycotoxins if at least one biomarker of exposure was determined in concentrations above the respective LOD.

Regarding the data treatment of non-detects (< LOD), and for biomarkers that presented more than 10% of positive samples, a multiple imputation based on linear regression was performed, based on 20 simulations, with a maximum of 100,000 draws set for case and parameters, to impute a value between zero and the respective LOD. For each result < LOD a mean value from the imputed values (n = 20) was determined. This approach keeps variability in the < LOD results, which is considered an advantage when compared with the substitution by a fixed value approach (LOD, ½ LOD or zero) (Vrijheid et al., 2017) and it is in agreement with the HBM4EU project (www.hbm4eu.eu) on

how to deal with left-censored data.

Descriptive statistics were performed (median, maximum and percentiles 90 and 95) in the data sets after a multiple imputation process. The normality of distributions was checked with the Shapiro-Wilk test, and since all were not following a normal distribution, non-parametric tests were used for further statistical analysis. A correlation among continuous variables (biomarkers results) was determined with the Spearman correlation coefficient (r_s) (≤ 0.2 = poor; $0.2 \leq 0.5$ = fair; $0.5 \leq 0.7$ = moderate; $0.7 \leq 0.9$ = very strong) (Akoglu, 2018; Chan, 2003). Datasets were analysed regarding differences between concentrations of biomarkers for 24h urine and first-morning urine samples (two related samples, Wilcoxon test) and between glucuronidation rates for male/female (independent samples, Mann-Whitney test). For those biomarkers (CIT and FB_1 in 24h urine samples; α-ZEL, DON-3G, CIT, FB_1 in first-morning urine samples) that presented more than 90% of negative samples, descriptive statistics were only performed for positive samples.

3. Results and discussion

3.1. Analytical validation

The results of the performance characteristics of the LC-MS/MS method, that allowed the identification and quantification of 37 mycotoxin biomarkers of exposure, were in agreement with the criteria mentioned in European Commission Decision (2002)/657/EC, and are presented in Table 2. All analytes presented good linear responses, apparent recovery ranged from 85.9% to 111.3%, and maximum inter-day precision and uncertainty were 15.9% and 20.8%. Thus, the analytical method was considered as fit for purpose.

3.2. Exposure to multiple mycotoxins

The analysis of biological samples from 94 participants revealed the presence of 11 and 12 out of 37 mycotoxin biomarkers of exposure in 24h urine and first-morning urine samples, respectively. NIV, 3-ADON, 15-ADON, OTα, T-2, T-2 triol, T-2 tetraol, HT-2, FB_2, FB_3, HFB_1, ZAN, β-ZEL, α-ZAL, β-ZAL, α-ZEL-GlcA, β-ZEL-GlcA, ZEN-14-Sulf, DAS,

Table 2
Performance characteristics of LC-MS/MS analytical method.

Analyte	Calibration range	R^2	LOD	LOQ	Apparent Recovery	RSD_r	RSD_R	SE	U (%)
	(μg/L)		(μg/L)	(μg/L)	(%)	(%)	(%)		
DON	0.50–25.00	0.99	0.5	1.0	103.3	5.5	6.6	7.4	14.8
DOM-1	0.50–25.00	0.99	0.3	0.5	101.3	2.8	4.5	0.5	10.0
DON-3G	0.50–25.00	0.99	0.5	1.1	97.8	0.9	3.7	8.7	5.3
3-ADON	0.50–25.00	0.99	0.4	0.8	94.6	5.6	8.4	10.3	12.8
15-ADON	0.50–25.00	0.99	0.4	0.8	108.4	7.4	9.2	10.1	13.9
DON-3-GlcA	0.50–25.00	0.99	0.5	1.0	111.3	7.2	10.1	9.9	20.8
DON-15-GlcA	0.50–25.00	0.99	0.5	0.9	108.6	8.1	8.4	10.5	17.6
ZEN	0.50–25.00	0.99	0.20	0.8	106.5	8.4	9.5	7.2	10.1
ZAN	0.50–25.00	0.99	0.15	0.32	102.1	9.1	9.9	8.6	10.3
α-ZEL	0.50–25.00	0.99	0.61	1.4	98.5	11.3	14.8	10.2	15.9
β-ZEL	0.50–25.00	0.99	0.91	2.1	89.2	12.6	13.7	11.0	15.2
α-ZEL-GlcA	0.50–25.00	0.99	1.40	3.0	102.1	8.4	9.5	12.3	11.0
β-ZEL-GlcA	0.50–25.00	0.99	1.72	3.5	109.6	14.2	15.8	13.4	17.5
α-ZAL	0.50–25.00	0.99	1.12	3.8	94.0	10.8	12.6	9.0	14.2
β-ZAL	0.50–25.00	0.99	1.6	4.2	90.7	9.3	10.7	8.5	12.4
ZEN-14-GlcA	0.50–25.00	0.99	0.3	1.3	92.3	6.2	7.5	5.9	9.2
ZEN-14-Sulf	0.50–25.00	0.99	2.6	4.9	97.2	11.0	13.5	10.5	14.6
OTA	0.05–2.50	0.99	0.01	0.02	104.5	9.2	13.0	5.0	15.9
OTα	0.05–2.50	0.99	0.01	0.02	94.2	14.2	15.6	9.4	18.2
CIT	1.00–50.00	0.99	0.5	1.0	103.2	9.5	12.6	7.4	14.2
FB_1	0.25–12.50	0.99	0.21	0.42	92.1	9.4	11.2	10.7	13.2
FB_2	0.25–12.50	0.99	0.18	0.36	102.7	6.3	7.8	5.1	8.9
FB_3	0.25–12.50	0.99	0.18	0.37	103.2	6.6	8.5	7.2	10.8
HFB_1	0.25–12.50	0.99	0.20	0.42	98.4	9.2	10.1	8.9	11.2
AOH	1.00–50.00	0.99	0.4	0.9	89.7	5.9	7.8	5.2	10.6
AME	1.00–50.00	0.99	0.5	1.0	87.0	8.2	9.6	6.2	12.4
NIV	0.50–25.00	0.99	0.2	0.4	105.9	4.5	7.8	6.1	8.3
FUS-X	0.50–25.00	0.99	0.4	0.8	99.5	10.2	11.0	7.5	12.9
DAS	1.00–50.00	0.99	0.8	1.5	87.3	11.0	12.4	9.7	13.1
ROQC	1.00–50.00	0.99	0.6	1.2	95.1	12.1	14.8	12.6	16.0
NEO	0.50–25.00	0.99	0.6	1.2	109.2	8.8	9.7	7.2	12.1
T-2	0.50–25.00	0.99	0.1	0.2	97.4	4.0	5.8	5.2	8.0
HT-2	0.50–25.00	0.99	0.2	0.4	110.0	12.3	14.5	11.8	16.8
T-2 triol	0.50–25.00	0.99	0.3	0.6	104.8	13.8	15.9	15.2	17.6
T-2 tetraol	0.50–25.00	0.99	0.4	0.8	98.2	12.9	14.6	15.0	17.4
PAT	1.00–50.00	0.99	0.5	1.0	85.9	7.2	9.3	8.2	11.7
STER	0.05–2.50	0.99	0.02	0.05	100.2	4.2	5.9	5.2	7.0

