Environment International 137 (2020) 105539

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Mycotoxin exposure assessments in a multi-center European validation study by 24-hour dietary recall and biological fluid sampling

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ARTICLE INFO

Handling Editor: Adrian Covaci

Keywords:
Mycotoxins
Exposure
Risk
Europe
Aflatoxins
Deoxynivalenol

ABSTRACT

The European Food Consumption Validation (EFCOVAL) project includes 600 men and women from Belgium, the Czech Republic, France, the Netherlands, and Norway, who had given serum and 24-hour urine samples, and completed 24-hour dietary recall (24-HDR) interviews. Consumption, according to 24-HDR, was matched against the European Food Safety Authority (EFSA) databases of mycotoxin contaminations, via the FoodEx1 standard classifications, producing an indirect external estimate of dietary mycotoxin exposure. Direct, internal measurements of dietary mycotoxin exposure were made in serum and urine by ultra-performance liquid chromatography coupled to tandem mass spectrometry. For the first time, mycotoxin exposures were thoroughly compared between two 24-HDRs, and two 24-hour urine samples collected during the same days covered by the 24-HDRs. These measurements were compared to a single-time point serum measurement to investigate evidence of chronic mycotoxin exposure. According to 24-HDR data, all 600 individuals were exposed to between 4 and 34 mycotoxins, whereof 10 found to exceed the tolerable daily intake. Correlations were observed between two time points, and significant correlations were observed between concentrations in serum and urine. However, only acetyldeoxynivalenol, ochratoxin A, and sterigmatocystin were found to have significant positive correlations between 24-HDR exposures and serum, while aflatoxin G1 and G2, HT-2 toxin, and deoxynivalenol were associated between concurrent 24-HDR and 24-hour urine. Substantial agreements on quantitative levels between serum and urine were observed for the groups *Type B Trichothecenes* and *Zearalenone*. Further research is required to bridge the interpretation of external and internal exposure estimates of the individual on a time scale of hours. Additionally, metabolomic profiling of dietary mycotoxin exposures could help with a comprehensive assessment of single time-point exposures, but also with the identification of chronic exposure biomarkers. Such detailed characterization informs population exposure assessments, and aids in the interpretation of epidemiological health outcomes related to multi-mycotoxin exposure.

1. Introduction

Over the course of a human lifetime, dietary exposure to mycotoxins is unavoidable (Andrade and Caldas, 2015; Abrunhosa et al., 2016; Eskola et al., 2019; Schatzmayr and Streit, 2013; Smith et al., 2016; Streit et al., 2013; Van Der Fels-Klerx et al., 2012). Even in the highly

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https://doi.org/10.1016/j.envint.2020.105539
Received 2 December 2019; Received in revised form 29 January 2020; Accepted 29 January 2020
Available online 05 February 2020

EU-regulated food supply and sanitary living conditions, agricultural products attract fungal colonization (178/2002/EC, 2002; 1126/2007/EC, 2007; 1881/2006/EC, 2006; 2006/576/EC, 2006; 2013/165/EU, 2013). Certain fungal secondary metabolites, termed mycotoxins, are reported in surveys of food crops, in harvest storage, in processed plant-based foods, and finally in human populations (Eskola et al., 2019; Blesa et al., 2012; Jodlbauer et al., 2000; De Boevre et al., 2012; Magan et al., 2011; Pleadin et al., 2015; Scudamore and Livesey, 1998; Solfrizzo et al., 2014; Heyndrickx et al., 2014). Chronic dietary exposure to multiple mycotoxins throughout the life-course of a human being is a reality (De Ruyck et al., 2015). The toxicological burden of mycotoxins has been described in many detailed *in vivo* studies, and ranges from gastrointestinal manifestations, genotoxicity, estrogenicity to death (Smith et al., 2016; De Ruyck et al., 2015; Adam et al., 2017; Probst et al., 2007; Kamala et al., 2018).

Mycotoxins in the food supply have the opportunity to be absorbed through the gastrointestinal tract, into the circulatory system. Some mycotoxins pass unchanged from the farm crop to the blood stream (Assuncao et al., 2015; Hartinger and Moll, 2011; Kostelanska et al., 2009; Vidal et al., 2016). The broad variation in physicochemical properties among mycotoxins, however, leads to a large variation in rates of absorption, metabolism and excretion (Kiessling et al., 1984). Indeed, humans significantly metabolize and excrete mycotoxins in a complex manner, resulting in disparate toxicokinetic profiles (Vidal et al., 2018; Vidal, 2018; Ringot et al., 2006; Degen et al., 2018). Subsequently, in assessments of human exposure to mycotoxins, metabolized forms (*i.e.*, biomarkers of exposure), need to be included when accurately calculating exposure (Berthiller et al., 2011; Nathanail et al., 2015). The identification and suitability of a reliable mycotoxin biomarker(s) are pertinent (Vidal et al., 2018; Vidal, 2018; Yang et al., 2015; Yang et al., 2015; Abnet et al., 2001; Dragusel, 2013). The production of glucuronic acid conjugates from deoxynivalenol (DON) and zearalenone (ZEN) are common examples (Maul et al., 2012; Pfeiffer et al., 2010; Vidal et al., 2016; Yang et al., 2017). Diacetoxyscirpenol (DAS) is metabolized into five different forms *in vitro* by human liver microsomes and mostly cleared from blood of pigs within 4–6 h (Yang et al., 2015; Bauer et al., 1985). Some mycotoxins undergo minimal endogenous metabolism and are detected in their original conformation, such as the non-polar fumonisins (FB), resulting in low rates of both absorption and metabolism (Hartinger and Moll, 2011; Stockmann-Juvala and Savolainen, 2008; Van der Westhuizen et al., 2013). In certain cases, mycotoxins may affect toxicity by their conjugation with cellular DNA, producing mycotoxin-DNA adducts such as aflatoxin B1 (AFB1)-N7-guanine (Wild et al., 1990). A comprehensive overview of mycotoxin biomarkers and their toxicokinetic profiles is detailed in Vidal (2018).

It becomes increasingly interesting to compare biological fluids, such as urine and blood, to correctly elucidate a comprehensive cross-section of internal mycotoxin exposures. Considering the chronic nature of dietary exposure, as well as the variety of a modern diet, modelling real-world exposure requires the simultaneous assessment of multiple mycotoxins (Warth et al., 2013). Determining and monitoring mycotoxin presence informs food safety assessments by indirectly evaluating a population's probable external exposure, or by directly quantifying internal exposure through biomarker analysis (Abrunhosa et al., 2016; Gerding et al., 2014; Heyndrickx et al., 2015; Wallin et al., 2015).

Recently, Gormley et al. (2019) described an increasing interest in using dietary biomarkers in order to address possible measurement errors in external exposure estimates (Gormley et al., 2019). Though dietary analyses are widely used and accessible tools for estimating population intake of dietary contaminants, complimentary assessments are required to elucidate the variance among populations. Therefore, for the first time, the agreement among different multi-mycotoxin internal exposure measurements and theoretical external exposure estimates was explored in a human population. Single time-point exposures were compared between 24-hour dietary recall (24-HDR) surveys and 24-hour urine collections from the same day. These measurements were repeated after one month and also compared to a single time point serum measurement to investigate evidence of and associations with chronic mycotoxin exposure. This pioneering work will further advance research on multiple mycotoxin monitoring on the population level.

Table 1
Demographic breakdown of the EFCOVAL project sample.

(*N* = 600)	Belgium	Czech Republic	France	the Netherlands	Norway
n	123	124	113	122	118
Age (years)	54.7 (5.2)	53.9 (5.9)	55.1 (5.7)	56.3 (5.1)	54.9 (6.4)
Female (%)	48.8	50.0	52.2	50.8	50.8
BMI (kg/m^2)	26.2 (4.0)	25.6 (3.2)	24.3 (3.1)	26.0 (4.4)	26.4 (4.2)

Legend: mean values are given, with standard deviations in parenthesis.

2. Methods

2.1. Study design

The comparison of cross-sectional survey methods includes human populations from five European nations, briefly characterized in Table 1. The population characterization, dietary data, and biological samples were obtained from the European Food Consumption Validation (EFCOVAL) project (Crispim et al., 2011; de Boer et al., 2011). EFCOVAL cataloged people's dietary intake, and also collected biological samples of 24-hour urine and non-fasted serum (de Boer et al., 2011). These biological samples were analyzed for multiple mycotoxins by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), followed by confirmatory analyses using high-resolution mass spectrometry (HRMS). The two days on which measurements were taken were expected to be directly comparable with urine measurements taken the same day. The mean exposure of the two days was used to compare against a serum sample taken some weeks prior to the 24-HDR's, and also against the mean of the two days' urine collections, allowing a three-way comparison of different exposure measurements (24-HDR vs. serum; 24-HDR vs. urine; serum vs. urine).

2.2. Study population

The EFCOVAL project was active from October 2006 until the end of March 2010. In this dataset, individuals were aged between 45 and 65 years only. Younger and older persons were considered more likely to experience confounding lifestyle factors (*i.e.*, no regular diets) (Huybrechts et al., 2011). The research participants in this work were 297 men and 303 women from Belgium, the Czech Republic, France, the Netherlands, and Norway, allowing consideration of the high diversity in dietary patterns around Europe. In EFCOVAL, efforts were made to ensure a balanced representation of sexes, levels of education, and regional population centers within each country. Exclusion criteria were as follows: using diuretics, following a prescribed medical diet, being enrolled in another study during the same time span, not being able to read or speak the national language, being pregnant, lactating, having diabetes mellitus or kidney disease, and donating blood or plasma during (or < 4 weeks before) the study (Huybrechts et al., 2011).

2.3. External exposure estimate

On two days, separated by at least one month, participants were asked to recall all consumed foods of the previous 24 h, *i.e.*, a 24-HDR interview (Crispim et al., 2011; Huybrechts et al., 2011). A European Food Safety Authority (EFSA) dynamic database, including the clustered mycotoxin occurrences of all mycotoxin detections filed by the EU Member States, was used as a basis for estimating mycotoxin levels in

the different foods reported in each 24-HDR from the EFCOVAL participants (Eskola et al., 2018; EFSA, 2010). This dataset comprised contamination data of foods with production years from 2001 to 2014, and sampling years from 1997 to 2014. Noteworthy, the country in which mycotoxins were detected in a food item was not considered, allowing a much larger list of food items for all countries in the study. More details on this dataset are enlisted in the EFSA (2010).

When reporting contaminant concentrations analyzed in monitoring programs, actual numeric values of concentrations were reported when the measurements exceed the limit of detection (LOD) or limit of quantification (LOQ). Therefore, a middle bound (MB)-concentration scenario was applied for non-detect samples. This implied that for commodities (*e.g.*, bread) for which there was at least one sample with a concentration value ≥ LOD or LOQ, all non-detect samples were assigned a concentration equal to half the limit value (1/2 LOD or LOQ). The remaining non-detect samples were assumed to contain no mycotoxins. This scenario was chosen as a more optimal approach, as opposed to assigning all non-detect samples a concentration equal to 0 μg/kg (*i.e.*, lower bound-scenario). Nevertheless, the lower bound-scenario was also used in this study for conducting sensitivity analyses.

Following procedures - similar to those that later were standardized by the European Prospective Investigation into Cancer (EPIC) Nutrient DataBase (ENDB) project-, food types were aggregated according to the FoodEx1 standard classifications and assigned a mycotoxin concentration equal to the median recorded by EFSA (Slimani et al., 2002; EFSA, 2016). Next, for all foods that had been recalled in the 24-HDR, the same food item (or a proxy/surrogate) was searched in the EFSA food occurrence database (FoodEx1 item). When all foods reported in a 24-HDR had been matched with an EFSA FoodEx1 item, then the mycotoxin data could be linked to the food intake data from EFCOVAL. Since the EFSA database included several samples that have been analyzed in one or more laboratories for one single FoodEx1 item, the median of the mycotoxin values analyzed for all these different samples was used to perform the linking with the food consumption data. The median approach was applied rather than the mean, in order to limit the impact of extreme values on the aggregated FoodEx1 values (EFSA, 2016).

After matching the mycotoxin occurrence data with the food consumption data, the mycotoxin intake was calculated by multiplying the portion size of the food with the mycotoxin concentration in the food. Hence, the total intake per day for each mycotoxin was calculated per individual as the sum of that particular mycotoxin derived from the different foods consumed during the day.

2.4. Serum sample preparation

Preceding the first 24-HDR interview by approximately one week (or two weeks in the Czech Republic), participants gave a non-fasted blood sample, from which serum was immediately isolated, aliquoted, and stored at −80 °C. Several sample preparation techniques that are reported in the literature, such as immuno-affinity column separation, salt-assisted liquid-liquid extraction, or general solid-phase extraction, were not considered feasible due to the relatively low volumes of EFCOVAL serum samples. As such, a protein precipitation technique was applied, as described below, on a total of 61 Dutch, 58 Belgian, 40 Czech, 78 Norwegian, and 32 French serum samples.

Serum samples were thawed at 4 °C, briefly vortexed, then 50 μL was transferred to a new polypropylene tube. To this, 100 μL of cold acetonitrile containing 1% (v/v) formic acid was added, the mixture was vortexed, then allowed to stand for one hour at 4 °C. These samples were centrifuged at 10,000*g* for 10 min, then 120 μL of the supernatant was transferred to an injection vial and evaporated to dryness under a steady stream of nitrogen in a water bath at 40 °C. The residue was dissolved by vortexing in 10 μL methanol containing 0.4% (v/v) formic acid, then 30 μL of ultrapure water containing 0.4% (v/v) formic acid was added and mixed, producing a dilution factor of 1. The used consumable materials and reagents are detailed in **Supplementary File S1**.

2.5. Urine sample preparation

During each of the two days for which participants had been asked for a 24-HDR, participants had collected their urine over the following 24-hour period. These urine samples were stored at −80 °C until use. The sample preparation procedure followed a recently published approach (Breidbach, 2017), slightly adapted for aqueous samples, as described below. A total of 62 (n = 31) Dutch, 96 (n = 59) Belgian, 79 (n = 40) Czech, and 76 (n = 58) Norwegian urine samples were successfully assayed on multiple mycotoxins; urine samples from France were not available.

After thawing at 4 °C, 500 μL of urine was added to 500 μL of ultrapure water containing 1% (v/v) formic acid, then 1 mL of ethyl acetate was added, and mixed thoroughly by overhead shaking for one hour. Next, 1 g of sodium sulfate was added, and shaken by hand for one minute, then crystallized for 20 min. The samples were centrifuged at 3000*g* for 3 min, then 320 μL of the organic phase was transferred to an injection vial and evaporated to dryness under a steady stream of nitrogen in a water bath at 40 °C. The residue was dissolved by vortexing in 40 μL methanol containing 0.4% (v/v) formic acid, then 120 μL of ultrapure water containing 0.4% (v/v) formic acid was added and mixed, producing a dilution factor of 1.