LOD = Limit of detection. LOQ = Limit of quantification. SE = Standard error of mean. RSD_r = relative standard deviation intra-day precision. RSD_R = relative standard deviation inter-day precision. U = measurement uncertainty. DON = Deoxynivalenol; DOM-1 = deepoxy-deoxynivalenol; 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; DON-3-GlcA = deoxynivalenol-3-glucuronide; DON-15-GlcA = deoxynivalenol-15-glucuronide; ZEN = zearalenone; ZAN = zearalenone; α-ZEL = alpha-zearalenol; β-ZEL = beta-zearalenol; α-ZAL = alpha-zearalanol; β-ZAL = beta-zearalanol; α-ZEL-GlcA = alpha-zearalenol-glucuronide; β-ZEL-GlcA = beta-zearalenol-glucuronide; ZEN-14-Sulf = zearalenone-14-sulphate; ZEN-14-GlcA = zearalenone-14-glucuronide; OTA = ochratoxin A; Otα = ochratoxin-alpha; CIT = citrinin; T-2 = toxin T-2; T-2 triol = toxin T-2 triol; T-2 tetraol = toxin T-2 tetraol; HT-2 = toxin HT-2; FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; FB_3 = fumonisin B_3; HFB_1 = hydrolysed -fumonisin B_1; DAS = Diacetoxyscirpenol; ROQC = roquefortine C; NEO = neosalinol; FUS-X = Fusarenon-X; STER = Sterigmatoscystin; PAT = Patulin; AME = Alternariol monomethyl ether; AOH = Alternariol, NIV = Nivalenol.

ROQC, NEO, FUS-X, STER, PAT, and AME were not detected in any of the analysed urine samples. Overall, only five (5.3%) 24h urine samples and 11 (11.7%) first-morning urine samples were negative for all the biomarkers determined in the present study. Table 3 presents the results of mycotoxin' biomarkers of exposure determined in the biological samples (24h urine and first-morning urine) of the 94 participants.

For the first time, within a human biomonitoring study, results confirmed the exposure of the Portuguese population to DON, ZEN, AOH, OTA, FB_1 and CIT, with detection and quantification of these mycotoxins and/or their metabolites in 24h and first-morning urine samples.

DON and its metabolites (DOM-1, DON-15-GlcA and DON-3-GlcA) were the most frequently detected biomarkers in 24h urine samples with 63% (DON), 41% (DOM-1), 52% (DON-15-GlcA), 44% (DON-3-GlcA) of positive samples. If considering DON and metabolites, 78% of participants were exposed to DON. In this study, it was also possible to confirm the exposure to DON-3G, with 20% of samples being positive for this modified mycotoxin. In first-morning urine samples, DON and metabolites were the second most detected biomarkers (DON 30%; DOM-1 32%; DON-3G 11%; DON-3-GlcA 24%; DON-15-GlcA 39%), confirming the results obtained for the 24h urine samples. The exposure of the Portuguese population to DON and DON-3G is confirmed for the first time, being DON identified within 37 analysed mycotoxins as one of the most prevalent. These results are in line with the results obtained from other human biomonitoring studies in Europe where DON and its metabolites were reported as one of the most frequent mycotoxins' biomarkers detected. These studies revealed the presence of DON and metabolites in 96% (DON 10.32 μg/L), 63% (DON 3.37 μg/L), 70–100% (DON 1.7 μg/L; DON-3-GlcA 4.4 μg/L; DON-15-GlcA 31.2 μg/L), 76% (total DON 5.3 μg/L), 93% (total DON 11.3 μg/L), 99% (total DON 4.9 μg/L), 74–91% (DON 2.1 μg/L; DON-3-GlcA 36.0 μg/L) of first-morning urine samples collected in Southern Italy (Solfrizzo et al., 2014), Sweden (Wallin et al., 2015), Belgium (Heyndrickx et al., 2015), Italy, United Kingdom (UK) and Norway (Brera et al., 2015) and Spain (Vidal et al., 2016), respectively. Turner et al. (2008) reported the presence of DON in 98.7% (8.9 μg/g crea) of 24h urine samples collected in the scope of a national survey in UK. Although reporting a similar frequency of positive samples for DON and metabolites, the concentrations reported in the present study are lower than those reported in the referred studies, meaning that the exposure of the

Table 3
Biomarkers of exposure to mycotoxins determined in paired urine samples of 94 participants in the National Food, Nutrition, and Physical Activity Survey of the Portuguese General Population (2015–2016). Results are presented as biomarker volume-weighted concentration (μg/L), biomarker creatinine adjusted concentration (μg biomarker/g crea) and as biomarker daily excretion when applicable (μg biomarker/day). Correlations (Spearman's coefficient) between biomarkers results for 24h urine and first-morning urine samples are presented for DON, ZEN, AOH and OTA.

Biomarkers	24h Urine (24U)							First-morning Urine (FMU)						Correlation coefficients 24U/FMU
	units	Median	Max	P90	P95	> LOD (n; %)	> LOQ (n; %)	Median	Max	P90	P95	> LOD (n; %)	> LOQ (n; %)	
DON	μg/L	2.51	36.31	8.72	16.80	59; 63%	59; 63%	0.38	9.40	2.95	5.35	28; 30%	23; 24%	0.318[a]
	μg/g crea	2.24	29.86	8.66	11.02			0.48	5.19	2.05	2.80			0.167
	μg/day	3.51	30.90	10.38	13.20			NA						–
DOM-1	μg/L	0.24	5.13	2.86	4.05	39; 41%	37; 39%	0.23	4.00	1.90	3.08	30; 32%	27; 28%	0.149
	μg/g crea	0.36	7.81	2.64	4.46			0.26	6.85	1.65	2.94			0.289
	μg/day	0.40	7.53	3.48	5.42			NA						–
DON-3G[b]	μg/L	0.34	2.09	0.84	1.20	19; 20%	7; 7%	0.75	2.70	2.58	–	10; 11%	2; 2%	–
	μg/g crea	0.37	1.70	0.93	1.10			0.58	1.91	1.82	–			–
	μg/day	0.46	2.36	1.07	1.51			NA						–
DON-3-GlcA	μg/L	0.33	34.67	11.78	18.53	41; 44%	35; 37%	0.25	10.30	2.05	15.34	23; 24%	18; 19%	0.367[a]
	μg/g crea	0.46	25.39	6.86	14.45			0.24	8.25	1.63	2.43			0.339[a]
	μg/day	0.49	37.91	10.15	17.59			NA						–
DON-15-GlcA	μg/L	1.73	204.17	53.76	76.61	49; 52%	48; 51%	0.39	36.48	15.34	22.28	37; 39%	36; 38%	0.168
	μg/g crea	1.32	167.90	48.56	63.26			0.64	25.78	10.77	17.23			0.251[a]
	μg/day	1.66	173.73	52.07	94.62			NA						–
ZEN	μg/L	0.17	3.98	1.51	2.93	45; 48%	24; 26%	1.30	11.51	3.05	3.85	54; 57%	50; 53%	0.166
	μg/g crea	0.28	7.59	1.76	3.28			1.01	8.28	3.72	5.38			0.293[a]
	μg/day	0.33	9.31	2.04	2.93			NA						–
ZEN-14-GlcA	μg/L	0.17	25.70	5.13	8.78	15; 16%	15; 16%	0.15	25.7	2.85	7.55	15; 16%	14; 15%	−0.010
	μg/g crea	0.18	39.78	3.19	9.10			0.13	12.42	4.17	6.62			0.240[a]
	μg/day	0.25	42.30	6.02	13.84			NA						–
α-ZEL[b]	μg/L	ND				0; 0%	0; 0%	2.70	8.60	–	–	5; 5%	4; 4%	–
	μg/g crea							2.14	6.14	–	–			–
	μg/day							NA						–
OTA	μg/L	0.006	1.23	0.045	0.060	17; 18%	16; 17%	0.007	0.610	0.065	0.083	25; 27%	23; 24%	0.073
	μg/g crea	0.006	1.37	0.038	0.060			0.008	1.900	0.046	0.062			0.342[a]
	μg/day	0.009	0.837	0.044	0.084			NA						–
AOH	μg/L	0.28	24.55	3.81	6.73	27; 29%	22; 23%	0.21	9.91	1.05	2.40	12; 13%	11; 12%	0.232[a]
	μg/g crea	0.37	16.94	2.20	9.28			0.18	4.28	0.72	2.32			0.349[a]
	μg/day	0.45	16.68	3.03	10.66			NA						–
CIT[b]	μg/L	0.85	1.20	–	–	2; 2%	1; 1%	0.75	1.00	–	–	2; 2%	0; 0%	–
	μg/g crea	1.42	2.22	–	–			1.03	1.75	–	–			–
	μg/day	1.26	1.93	–	–			NA						–
FB_1[b]	μg/L	0.33	0.48	–	–	7; 7%	2; 2%	0.24	0.53	–	–	3; 3%	1; 1%	–
	μg/g crea	0.24	0.31	–	–			0.14	0.42	–	–			–
	μg/day	0.47	0.56	–	–			NA						–

[a] Correlations with statistical significance ($p < 0.05$).

[b] Descriptive statistics were only performed for positive samples; NA = Not applicable; ND = Not Detected; DON = Deoxynivalenol; DOM-1 = deepoxy-deoxynivalenol; DON-3G = deoxynivalenol-3-glucoside; DON-3-GlcA = deoxynivalenol-3-glucuronide; DON-15-GlcA = deoxynivalenol-15-glucuronide; ZEN = zearalenone; α-ZEL = alpha-zearalenol; ZEN-14-GlcA = zearalenone-14-glucuronide; OTA = ochratoxin A; CIT = citrinin; FB_1 = fumonisin B_1; AOH = Alternariol, NIV; LOD = Limit of Detection; LOQ = Limit of Quantification.

Portuguese population to DON is probably lower than in other European countries. The presence of DOM-1 was also referred by Brera et al. (2015) (3.2–14.8%; 0.03 μg/g crea), Wallin et al. (2015) (8%; 0.18 μg/L) and Vidal et al. (2016) (96%; 4.9 μg/L), with median concentrations similar to our study (0.23 μg/L). Vidal et al. (2016) reported the presence of DON-3G in first-morning urine samples in higher frequency (74%; 1.6 μg/L) but in similar concentrations. Correlation between concentrations of DON and metabolites in 24h and first-morning urine samples was fair and significant for DON ($r_s = 0.318$, $p = 0.002$) and DON-3-GlcA ($r_s = 0.367$; $p = 0.000$). For DON-15-GlcA the correlation determined was poor ($r_s = 0.168$) without statistical significance. Statistical significant differences between volume-weighted concentrations of biomarkers in 24h urine and first-morning urine samples were determined for DON ($p = 0.000$), DON-3-GlcA ($p = 0.000$), DON-15-GlcA ($p = 0.000$) and DOM-1 ($p = 0.047$). Similar results were observed when comparing creatinine-adjusted biomarker concentrations (data not shown). This highlights that the type of urine sample collected for a biomonitoring study may influence concentrations of DON and metabolites. The intervention study on human metabolism of DON and DON-3G reported by Vidal et al. (2018a) referred that excretion occurs within 24h, and a large amount of the total DON was excreted in the first hours (< 6h), meaning that if urine samples are collected only in the morning or in a random sampling during the day, they will mainly represent the DON and DON-3G intake from the last 6h (Vidal et al., 2018a). Results obtained in the present study showed the importance of urine sampling to assess DON exposure. The concentration of DON and metabolites had lower concentrations in the first-morning urines than in 24h urines suggesting that morning urines mainly reflect the DON exposure from previous dinner (usually the last meal before morning sample collection) and is not representative of the overall daily exposure.