Urinary osmolality was used to determine relative dilution factors for urine samples. This was measured by a Model 3320 Osmometer from Advanced Instruments, Inc. (Massachusetts, USA). Six-fold replicate measurements of osmolality in control samples and calibrant solutions returned standard deviations less than 1.5% of the observed average. Therefore, samples were measured in duplicate, and the average recorded osmolality was used for normalization of detected mycotoxin concentrations. The used consumable materials and reagents are detailed in **Supplementary File S1**.

2.6. UPLC-MS/MS And HRMS analysis

Analytical reference standards were not commercially available for some metabolized forms of mycotoxins of interest. Therefore, phase I metabolites aflatoxin Q1 (AFQ1), and monoacetoxyscirpenol (MAS), as well as the phase II metabolites deoxynivalenol-3-glucuronide (DON3GlcA) and deoxynivalenol-15-glucuronide (DON15GlcA) were produced by incubating analytical standards with liver microsomes, according to a previously described method (Yang et al., 2015; Yang et al., 2017) (**Supplementary File S2**). The instrumental parameters are detailed in **Supplementary File S2**. Observed mass transitions and target-specific parameters are listed in Table 2.

The limit of quantification (LOQ) was set as the lowest concentration analytical standard observed above the limit of detection (LOD), in turn, set at an extrapolation of the linear dose-response to three standard deviations above the mean background noise signal (Table 2). Sample signals identified above LOQ were subsequently normalized against internal standard signals to counter matrix effects, and in the case of urine samples, further normalized by urinary dilution coefficients.

2.7. Mycotoxin grouping

Through each of the three measurement points, some mycotoxins may be represented by various forms, which were detected individually. For example, deoxynivalenol-3-glucoside (DON3Glu) in the food may be cleaved to DON in the gut and further metabolized to DON15GlcA in the urine. Additionally, some co-produced mycotoxins were grouped to account for unreported specificity of mycotoxin, contaminating the diet of the population. Hence, in addition to individual mycotoxins, groups of related mycotoxins were used to facilitate direct comparison between the three types of exposure measurement.

The group *Aflatoxins* includes AFB1, aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin Q1 (AFQ1), and aflatoxin

Table 2
LC-MS/MS parameters for targeted mycotoxin detection.

Target		RT	Molecular ion	*m/z*	Cone (V)	Quantifier	C.E. (Q) (eV)	Qualifier	C.E. (q) (eV)	q/Q	LOQ (ng/L)	LOD (ng/L)
Patulin	PAT	2.32	$[M+H]^+$	155.05	30	81.10	10	71.15	10	0.869	50	25
T-2 toxin tetraol	T2(OH)4	2.49	$[M+H]^+$	299.15	30	175.15	18			0.507	1000	944
			$[M+NH_4]^+$	316.20	30			173.25	18			
Nivalenol	NIV	2.51	$[M+H]^+$	313.15	35	175.20	12	125.20	10	0.449	336	305
Deoxynivalenol-3-glucuronic acid	DON-15-GlcA	2.84	$[M-H]^-$	471.00	60	175.05	40	149.90	45	0.776		
Deoxynivalenol-15-glucuronic acid	DON-15-GlcA	3.06	$[M-H]^-$	471.00	60	149.90	45	217.00	40	0.512		
Deoxynivalenol-3-glucoside	DON-3-G	3.21	$[M+Na]^+$	481.20	40	451.25	25	433.00	25	0.418	73	46
Deoxynivalenol	DON	3.23	$[M+H]^+$	297.15	30	175.30	20	189.25	15	0.411	168	108
^{13}C-deoxynivalenol (IS)	13C-DON	3.23	$[M+H]^+$	312.10	30	262.90						
Ergometrine	Em	3.42	$[M+H]^+$	325.60	30	223.00	20	265.00	18	0.883	99	60
Deepoxydeoxynivalenol	DOM	4.05	$[M+H]^+$	281.15	30	109.25	9	137.30	9	0.798	168	96
Methyl ergometrine (IS)	MeEm	4.13	$[M+H]^+$	340.20	30	208.25	27					
Neosolaniol	NEO	4.16	$[M+NH_4]^+$	400.20	40	185.30	18	215.20	18	0.794	22	18
Ergometrinine	Emn	4.18	$[M+H]^+$	325.60	30	223.00	20	265.00	18	0.269	99	83
Verrucarol	VER	4.46	$[M+H]^+$	267.15	30	185.25	12	159.15	16	0.814	31	29
3-acetyl-deoxynivalenol	3AcDON	4.89	$[M+H]^+$	339.15	35	203.25	15	137.20	10	0.950	280	164
15-acetyl-deoxynivalenol	15AcDON	4.89	$[M+H]^+$	339.15	35	137.20	10	203.25	15	0.950	280	136
Aflatoxin B1-lysine adduct	AFB1-Lys	5.40	$[M+H]^+$	457.30	30	394.20	20	310.90	33	0.034		
Aflatoxin Q1	AFQ1	5.55	$[M+H]^+$	329.05	40	273.10	20	259.10	20	0.165		
Aflatoxin G2	AFG2	5.56	$[M+H]^+$	331.10	40	245.10	30	285.10	25	0.981	3	1
Aflatoxin M1	AFM1	5.65	$[M+H]^+$	329.05	40	273.10	22	311.10	25	0.356	5	5
Ochratoxin alpha	OTα	5.74	$[M+H]^+$	257.00	30	239.10	10	221.10	20	0.340	420	262
Aflatoxin G1	AFG1	5.99	$[M+H]^+$	329.05	40	243.10	25	283.05	25	0.442	9	1
Aflatoxin B2	AFB2	6.53	$[M+H]^+$	315.00	40	287.00	26	259.00	28	0.945	9	5
Tenuazonic acid	TA	6.60	$[M-H]^-$	196.10	55	139.00	20	112.00	24	0.517	250	180
Ergosinine	Esn	6.75	$[M+H]^+$	548.10	30	276.90	23	319.90	20	0.758	889	99
Ergosine	Es	6.99	$[M+H]^+$	548.10	30	276.90	23	319.90	20	0.698	209	99
Aflatoxin B1	AFB1	7.05	$[M+H]^+$	313.05	40	285.05	20	269.05	30	0.585	3	2
Diacetoxyscirpenol	DAS	7.13	$[M+NH_4]^+$	384.20	40	199.25	18	183.25	20	0.575	22	9
Ergotaminine	Etn	7.21	$[M+H]^+$	582.10	30	277.00	23	297.00	23	0.023	296	99
Citrinin	CIT	7.33	$[M+H]^+$	251.10	30	233.10	12	205.15	25	0.427	61	38
T-2 toxin triol	T2(OH)3	7.36	$[M+Na]^+$	405.20	40	303.15	14	125.25	14	0.062	202	86
Ergotamine	Et	7.40	$[M+H]^+$	582.10	30	277.00	23	268.00	23	0.050	296	99
Ergocornine	Eco	7.43	$[M+H]^+$	561.50	35	267.90	20	347.90	20	0.204	296	99
Dihydro ergotamine (IS)	DHEt	7.52	$[M+H]^+$	584.30	30	270.30	24					
Zearalenone-14-glucuronic acid	ZEN-14-GlcA	7.81	$[M+H]^+$	495.00	20	319.00	10	283.00	20	0.796		
Ergokryptine	Ek	8.48	$[M+H]^+$	576.10	30	268.00	23	305.00	23	0.073	296	99
Ergocristine	Ecr	8.58	$[M+H]^+$	610.10	35	305.00	22	268.00	22	0.114	296	99
Ergocorninine	Econ	8.60	$[M-H_2O+H]^+$	544.10	35	276.95	25	304.90	25	0.383	99	50
Roquefortine C	ROQ-C	8.80	$[M+H]^+$	390.20	40	193.30	25	322.15	20	0.537	22	10
^{13}C-HT-2 toxin (IS)	13C-HT2	8.83	$[M+H]^+$	464.30	40	102.90	26					
HT-2 toxin	HT2	8.83	$[M+NH_4]^+$	447.20	40	285.10	20			0.085	36	22
			$[M+Na]^+$	442.25	40			185.14	11			
Hydrolysed fumonisin B1	H-FB1	8.98	$[M+H]^+$	406.35	40	334.30	19	236.15	19	0.342	125	63
Alternariol	AOH	9.02	$[M+H]^+$	259.05	30	213.10	25	241.10	25	0.487	61	15
Ergokryptinine	Ekn	9.07	$[M+H]^+$	576.10	30	305.00	23	348.00	19	0.827	99	50
Ergocristinine	Ecrn	9.24	$[M+H]^+$	610.10	35	305.00	22	325.00	22	0.401	296	99
Beta zearalenol	β-ZEL	9.51	$[M+H]^+$	321.15	35	175.35	22	177.25	18	0.150	370	185
Fumonisin B1	FB1	9.55	$[M+H]^+$	722.40	40	352.20	20	334.40	20	0.445	81	58
T-2 toxin	T2	9.87	$[M+NH_4]^+$	489.20	40	245.30	25			0.741	13	8
			$[M+Na]^+$	484.25	40			185.35	21			
Alpha zearalenol	αZEL	10.39	$[M+H]^+$	321.15	35	177.05	17	175.15	22	0.221	124	75
Zearalanone	ZAN	10.47	$[M+H]^+$	321.15	35	189.20	17	187.20	22	0.593	125	119
Fumonisin B3	FB3	10.56	$[M+H]^+$	706.40	40	336.20	30	354.45	28	0.579	124	48
Zearalenone	ZEN	10.71	$[M+H]^+$	319.15	35	187.20	17	185.20	19	0.314	101	61
Sterigmatocystin	STE	11.09	$[M+H]^+$	325.05	40	281.10	35	310.05	22	0.904	5	3
Ochratoxin A	OTA	11.10	$[M+H]^+$	404.10	40	239.10	22	221.10	35	0.456	11	6
^{13}C-ochratoxin A (IS)	13C-OTA	11.10	$[M+H]^+$	422.00	40	377.00	20					
Alternariol methyl ether	AME	11.19	$[M+H]^+$	273.10	30	258.10	22	230.10	28	0.835	63	37
Fumonisin B2	FB2	11.28	$[M+H]^+$	706.40	40	336.20	32	354.30	35	0.406	134	86
Enniatin B	EnB	11.85	$[M+H]^+$	640.40	30	196.25	21	214.25	26	0.474	1000	570