ZEN was the second most frequent detected mycotoxin with 48% of positive 24h urine samples. In first-morning urine samples, ZEN was the most frequent detected mycotoxin (57%). Regarding the metabolites, ZEN-14-GlcA was detected in the same proportion for 24h urine and first-morning urine samples, and α-ZEL was only detected in 5% of first-morning urine samples. The presence of α-ZEL and ZEN-14-GlcA in analysed urine samples agreed with previously reported *in vitro* studies which observed that α-ZEL is the main phase I metabolite and ZEN-14-GlcA is the main phase II metabolite produced by human liver cells (Yang et al., 2017). Statistically significant differences ($p < 0.05$) with poor and fair correlation were observed for ZEN and ZEN-14-GlcA concentrations in 24h urine and first-morning urine samples, respectively. When comparing creatinine-adjusted biomarker concentrations, the correlations between concentrations in 24h urine and first-morning urine samples were higher for ZEN ($r_s = 0.293$; $p = 0.004$) and ZEN-14-GlcA ($r_s = 0.240$; $p = 0.02$). These results allow hypothesizing that exposure assessment from urinary biomarkers of ZEN and other mycotoxins like DON characterized by a fast excretion are influenced by the type of urine sample chosen. The excretion of DON and ZEN it is possibly better represented with the collection of 24h urine samples; however the dilution effect derived from collection of a whole-day urinary volume may influence the results obtained. The presence of ZEN and metabolites have been reported in previous biomonitoring studies developed across Europe (Ali and Degen, 2018; Heyndrickx et al., 2015; Solfrizzo et al., 2014; Wallin et al., 2015). Rodríguez-Carrasco et al. (2014) did not find positive urine samples for ZEN in Spain; however, the LOD reported is about 10-fold higher than the LOD reported here, which may explain the absence of positive samples in a neighbouring country. In Belgium less than 1% of urine samples revealed positive results, however it should be noted that it was possible to detect β-ZEL-14-GlcA (α-ZEL 5.0 μg/L; β-ZEL-14-GlcA 0.8 μg/L) (Heyndrickx et al., 2015). Contrary, higher percentages of positive samples were reported in studies developed in Italy (ZEN 100%, 0.056 μg/L; α-ZEL 100%, 0.074 μg/L; β-ZEL 98%, 0.088 μg/L), Sweden (ZEN 37%, 0.03 μg/L; α-ZEL 21%, 0.03 μg/L; β-ZEL 18%, 0.02 μg/L) and Germany (ZEN 100%, 0.07 μg/L; α-ZEL 100%, 0.13 μg/L; β-ZEL 100%, 0.03 μg/L) (Solfrizzo et al., 2014; Wallin et al., 2015; Ali and Degen, 2018). However, it should be highlighted that in the studies developed in Italy, Sweden and Germany, the analytical determinations were preceded by enzymatic hydrolysis with β-glucuronidase, which may partially explain the higher number of positive samples in these studies.

OTA was detected in 18% and 27% of 24h urine and first-morning urine samples, respectively, confirming the exposure of the Portuguese population to this mycotoxin. Correlation between OTA levels in 24h urine and first-morning urine samples was poor ($r_s = 0.073$, $p = 0.482$) for volume-weighted concentrations and fair ($r_s = 0.342$, $p = 0.001$) for creatinine adjusted concentrations. Results from 24h urine and first-morning urine samples presented statistical significant differences ($p = 0.000$). Since OTA is characterized by a slow excretion (Studer-Rohr et al., 2000) it wouldn't be expectable to have such differences between concentrations in 24h urine or first-morning urines. The range of concentrations determined in first-morning urine samples (0.007–0.610 μg/L) agrees with those observed in previous studies assessing exposure of Portuguese population to OTA (0.008–0.208 μg/L, first-morning or spot urine samples) (Duarte et al., 2010, 2012; 2009; Manique et al., 2008; Pena et al., 2006). However, the percentage of positive samples is higher in the previous reports than in the present study being important to assess the determinants of these exposures as a cause of these differences. When comparing the present data with other biomonitoring studies developed in Europe, it is possible to observe a lower extent of exposure in our population than in other European countries. Positive samples for OTA were detected in 35% (0.015 μg/L), 51% (0.46 μg/L), 100% (0.061 μg/L) and 100% (0.041 μg/L) of analysed samples in Belgium, Sweden, Italy and Spain, respectively (Heyndrickx et al., 2015; Solfrizzo et al., 2014; Vidal et al., 2016; Wallin et al., 2015). It should be also emphasized that the median levels of OTA determined in the present study are 3–10 fold lower than the ones determined in the referred studies.

AOH was detected in 29% and 13% of the 24h and first-morning urine samples, respectively. To our knowledge, it is the first time that AOH is quantified in urine samples collected in Europe. Results obtained for the two types of urine samples presented fair correlation coefficients ($r_s = 0.232$; $p = 0.025$ for volume-weighted concentrations; $r_s = 0.349$; $p = 0.001$ for creatinine adjusted concentrations) and statistically significant differences between them ($p = 0.000$). This fact indicates that, similarly to ZEN and DON, the type of urine sample used in the biomonitoring study may influence the concentration of *Alternaria* toxins. Šarkanj et al. (2018) already reported the detection of AOH in urine samples from Nigeria, with lower concentrations (0.03 μg/L) and lower LOD (0.01 μg/L) than the ones reported in this study (Table 2). Another *Alternaria* toxin, tenuazonic acid, was determined in German individuals ($n = 48$) with 98% of positive samples; all together these results showed that exposure to *Alternaria* toxins is a reality that urge to be clarified (Hövelmann et al., 2016).

For the remaining mycotoxins, low percentages of positive samples were found. CIT was detected in 2% of both types of urine samples. The occurrence of CIT in foods is often reported as co-existing with OTA (Ostry et al., 2013), thus being expected that the exposure of population determined within biomonitoring studies would present a similar pattern. In the present study, it was verified the opposite; samples that were positive for CIT were negative for OTA. Previous studies in Belgium and Germany had reported 59% (0.017 μg/L) (Heyndrickx et al., 2015) and 82% (0.03 μg/L) (Ali et al., 2015) of positive samples for CIT within the same range of concentrations. One hypothesis for this difference in population exposure is the pattern of food contamination that is probably different among countries from South, Western and Central Europe; however, there is no available data regarding the occurrence of CIT in foods marketed in Portugal to support this hypothesis. The low percentage of CIT present in Portuguese urine samples could explain the absence of correlation between CIT and OTA that was found in other studies.

Regarding FB_1, 7% and 3% of 24h urine and first-morning urine samples, respectively, were positive for FB_1. The biomarkers FB_2, FB_3 and the metabolite HFB_1 were not detected in any of the analysed samples. Silva et al. (2010) already performed a biomonitoring study to characterize the exposure of population from the central region of Portugal, and all the analysed samples were negative for FB_1 and FB_2. The difference in the results may be explained by the LOD that was about 20 times higher than the LOD reported in this study (Table 2). Gerding et al. (2014) found no positive samples for FB_1 using lower LODs. On the contrary, Solfrizzo et al. (2014) found 52% (0.029 µg/L) of positive samples for FB_1 in a range of concentrations similar to the range reported in the present study, being these results attributed to the presence of maize in traditional Italian dishes such as polenta.

3.3. Glucuronidation as metabolic pathway for DON and ZEN

The availability of DON and ZEN glucuronide standards guaranteed the determination of these metabolites in urine samples. The mean rate of glucuronidation was determined for those participants with positive 24h urine samples for both glucuronides and the respective parent compounds (DON, n = 43; ZEN, n = 13) (Fig. 1; Supplementary file S1).

Regarding DON, these results confirmed that glucuronidation is in fact a major metabolic pathway for detoxification of this mycotoxin (Table 3). Besides the high percentage of positive samples, the correlation coefficients between DON and DON-3-GlcA or DON and DON-15-GlcA are very strong, meaning that these metabolites are relevant urinary biomarkers of exposure for DON. Correlation coefficients were 0.791 and 0.744 (DON vs DON-3-GlcA) and 0.788 and 0.649 (DON vs DON-15-GlcA) for volume-weighted concentrations and creatinine adjusted concentrations, respectively. The rate of glucuronidation for total DON-GlcA (DON-3-GlcA + DON-15-GlcA) determined in this study for males and females, 79% (range 37–100%) is similar to other published studies: 76% (Warth et al., 2013), 91% (Turner et al., 2011), 92% (Heyndrickx et al., 2015) and 83% (Deng et al., 2018). Similarly to the findings reported by Vidal et al. (2018a), DON-15-GlcA is the major glucuronide excreted in urine with concentrations 8 times higher than DON-3-GlcA (9.3 for males and 6.6 for females). Vidal et al. (2018a) reported a lower ratio (4/1) but for 20 participants; in the present study the ratio was determined for 43 participants. In this study, there were no statistically significant differences found between males and females, even though females (n = 19) presented lower glucuronidation mean rates than males (n = 24) (Fig. 1). The absence of differences between males/females was previously reported (Deng et al., 2018; Solfrizzo et al., 2014; Turner et al., 2010). On the contrary, Vidal et al. (2018a) reported statistically significant differences between males and females for total DON excretion and glucuronidation rates after DON administration to the volunteers (1 µg DON/kg body weight), with women having a higher excretion of total DON.

Regarding ZEN, its metabolite ZEN-14-GlcA was determined in 16% of 24h urine samples, confirming that glucuronidation is a metabolic pathway for this mycotoxin, with high correlation coefficients between ZEN and ZEN-14-GlcA (0.509 and 0.653, for volume-weighted concentrations and creatinine adjusted concentrations, respectively). These results enabled for the first time the determination of a conjugation rate for ZEN. The mean conjugation rate determined was 85%, with no statistically significant differences between males (n = 7) and females (n = 6) (Fig. 1). These results pointed out the need for further studies with the development of intervention studies to clarify the toxicokinetics of ZEN.

3.4. Probable daily intake and risk characterization

Since there are no Health Based Guidance Values (HBGV) established for mycotoxins in biological samples, PDI was calculated from the concentration of individual 24h urine biomarkers concentrations and compared with established values for the safe intake such as TDI or PTWI, assuming that all exposure is a result of intake. The availability of 24h urines samples eliminates the need for assumptions on this parameter that is a major source of uncertainty in a risk assessment perspective. Since the assessed mycotoxins are not classified as carcinogens, risk characterization was performed with the Hazard Quotient approach, following the recommendations from EFSA (EFSA, 2013).