Legend: retention time (RT), mass to charge ratios (*m/z*) for precursor ion and two fragment ions (targets used for internal standards (IS) only recorded one dominant transition), as well as cone voltage (V) and collision energies (C.E.) (eV) required for optimal signal strength; LOD (limit of detection) and LOQ (limit of quantification)

B1-lysin (AFB1-Lys). The group *Alternaria toxins* includes altenuene, altertoxin, alternariol methyl ether (AME), alternariol (AOH), tentoxin, and tenuazonic acid (TA) (only AME, AOH, and TA were measured by UPLC-MS/MS). The group *Diacetoxyscirpenol* includes diacetoxyscirpenol (DAS), mono-acetoxyscirpenol (MAS), and neosolaniol (NEO). The group *Enniatins* includes enniatin A, enniatin A1, enniatin B, and enniatin B1 (EnB1) (only EnB1 was measured by LC-MS/MS). The group *Ergot alkaloids* includes ergocornine (Eco), ergocristine (Ecr), ergokryptine (Ek), ergometrine (Em), ergosine (Es), ergotamine (Et), and their respective inin-epimers (Econ, Ecrn, Ekn, Emn, Esn, and Etn,

respectively). The group *Fumonisins* includes fumonisin B1 (FB1), fumonisin B2 (FB2), and fumonisin B3 (FB3). The group *Ochratoxins* includes ochratoxin A (OTA) and ochratoxin α (OTα). The group *T2 & HT2* includes HT-2 toxin (HT2), T-2 toxin (T2), T-2 toxin triol (T2OH3) and T-2 toxin tetraol (T2OH4). The group *Type-B Trichothecenes* includes 3-acetyl deoxynivalenol (3AcDON), 15-acetyl deoxynivalenol (15AcDON), deoxynivalenol (DON), DON3Glu, deepoxydeoxynivalenol (DOM), deoxynivalenol-3-glucuronide (DON3GlcA), deoxynivalenol-15-glucuronide (DON15GlcA), fusarenone X (F-X), and nivalenol (NIV). The group *Zearalenone* includes alpha zearalenol (αZEL), beta zearalenol (βZEL), zearalanone (ZAN), and zearalenone (ZEN).

2.8. Statistical comparison between mycotoxin exposure measurements

The software package R 3.5.0 (Vienna, Austria) was used for statistical analysis. The percentage agreement in recurring positive or negative detections between measures, as well as Cohen's kappa, were calculated to describe agreement between time points or between types of measurement in terms of positive/negative screening. Cohen's kappa was interpreted according to the guidelines published by Landis and Koch (1977). The number of intersecting positive detections was counted, and their levels were compared by percentage coefficient of variance for measurements of the same type, or Spearman's test for correlation between different types of measurements. Finally, after treating all negative detections as zero, Spearman's test for correlation was applied to compare serum, mean 24-HDR, and mean urine against each other.

For quantitative comparisons, the 24-HDR dataset was used as 'ng per day per kilogram bodyweight', while serum and urinary mycotoxin concentrations were expressed as 'ng/L'. Generalized linear models were constructed for multivariate analysis on log-transformed concentration data, in order to satisfy assumptions of Gaussian distribution. Cohen's kappa was also applied to test for agreement over individuals exceeding median mycotoxin concentrations.

Exposures to groups of mycotoxins were assessed by Kruskal-Wallis *H* test for significant differences between different age brackets, body mass index (BMI), or geographic regions; significant differences between genders was assessed by Wilcoxon signed-rank test. Null hypotheses were rejected, and association considered significant at (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$.

3. Results

3.1. Mycotoxin prevalence

According to their reported diet, all of the EFCOVAL cohort's 600 individuals were exposed to dietary mycotoxins during the two sampling days, as listed in Table 3. Of particular note, the carcinogen AFB1, along with other aflatoxins, and DON, AcDON, OTA, and ZEN were calculated to be present in at least one 24-HDR for all 600 individuals; OTA was present in every individual's diet on both days. Individuals with particularly high exposures to HT2, PAT, STE, T2, or the groups *Aflatoxins*, *Diacetoxyscirpenol*, *Ergot alkaloids*, *Fumonisins*, *Ochratoxin A*, and *Zearalenone*, were all found to exceed the tolerable daily intakes (TDI) per kg body weight.

Serum measurements were able to detect at least one mycotoxin in 261 (97%) out of 268 samples, and 187 (99%) individuals out of 188 were observed to have mycotoxins in their urine, as listed in Table 4. The most prevalent groups were *Aflatoxins* (serum 57%; urine 51%), *Ergot Alkaloids* (serum 43%; urine 56%), *Fumonisins* (serum 42%; urine 40%), *Ochratoxins* (serum 42%; 48%), and *Type B Trichothecenes* (serum 42%; urine 52%). Additionally, PAT (70%) was frequently detected in serum, but was not detected in urine.