Human data available so far regarding mycotoxins' excretion rates

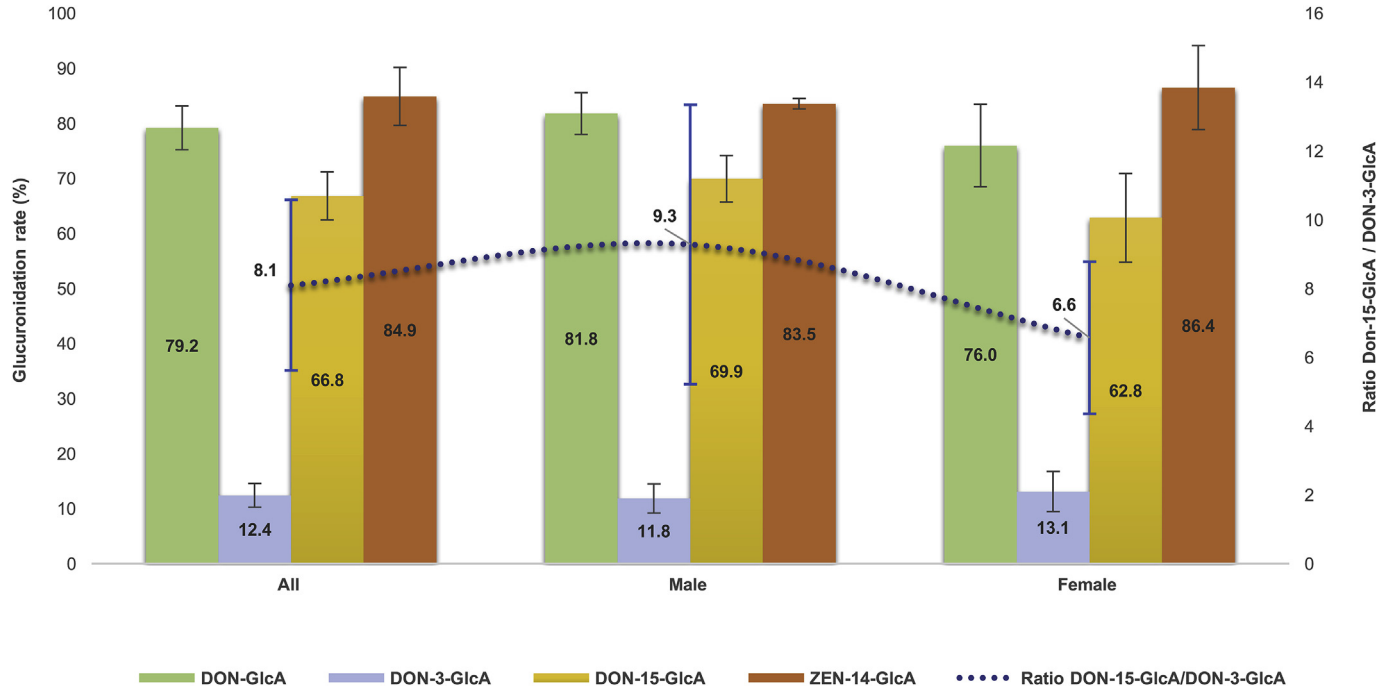

Fig. 1. Mean rates of glucuronidation for DON and ZEN (primary Y-axis), and ratio of DON glucuronides (secondary Y-axis), grouped by gender, determined in 24h urines. Error bars represent standard deviation.

are scarce due to the characteristics and health effects attributed to these compounds, making difficult the development of human intervention studies. Until now, data on excretion rates derived from human intervention studies is available for DON, FB_1, CIT, OTA and ZEN. However, studies developed to determine the excretion rate for ZEN, OTA and CIT included one (Warth et al., 2013 for ZEN and Studer-Rohr et al., 2000 for OTA) or two participants (Degen et al., 2018 for CIT), respectively, thus not covering inter-individual variability and the possible influence of age and sex in the excretion. For AOH the available data is very recent and was derived from an animal study performed in rats (Puntscher et al., 2019). Consequently, risk characterization may be hampered by high uncertainty. However, even being affected by uncertainty, these estimations provide an orientation and a first approach to the risk related to exposure to mycotoxins.

In the present study, the PDI for each mycotoxin was estimated considering available data for excretion rates and a risk characterization was performed. Results for estimated PDI of mycotoxins and respective HQ are presented in Table 4. Fig. 2 represents the HQ determined for all the mycotoxins, ordered from lowest to highest.

According to Table 4, for DON, a median PDI of 0.209 and 0.185 μg/kg bw/day and a maximum PDI of 3.619 and 3.203 μg/kg bw/day were estimated considering excretion rates of 64.0% (Vidal et al., 2018a) and 72.3% (Turner et al., 2010), respectively. In both scenarios a percentage of participants (9–10%) exceeding the TDI was shown (Fig. 2). DON was identified before as a health concern due to the prevalence of exposure exceeding the TDI (EFSA, 2017). Human biomonitoring studies developed in Spain, Italy, Belgium, UK, Croatia and Austria observed DON exposure above TDI for 8% (Rodríguez-Carrasco et al., 2014), 6% (Solfrizzo et al., 2014), 29% (Heyndrickx et al., 2015), 17% (Turner et al., 2010), 48% (Šarkanj et al., 2013) and 33% (Warth et al., 2012) of participants, respectively. Results obtained with the present study reinforce this pattern. The extended human exposure to DON in Europe could be associated to the frequent occurrence of DON in cereal-based products; in Spain 80–100% of wheat based products were contaminated by DON (Rodríguez-Carrasco et al., 2014), while in Belgium 96% of bread was contaminated by DON (Vanheule et al., 2014). In Portugal, the occurrence of DON is also frequent and some cereal samples presented concentrations above the legal EU limits (Abrunhosa et al., 2016).

Regarding FB_1 the exposure was considered as not safe for the seven participants with positive samples due to exceeding of the respective TDI, independently of the excretion rate used for calculations. The estimated PDI and respective HQ presented a 7-fold difference when considering different excretion rates. Solfrizzo et al. (2014) reported no participants exceeding the TDI, but considering the TDI of 2.0 μg/kg bw/day for FB_1. Since a low urinary recovery and a high inter-individual variability in absorption and excretion of FB_1 has been reported, the transfer of urinary concentrations to estimated intake should be considered carefully (Van Der Westhuizen et al., 2011).

Regarding OTA, a median PDI of 0.005 μg/kg bw/day was estimated and 14% of participants are probably exceeding the PTWI (Table 4, Fig. 2). Other authors (Solfrizzo et al., 2014; Degen, 2016) have referred the uncertainty associated with the conversion of OTA urinary concentrations to intake data due to extensive binding of OTA to plasma proteins, enterohepatic recirculation, and transport proteins involved in absorption and/or excretion. Additionally, the excretion rate determined for OTA by Studer-Rohr et al. (2000) was derived from only one individual. However, and in spite of being data derived from animal studies, excretion rate determined by Gambacorta et al. (2013) corroborate this value (2.6%). The median estimated intake determined here was in the same order of magnitude of the estimation of Abrunhosa et al. (2016) for Portuguese population based on occurrence and consumption data (0.001 μg/kg bw/day). The exceedance of PTWI for OTA was referred by Vidal et al. (2016) in Spain and Solfrizzo et al. (2014) in Italy.

Regarding ZEN, it was estimated a median PDI of 0.081 μg/kg bw/day and 24% of participants exceeding the TDI (Table 4, Fig. 2). In Germany, Ali and Degen (2018) observed only one participant that exceeded the TDI for ZEN, with the overall studied population presenting an exposure considered as safe. Solfrizzo et al. (2014) did not found any participants exceeding the TDI for ZEN (TDI = 0.200 μg/kg bw/day in 2014), using the excretion rate derived from Gambacorta et al. (2013) study on piglets (36.8%). If we consider this excretion rate obtained from animals, the median and maximum estimated PDI would be 0.083 μg/kg bw/day and 4.441 μg/kg bw/day, with 7% of participants exceeding TDI for ZEN. Therefore, in spite of all the uncertainties associated with these estimations, the exposure of Portuguese population to ZEN is of concern and should be carefully re-evaluated when more robust data on toxicokinetics is available.

Regarding CIT, only two female participants presented positive 24h urine samples and one of them slightly exceeded the respective TDI with an HQ of 1.04. However, these results should be considered carefully since data on excretion rate for CIT was obtained from a study with only two participants (two females ingested two doses) and with a high variability in results (Degen et al., 2018).

For AOH and other *Alternaria* toxins no toxicological data are yet

Table 4
Estimate of Probable Daily Intake and Risk Characterization of exposure of the Portuguese population to mycotoxins using urine biomarkers.

Mycotoxins	Excretion Rate			PDI (μg/Kg bw/day)				HBGV	HQ				% of participants with HQ > 1
	Reference	ER, %	Number of individuals	Med	P90	P95	Max	(μg/Kg bw/day or/week)	Med	P90	P95	Max	
DON	Vidal et al. (2018a)	64%	20	0.209	0.990	1.584	3.619	1.00 (TDI)	0.21	0.99	1.58	3.62	10
	Turner et al. (2010)	72.3%	35	0.185	0.876	1.402	3.203		0.19	0.88	1.40	3.20	9
FB_1[a]	Van Der Westhuizen et al. (2011)	0.075%	22	7.685	8.826	9.175	9.524	1.00 (TDI)	7.69	8.83	9.18	9.52	–
	Riley et al. (2012)	0.5%	8	1.153	1.324	1.376	1.429		1.15	1.32	1.38	1.43	–
CIT[a]	Degen et al. (2018)	14.75%	2	0.140	0.194	0.201	0.208	0.20 (TDI)	0.70	0.97	1.01	1.04	–
ZEN	Warth et al. (2013)	9.6%	1	0.081	0.648	1.426	4.347	0.25 (TDI)	0.32	2.59	5.70	17.39	24
OTA	Studer-Rohr et al. (2000)	2.5%	1	0.005	0.026	0.037	0.597	0.12 (PTWI)	0.27	1.53	2.18	35.10	14
AOH[b]	Puntscher et al. (2019)	8.3%	[c]	0.074	0.450	2.048	2.451	–	–	–	–	–	–

PDI = Probable Daily Intake; HBGV = Health Based Guidance Vaue; HQ = Hazard Quotient; Med = Median; P90 = Percentile 90; P95 = Percentile 95; Max = Maximum; DON = Deoxynivalenol; ZEN = Zearalenone; OTA = Ochratoxin A; CIT = Citrinin; FB_1 = Fumonisin B_1; AOH = Alternariol; TDI = Tolerable Daily Intake; PTWI = Provisionally Tolerable Weekly Intake (divided by 7 for HQ calculation purposes).

[a] Mycotoxins with positive samples below 10% - Probable Daily Intake and Hazard Quotient determined only for positive samples (CIT, n = 2; FB_1, n = 7). % of participants exceeding TDI (HQ > 1) not determined.

[b] HBGV available (Threshold of Toxicological Concern) with high uncertainty associated for risk characterization - Hazard Quotient not determined.

[c] Puntscher et al. (2019) – Study performed in rats.

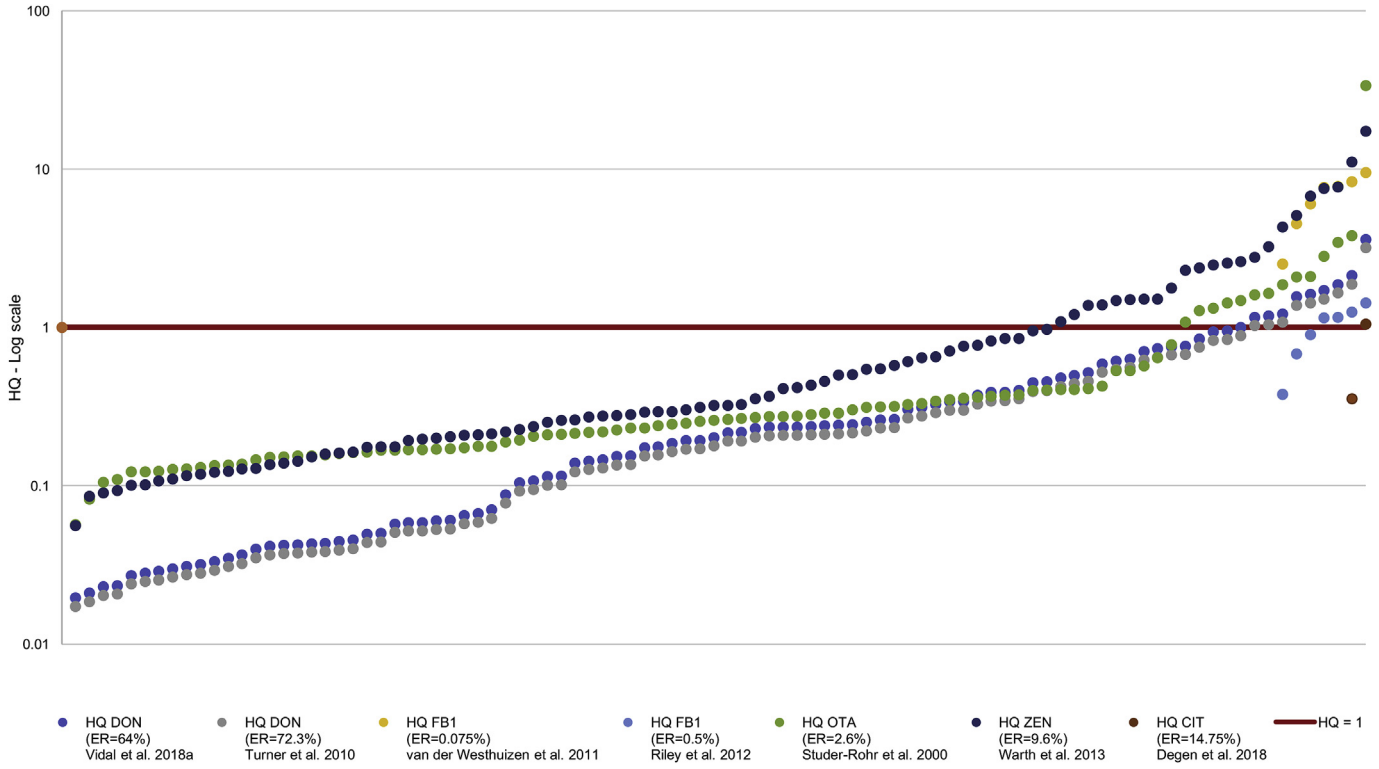

Fig. 2. Hazard quotient determined and sorted by ascending order for DON, FB_1, ZEN, OTA and CIT.