3.2. Co-exposure to multiple mycotoxins

As shown in Fig. 1, analysis of 24-HDR determined co-exposure to a minimum of 4 different mycotoxins, with one individual reporting a day's food intake up to 34 different mycotoxins. The most common number of calculated co-exposures from dietary intake in a single day was 12 mycotoxins. Blood samples were observed to contain up to 12 mycotoxins, with 4 co-exposures in 20% of samples being most common. Only 5% of the population tested positive for only one mycotoxin. In urine samples, up to 13 individual targets were co-detected, with a mode of 5 co-detections in 18% of samples, and 4% returning a single detection, only.

Grouping the mycotoxins, as described above, resulted in 8 to 13 co-exposed groups in the dietary analysis, while blood and urine were found contaminated by up to 9 or 8 groups, with 4 or 5 groups being most common, respectively. Among samples that tested positive for at least one mycotoxin, 8% of blood and 4% of urine samples were found contaminated with only 1 group of mycotoxins.

3.3. Demographic variables and mycotoxin exposure

As illustrated in Fig. 2, distributions of detected exposure levels for most groups of mycotoxins were found by Kruskal-Wallis *H* test to be significantly different between countries. Further, several mycotoxin groups (*Aflatoxins*, *DAS*, *Ergot Alkaloids*, *Ochratoxins*, and *T2 & HT2*) were found to significantly differ between countries according to all three measurement types. Nevertheless, few consistent patterns of geographic distribution were observed between measurement types, and the frequent occurrence of outlier data points more than two interquartile ranges away from the median indicate high degrees of variability in detected exposure levels even within countries.

As depicted in **Supplementary File S3**, exposures to groups of mycotoxins were not widely correlated with participants' age, nor body mass index (BMI) according to measurements in biological fluids. Kruskal-Wallis *H* tests identified significant differences in the 24-HDR calculated exposures for some mycotoxin groups and decennial age strata (*Aflatoxins*, *Enniatins*, and *Ochratoxins*), BMI (*Aflatoxins*, *Alternaria Toxins*, *DAS*, *Ochratoxins*, PAT, *T2 & HT2*, *Type B Trichothecenes*, and *Zearalenone*), and gender (*Aflatoxins*, *Alternaria Toxins*, *Ochratoxins*, PAT, STE, and *Type B Trichothecenes*). However, these observations were not consistent across different measurement types.

3.4. Repeated 24-HDR mycotoxin exposure estimates (2× 24-HDR)

Table 5 represents correlations between mycotoxin exposures assessed on two different time points for the EFCOVAL-individuals who completed a 24-HDR on two occasions. Significant correlations for the two 24-HDR were found by Spearman's test for almost all individual and grouped mycotoxins, excepting only CIT, DAS, Ek, PAT, T2, TA, and all individual or grouped members of *Fumonisins* and *Zearalenone* (Table 5).

Supplementary analyses were run to investigate agreement for mycotoxin detection between these two different time points. At least 99% agreement between detection rates at both 24-HDR time points was observed for the mycotoxins AFB1, OTA, PAT, DON, and ZEN, as well as the groups *Aflatoxins*, *Ochratoxins*, *Type B Trichothecenes*, and *Zearalenone* (**Supplementary Table S3**). Nevertheless, screening detection rates were in less than moderate agreement (Cohen's $K \leq 0.4$, Table 5). Noteworthy, when one measurement has little to no variation (*e.g.*, all subjects scoring positive for the mycotoxin), the agreement corrected for chance (K) is considered to be 0, or close to 0, and will require many successive ratings in order to mitigate the chance variable and indicate correlation. This explains the low K-values for mycotoxins, where the agreement is close to 100%.

The amounts calculated to contaminate each individual's diet were found to vary considerably depending on the mycotoxin, with average

Table 3
Dietary mycotoxin exposure estimates as the mean of two 24-hour dietary recall surveys, recorded one month apart.

Mycotoxins		n = 600	Min	Q1	Median	Q3	Max	Min	Q1	Median	Q3	Max	TDI
		Detections (%)	**Mean ng intake per day**					**ng intake per kg body weight (b.w.)**					ng/kg b.w./day
Aflatoxins	**Group Total**	600 (100.0)	9.12	256	647	1330	9520	**0.140**	**3.39**	**8.70**	**18.5**	**107**	n.a.
	AFB1	600 (100.0)	1.75	46.4	429	911	5210	**0.027**	**0.584**	**5.61**	**13.6**	**81.4**	n.a.
	AFB2	600 (100.0)	0.413	15.3	23.0	35.0	473	**0.008**	**0.198**	**0.317**	**0.483**	**5.32**	n.a.
	AFG1	600 (100.0)	1.12	15.8	24.3	35.2	1060	**0.015**	**0.209**	**0.321**	**0.488**	**16.6**	n.a.
	AFG2	600 (100.0)	0.957	15.4	23.7	35.8	129	**0.014**	**0.197**	**0.322**	**0.499**	**2.02**	n.a.
	AFM1	588 (98.0)	0.02	0.749	1.98	14.1	433	**$2.2e^{-4}$**	**0.010**	**0.027**	**0.178**	**6.10**	n.a.
Alternaria Toxins	**Group Total**	598 (99.7)	179	4770	7980	12,100	57,400	2.41	66.1	109	162	787	n.a.
	Altenuene	598 (99.7)	9.00	340	645	1010	4150	0.108	4.39	8.52	14.0	57.5	n.a.
	AOH	598 (99.7)	13.0	405	699	1220	8640	0.161	5.60	9.78	16.6	97.2	n.a.
	AME	598 (99.7)	4.73	114	218	521	8300	0.061	1.57	2.98	6.93	93.4	n.a.
	Altertoxin I	554 (92.3)	5.00	565	1110	1630	4150	0.069	7.78	14.7	22.0	70.6	n.a.
	Tentoxin	597 (99.5)	1.20	171	328	561	6060	0.014	2.24	4.37	7.79	94.7	n.a.
	TA	598 (99.7)	17.8	2360	397	6440	39,700	0.239	32.8	54.8	86.6	557	n.a.
Beauvericin	264 (44.0)	0.30	5.50	11.0	20.8	135	0.004	0.070	0.161	0.307	1.44	n.a.	
CIT	7 (1.2)	2.50	26.6	30.2	30.5	84.0	0.039	0.329	0.356	0.391	1.45	n.a.	
DAS	6 (1.0)	17.0	586	1130	2040	8910	0.315	8.00	15.3	27.0	**97.4**	60	
Enniatins	**Group Total**	330 (55.0)	4.38	130	302	972	281,000	0.055	1.70	4.39	13.0	5160	n.a.
	Enniatin A	330 (55.0)	0.30	7.93	18.9	40.3	234,000	0.004	0.113	0.261	0.565	4290	n.a.
	Enniatin A1	330 (55.0)	1.53	39.8	96.2	207	25,000	0.019	0.565	1.35	2.76	459	n.a.
	Enniatin B	330 (55.0)	0.60	17.9	43.1	227	43,600	0.008	0.236	0.639	2.96	588	n.a.
	EnB1	330 (55.0)	1.95	55.4	126	381	16,100	0.025	0.745	1.85	5.10	244	n.a.
Ergot Alkaloids	**Group Total**	388 (64.7)	60	1170	3070	10,200	55,700	0.698	15.2	41.4	135	**731**	600
	Eco	313 (52.1)	5.00	84.0	200	904	9280	0.058	1.19	2.96	13.2	118	600
	Econ	290 (48.3)	4.44	60.0	138	442	3750	0.058	0.786	1.88	6.14	42.2	600
	Ecr	388 (64.7)	5.00	122	500	1500	12,400	0.058	1.56	6.91	21.2	168	600
	Ecrn	290 (48.3)	4.44	60.0	142	527	3750	0.058	0.786	2.02	6.68	42.2	600
	Ek + Ekn	148 (24.7)	250	800	1670	3290	12,400	2.87	10.0	24.0	49.4	168	600
	Em	274 (45.7)	5.00	68.0	155	740	9280	0.058	0.971	2.22	10.1	118	600
	Emn	251 (41.8)	3.83	45.0	106	500	4690	0.050	0.600	1.44	6.72	52.7	600
	Es	313 (52.2)	5.00	68.3	200	905	9280	0.058	0.981	2.71	12.1	118	600
	Esn	290 (48.3)	5.00	60.0	149	600	3750	0.058	0.786	2.10	8.15	42.2	600
	Et	360 (60.0)	5.00	115	500	1600	14,300	0.058	1.47	6.98	22.9	232	600
	Etn	251 (41.8)	5.00	48.0	117	427	3750	0.058	0.646	1.47	6.02	42.2	600
Fumonisins	**Group Total**	572 (95.3)	6.00	1680	3270	6230	247,000	0.095	23.1	43.7	85.4	**2450**	2000
	FB1	571 (95.2)	3.00	778	1540	2960	201,000	0.048	10.8	20.7	39.8	1990	2000
	FB2	571 (95.2)	3.00	662	1300	2160	32,200	0.048	8.82	17.5	29.6	319	2000
	FB3	225 (37.5)	13.3	131	499	1130	13,300	0.164	1.99	6.67	15.0	132	2000
OTA	600 (100.0)	26.9	237	401	619	4850	0.387	3.07	5.30	8.65	**76.3**	14	
PAT	311 (51.8)	27.5	1430	2180	3250	393,000	0.388	19.1	29.2	45.2	**6150**	400	
STE	68 (11.3)	37.5	125	191	282	1280	**0.583**	**1.84**	**2.47**	**4.07**	**11.3**	n.a.	
T2 & HT2	**Group Total**	591 (98.5)	2.42	1100	2220	4040	26,500	0.029	14.5	30.6	55.4	**290**	60
	HT2	591 (98.5)	1.27	470	980	1900	13,200	0.015	6.38	13.5	25.8	**144**	60
	T2	589 (98.2)	1.15	187	465	1500	13,200	0.014	2.55	6.41	20.1	**144**	60
Type B Trichothecenes	**Group Total**	600 (100.0)	279	9680	17,000	27,700	89,000	3.76	138	234	234	973	1000
	3AcDON	600 (100.0)	18.0	723	1460	2370	10,100	0.191	9.79	19.5	31.9	113	1000
	15AcDON	600 (100.0)	18.0	748	1450	2310	7290	0.191	9.84	19.4	31.8	95.4	1000
	DON	600 (100.0)	42.0	5770	11,400	20,800	86,900	0.566	79.7	155	274	950	1000
	DON3Glu	124 (20.7)	4.00	1290	2910	4810	15,300	0.067	17.8	40.0	63.5	237	1000
	F-X	156 (26.0)	6.38	330	538	922	2913	0.093	4.32	7.43	11.8	32.8	1000
	NIV	596 (99.3)	7.30	471	816	1360	5180	0.089	6.42	11.1	19.6	71.0	1000
Zearalenone	**Group Total**	600 (100.0)	28.0	630	1070	2760	39,100	0.377	8.70	15.0	36.6	**639**	250
	αZEL	41 (6.8)	0.50	16.0	25.0	62.5	481	0.008	0.275	0.347	0.801	6.25	250
	βZEL	41 (6.8)	1.52	25.0	40.0	62.5	481	0.025	0.342	0.610	0.864	6.25	250
	ZAN	11 (1.8)	39.0	47.4	54.6	66.0	94.8	0.486	0.743	0.841	1.02	1.30	250
	ZEN	600 (100.0)	28.0	564	951	2330	38,500	0.377	7.76	13.1	30.1	**629**	250