available to support the establishment of a TDI. Additionally, there is no legislation for the occurrence of these mycotoxins in food and feed (Arcella et al., 2016; Solfrizzo, 2017) and regarding toxicokinetics, the only study available so far was performed in rats (Puntscher et al., 2019). The only guidance value available is the Threshold of Toxicological Concern (TTC) established by EFSA, set at 2.5 ng/kg bw/day (Arcella et al., 2016). Since the TTC is used to rank substances to prioritize the need for further risk assessment, and is not related with substance-specific data, the associated uncertainty hampers a proper and accurate risk characterization (EFSA, 2012b; Munro et al., 2008). For these reasons, risk characterization regarding the exposure to AOH was not performed and estimation of PDI should be considered carefully and re-evaluated as soon as new data on toxicokinetics is available. However, it should be emphasized that exposure to AOH is in fact a reality for the Portuguese population. In the present study, 29% of 24h-urine samples were positive. These results will reinforce the need for further risk assessment and for the establishment of a TDI for *Alternaria* toxins.

3.5. Strengths and limitations

To the best of our knowledge, the present study considered for the first time paired 24h and first-morning urine samples from the same participant for the study of urinary mycotoxins biomarkers. Taking advantage of this, more data on excretion of mycotoxins was generated contributing for a better clarification of the toxicokinetics of some mycotoxins. Another important aspect is the sample size since it includes 94 participants from North and Central regions of Portugal. The collection of 24h urine samples and anthropometric data for the 94 participants at individual level was fundamental to reduce the uncertainty associated with the reported findings; there was no need to use assumptions for body weight and urinary daily volume when estimating the PDI, making these estimations more accurate. The analytical method used for determination of urinary mycotoxins biomarkers is also a strength of this study; it provided quantitative results for 37 mycotoxins and metabolites in a single run using isotope-labelled internal standards. Regarding DON, ZEN and FB_1, several metabolites were included allowing their direct quantification. Low instrumental limits allowed to reduce the amount of left-censored data (< LOD) and for these results, a multiple imputation method for substitution was used instead of replacing all results for a fixed value, with the advantage of keeping variability in the lower values of the distribution. Finally, it is the first time that 37 mycotoxins and metabolites were reported for Portuguese population.

Some limitations should also be considered within human biomonitoring studies to assess human exposure to mycotoxins, and particularly in this study. Regarding the estimation of PDI, some excretion rates used for calculations were obtained from human studies with one or two participants (ZEN, OTA and CIT), thus not covering the possible influence of dose, age and sex (Warth et al., 2013; Studer-Rohr et al., 2000; Degen et al., 2018). Excretion rate for AOH was obtained from an animal study, increasing the associated uncertainty (Puntscher et al., 2019) However, these are the best data available until now. The lack of full knowledge on mycotoxins metabolism was already identified as source of uncertainty (de Nijs et al., 2016). The absence of HBGV for mycotoxins in urine samples hampers the direct calculation of HQ, affecting the uncertainty associated to the conclusions derived from these results (Zidek et al., 2017). Results for risk characterization of exposure to ZEN, CIT, OTA and AOH should be evaluated carefully and only provide an orientation for future studies. The need for analytical standards for mycotoxins' metabolites and highly sensitive analytical methods for detection of low exposures were other aspects identified as limitations in human biomonitoring studies (Vidal et al., 2018b); in the present study, the metabolite dihydro-citrinone, however being important to more accurately determine the human exposure to CIT, was not determined. The LODs achieved for α-ZEL-GlcA and β-ZEL-GlcA were higher than for ZEN, α-ZEL and β-ZEL, contributing to an eventual underestimation of exposure to ZEN. For mycotoxins with fast excretion rates, it could be important in the future to consider a different sampling design to quantify exposure over time in order to have a more

accurate estimation of individual exposure (Calafat, 2016). Therefore, the development of future studies is strongly encouraged.

4. Conclusions

The present study aimed, for the first time and within a human biomonitoring study, to assess the exposure of the Portuguese population to mycotoxins through the determination of 37 mycotoxins' urinary biomarkers. Results indicate that the Portuguese population is in some extent exposed to six mycotoxins (DON, ZEN, OTA, AOH, FB_1 and CIT) through the identification and quantification of 12 urinary biomarkers, within parent toxins and metabolites. To our knowledge, this is the first human biomonitoring study for mycotoxins' biomarkers using paired 24h and first-morning urine samples for the same participant with inclusion of 94 individuals. These sampling characteristics allowed to have a more clear perspective on the relation of concentrations between the two types of urine samples, and associated statistical significant differences. It was possible to confirm that exposure assessment of mycotoxins with fast excretion rates, such as ZEN, DON and AOH, is influenced by the type of urine sample chosen. The number of participants also allowed to gather more information on glucuronidation rates for DON and ZEN.

Risk characterization revealed that exposure of the Portuguese population to OTA, DON, ZEN and FB_1 is above safety limits and may represent a health concern. Nevertheless, these results should be interpreted carefully due to uncertainty related to toxicokinetics data used in the estimations.

Although still presenting some limitations, human biomonitoring studies are becoming fundamental for an accurate exposure assessment, and consequently imperative for a wide use in the risk assessment and public health domain. The establishment of biomonitoring equivalents for mycotoxins in urine is considered a major goal for further studies in this area, in order to achieve a more accurate risk characterization, reducing the associated uncertainties. The present study contribute with reliable and evidence-based results, and confirmed that mycotoxins represent a burden and are part of the human exposome of the Portuguese population. Further studies are needed to shed a light on the determinants of exposure in order to contribute for the promotion of public health measures to reduce the mycotoxins' exposure in Portugal.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.06.010.

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