Legend: Group Totals may include non-specifically reported mycotoxin contaminants from the EFSA database. For mycotoxins both with published and no described tolerable daily intakes (TDI), outstanding values are in grey-bold. n.a.: no TDIs are available or described. Mycotoxin abbreviations are as follows: 3-acetyl deoxynivalenol (3AcDON); 15-acetyl deoxynivalenol (15AcDON); aflatoxin B1 (AFB1); aflatoxin B2 (AFB2); aflatoxin G1 (AFG1); aflatoxin G2 (AFG2); aflatoxin M1 (AFM1); alpha zearalenol (αZEL); alternariol (AOH); alternariol methyl ether (AME); beta zearalenol (βZEL); citrinin (CIT); creatinine (CTN); deepoxydeoxynivalenol (DOM); deoxynivalenol (DON); deoxynivalenol-3-glucoside (DON3Glu); diacetoxyscirpenol (DAS); dihydroergotamine (DHEt); enniatin B1 (EnB1); ergocornine (Eco); ergocristine (Ecr); ergokryptine (Ek); ergometrine (Em); ergosine (Es); ergotamine (Et); and their respective epimers (Econ; Ecrn; Ekn; Emn; Esn; and Etn; respectively); fumonisin B1 (FB1); fumonisin B2 (FB2); fumonisin B3 (FB3); fusarenone X (F-X); HT-2 toxin (HT2); methylergometrine (MeEm); neosolaniol (NEO); nivalenol (NIV); ochratoxin A (OTA); ochratoxin α (OTα); patulin (PAT); roquefortine C (ROQ-C); sterigmatocystin (STE); T-2 toxin (T2); T-2 toxin tetraol (T2OH4); T-2 toxin triol (T2OH3); verrucarol (VER); zearalanone (ZAN); zearalenone (ZEN).

Table 4
Mycotoxin detections in serum samples obtained one or two weeks prior to the first or two pooled 24-hour urine collections, separated by one month are detailed. All concentrations are in ng/L.

Mycotoxins		*n* = 268	Serum					*n* = 188	Mean of Two 24hr Urine Collections				
		Detections (%)	Min	Q1	Median	Q3	Max	Detections (%)	Min	Q1	Median	Q3	Max
Aflatoxins	**Group Total**	154 (57.2)	0.2	16.5	37.9	83.8	8720	95 (50.5)	1e-2	11.7	33.6	155	4730
	AFB1	39 (14.5)	0.1	1.50	9.30	43.4	141	20 (10.6)	1e-2	0.3	5.5	73.6	236
	AFB2	73 (27.1)	1.3	17.5	32.7	62.6	8710	44 (23.4)	1.8	11.7	25.8	140	703
	AFG1	7 (2.6)	3.7	10.3	13.2	122	393	2 (1.1)	23.9	36.8	49.7	62.6	75.4
	AFG2	44 (16.4)	3.5	23.8	63.7	88.5	256	21 (11.2)	1.5	8.2	20.2	53.4	2580
	AFM1	55 (20.4)	0.7	5.3	12.5	58.5	172	33 (17.6)	0.4	10.4	21.5	550	4730
Alternaria Toxins	**Group Total**	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	53 (28.2)	25.0	1860	4040	10,200	28,500
	AME	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	14 (7.4)	134	5430	16,400	20,100	22,400
	AOH	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	14 (7.4)	25.0	319	596	972	1570
	TA	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	39 (20.7)	154	2180	3500	6500	28,500
Citrinin	39 (14.5)	65.1	131	383	9370	59,400	27 (14.4)	60.2	186	618	999	8470	
Diacetoxyscirpenol	**Group Total**	82 (30.5)	0.5	9.8	29.9	123	7940	36 (19.1)	0.3	71.4	132	292	3390
	DAS	61 (22.7)	2.7	13.8	69.7	203	7940	25 (13.3)	4.7	80.8	130	184	504
	NEO	24 (8.9)	0.5	1.5	5.2	20.9	86.3	12 (6.4)	0.3	3.1	168	1550	3390
EnB1	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	81 (43.1)	296	844	1660	7590	17,900	
Ergot Alkaloids	**Group Total**	116 (43.1)	3.5	30.1	63.0	155	3240	106 (56.4)	0.1	37.2	191	942	31,100
	Eco	14 (5.2)	66.6	86.9	158	534	2460	16 (8.5)	16.8	38.9	70.7	436	868
	Econ	38 (14.1)	3.5	27.4	44.4	70.7	787	22 (11.7)	9.6	50.1	74.8	405	1440
	Ecr	19 (7.1)	44.8	73.7	126	163	531	14 (7.4)	16.4	59.2	121	515	1100
	Ecrn	23 (8.6)	7.2	12.1	14.9	18.2	111	18 (9.6)	6.5	14.7	139	752	1250
	Ek	4 (1.5)	11.8	62.8	81.6	84.7	88.9	9 (4.8)	5.4	305	381	626	1090
	Ekn	22 (8.2)	13.3	27.7	46.4	127	431	16 (8.5)	14.4	58.7	482	703	1600
	Em	12 (4.5)	10.7	21.2	26.5	76.5	439	6 (3.2)	15.5	71.5	637	1570	2530
	Emn	19 (17.1)	40.9	59.5	72.6	109	629	10 (5.3)	9.0	1210	5100	9240	29,700
	Es	32 (11.9)	6.2	7.9	10.1	27.8	735	10 (5.3)	2.1	7.5	19.3	99	990
	Esn	30 (11.1)	25.5	30.4	36.6	56.8	468	17 (9.0)	11.1	18.2	33.6	165	3880
	Et	14 (5.2)	27.4	34.1	44.3	57.5	1400	9 (4.8)	0.1	24.7	45.0	104	990
	Etn	1 (0.4)	n.a.	n.a.	61.2	n.a.	n.a.	10 (5.3)	8.1	42.8	75.4	119	236
Fumonisins	**Group Total**	113 (42.0)	683	4370	5370	9250	84,100	76 (40.4)	8.6	773	2770	6200	40,300
	FB1	80 (29.7)	1070	4280	4990	7110	84,100	49 (26.0)	8.6	1350	3230	5430	23,900
	FB2	43 (16.0)	683	4680	5290	5750	31,100	35 (18.6)	38.7	452	2790	5060	25,200
	FB3	14 (5.2)	468	1050	1180	3340	43,000	6 (3.2)	915	1190	3460	7190	17,100
Ochratoxins	**Group Total**	113 (42.0)	81.1	230	548	985	167,000	90 (47.9)	0.8	477	5820	18,700	1,610,000
	OTA	104 (38.7)	68.1	210	454	770	16,500	76 (40.4)	0.8	210	1640	16,100	1,610,000
	OTα	13 (4.8)	3520	4640	29,300	70,800	167,000	48 (25.5)	1.6	1510	3820	12,400	807,000
PAT	187 (69.5)	0.1	37.2	191	942	31,100	0 (0.0)						
ROQ-C	64 (23.8)	2.3	4.3	5.3	10.1	300	13 (6.9)	2.1	3.0	9.4	1810	39,800	
STE	24 (8.9)	15.4	20.0	61.3	315	420	30 (16.0)	4.7	51.5	2020	3670	8280	
T2 & HT2	**Group Total**	98 (36.4)	20.3	55.5	93.9	793	19,200	56 (29.8)	6e-2	44.4	93.5	785	39,600
	HT2	38 (14.1)	20.3	41.0	873	2440	19,200	12 (6.4)	30.0	54.6	484	1090	4660
	T2	68 (25.3)	36.0	54.3	79.1	151	3330	41 (21.8)	6e-2	36.2	55.8	94.7	779
	T2OH3	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	2 (1.1)	1020	10,700	20,300	30,000	39,600
	T2OH4	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	8 (4.3)	703	17,400	24,400	26,700	37,100
Type B Trichothecenes	**Group Total**	113 (42.0)	4.7	230	645	2350	768,000	97 (51.6)	7.5	338	3490	15,600	82,100
	AcDON	22 (8.2)	4.7	27.4	41.1	53.9	1820	44 (23.4)	6.2	104	12,700	21,800	56,900
	DOM	18 (6.7)	378	712	1210	2240	313,000	12 (10.1)	75.6	763	3410	9730	32,700
	DON	38 (14.1)	9.2	70.4	223	431	1390	46 (24.5)	1.4	224	494	3700	9070
	DON3Glu	26 (9.7)	201	286	483	895	10,500	12 (6.4)	996	1650	2130	2800	37,200
	F-X	14 (5.2)	99.0	708	876	1180	15,100	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.
	NIV	22 (8.2)	2710	5340	8090	31,400	454,000	6 (3.2)	2660	7410	9960	17,500	19,600
VER	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	4 (2.1)	1.6	2.5	2.8	3.3	4.7	
Zearalenone	**Group Total**	81 (30.1)	3.9	10,700	20,300	37,600	266,000	52 (27.7)	5.2	3800	13,800	29,700	501,000
	αZEL	36 (13.3)	11,900	30,200	36,100	47,200	175,000	17 (9.0)	549	19,200	27,800	50,900	501,000
	βZEL	46 (17.1)	236	10,100	12,000	14,300	256,000	34 (18.1)	27.6	4860	9070	20,600	116,000
	ZAN	0 (0.0)	n.a.	n.a.	n.a.	n.a.	n.a.	3 (1.6)	2870	14,700	26,500	61,000	95,400
	ZEN	5 (1.9)	3.9	125	157	266	9890	6 (3.2)	5.19	28.0	769	2110	2950

Legend: Mycotoxin abbreviations are as follows: 3-acetyl deoxynivalenol (3AcDON); 15-acetyl deoxynivalenol (15AcDON); aflatoxin B1 (AFB1); aflatoxin B2 (AFB2); aflatoxin G1 (AFG1); aflatoxin G2 (AFG2); aflatoxin M1 (AFM1); alpha zearalenol (αZEL); alternariol (AOH); alternariol methyl ether (AME); beta zearalenol (βZEL); citrinin (CIT); creatinine (CTN); deepoxydeoxynivalenol (DOM); deoxynivalenol (DON); deoxynivalenol-3-glucoside (DON3Glu); diacetoxyscirpenol (DAS); dihydroergotamine (DHEt); enniatin B1 (EnB1); ergocornine (Eco); ergocristine (Ecr); ergokryptine (Ek); ergometrine (Em); ergosine (Es); ergotamine (Et); and their respective epimers (Econ; Ecrn; Ekn; Emn; Esn; and Etn; respectively); fumonisin B1 (FB1); fumonisin B2 (FB2); fumonisin B3 (FB3); fusarenone X (F-X); HT-2 toxin (HT2); methylergometrine (MeEm); neosolaniol (NEO); nivalenol (NIV); ochratoxin A (OTA); ochratoxin α (OTα); patulin (PAT); roquefortine C (ROQ-C); sterigmatocystin (STE); T-2 toxin (T2); T-2 toxin tetraol (T2OH4); T-2 toxin triol (T2OH3); verrucarol (VER); zearalanone (ZAN); zearalenone (ZEN).

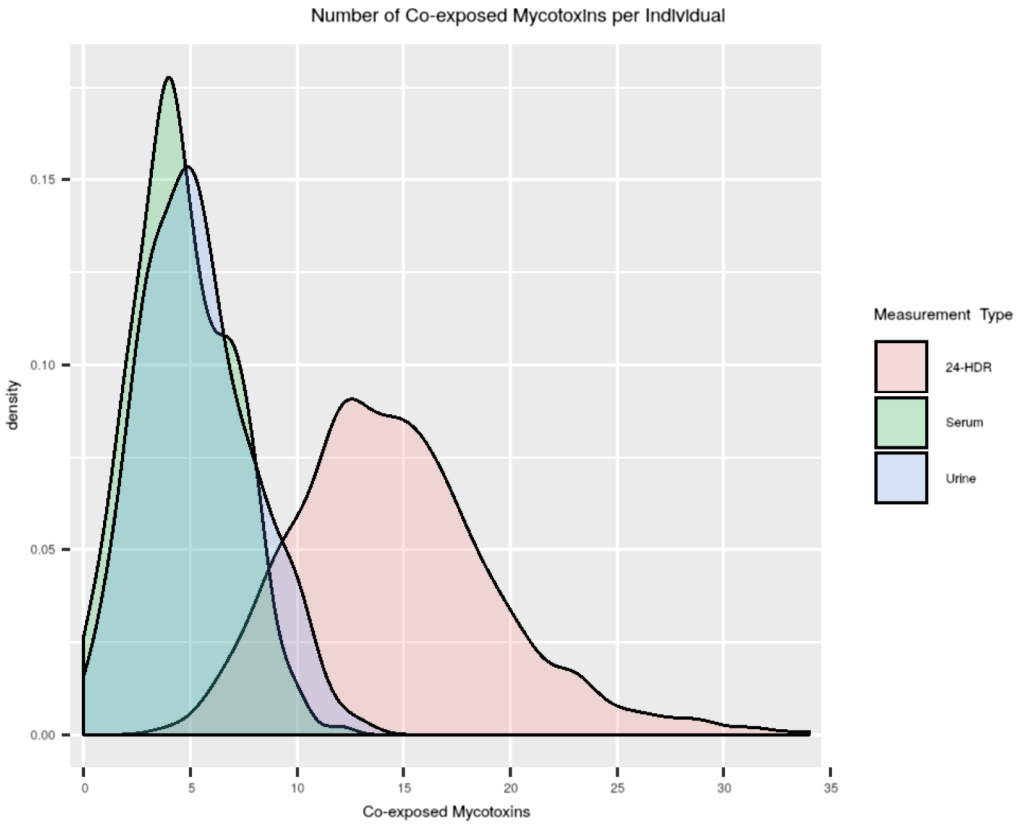

Fig. 1. Kernel density estimate of the number of individual mycotoxins detected together in individual samples according to three methods of assessment: 24-HDR; 24-hour pooled urine and serum. Legend: The density of co-exposure levels within the population is expressed as a decimal fraction for direct comparability between measurement types.

coefficients of variance ranging from 31% for STE to 82% for T2. Nevertheless, incidences of concentrations above the median were moderately to strongly associated ($0.6 \leq K < 0.8$) for AFB1 (0.61), Econ (0.63), Ecrn (0.63), Es (0.64), Esn (0.72), and βZEL (0.62) (**Supplementary File S4**).

3.5. Urinary mycotoxin detections in repeated sampling (2× 24HR Urine)

Mycotoxins were determined in two separately pooled, 24-hour collection urine samples from 125 individuals, obtained at least one month apart. Detection rates across the two time-points were evaluated by Cohen's K and moderate agreements ($0.4 < K \leq 0.6$) were observed for Esn, OTA, and ROQ-C as listed in Table 5. However, less than half of all mycotoxins were found to have better than 90% agreement in terms of screening between the two time points. On average, only 7.4% of individuals tested positive for the same groups of mycotoxins in both urine samples. Further, the mean percentage coefficient of variance between the concentrations detected at each time point were found to exceed 50% for almost all mycotoxins, except βZEL (40%), AOH (16%), DON (33%), and T2 (37%) (**Supplementary File S4**). Incidence of concentrations above the median were strongly associated ($K = 0.81$) between time points for EnB1, and moderately associated for the groups *Aflatoxins* and *T2 & HT2*, as well as T2 individually (**Supplementary File S4**). Nevertheless, counting negative detections as 0, and applying Spearman's test identified αZEL, AFG2, AFM1, EnB1, Es, Esn, OTA, ROQ-C, T2, 15AcDON, and DON as significantly correlated between time points (Table 5).

3.6. Comparison of mycotoxin detections between 24-HDR and 24-hour pooled urine collection (24-HDR VS 24HR Urine)

Participants collected urine for 24 h before submitting a 24-HDR covering that day's diet. Detection rates of grouped mycotoxins were only in slight or negative agreement, with Cohen's K calculated at less than 0.2 for all mycotoxins (Table 5). There was at least 80% agreement in detection rates between the two measurement types for the individual mycotoxins CIT, FB2, STE, DON3Glu, αZEL, βZEL, and ZAN, though the average agreement for all mycotoxins was only 33%. For quantitative analyses, some significant correlations were found for AFG1 (0.14), AFG2 (0.12), HT2 (0.12), DON (0.16) (Table 5). However, applying Spearman's test to intersecting positive detections only, there was no significant positive correlation observed between actual levels measured of any individual or grouped mycotoxins.

For 188 participants, mycotoxin detections at up to two time points could be averaged, and the mean 24-HDR concentrations compared to the mean urine concentrations, as listed in Table 6. The two types of measurements did not agree more than slightly ($K \leq 0.2$) on detection rates according to Cohen's K, despite high percentage agreement for CIT (84%), FB3 (80%), αZEL (85%), and ZAN (96%) (Table 6). Spearman's test did not find any significant positive correlation between any detected levels (Table 6).

3.7. Comparison of mycotoxin detections between mean 24-HDR and serum

The mycotoxin detection profiles obtained from 268 individuals' serum samples were compared with 24-HDR-mycotoxin exposure estimates, as listed in Table 6. Rates of detection agreed slightly or negatively ($K < 0.1$), according to Cohen's K for any mycotoxins, except for STE ($K = 0.12$), which also had 81% agreement between the measurement types (Table 6). Further, Spearman's test found significant correlations for Et ($\rho = 0.15$), OTA ($\rho = 0.13$), STE ($\rho = 0.13$), and AcDON ($\rho = 0.15$). After grouping mycotoxins, detected levels were not found by Spearman's test to be significantly positively associated ($p < 0.05$), nor was the incidence of levels above the median found by Cohen's kappa to agree between mean 24-HDR and serum (**Supplementary File S5**).

Fig. 2. Comparison of mycotoxin groups' exposure concentrations detected in samples from each country. Legend: The mycotoxin exposure is annotated as the exposure levels of the mycotoxin groups. Significantly different distributions were identified by the Kruskal-Wallis *H* test. The probability cut-offs for significantly independent groups were set at (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. The boxplots of mycotoxin concentrations illustrate the interquartile range as a box, with a black bar indicating the median, and dashed extensions to the lowest and highest detected concentrations, excepting outliers further than 1.5 interquartile ranges above the upper quartile or below the lower quartile. Additionally, a scatterplot alongside serves to illustrate sample size, patterns of distribution, and outliers. Each mycotoxin group was assessed by Kruskal-Wallis H test for significant differences between demographic groups; significant differences between genders was assessed by Wilcoxon signed-rank test. Null hypotheses were rejected, and association considered significant at (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$; (****) $p < 0.0001$.

3.8. Comparison of mycotoxin detections between serum and urine (serum VS mean 24HR urine)

Levels of mycotoxins in 147 serum samples, which were taken on average one or two weeks prior to the first urine collections, were compared to the average in urine from the same individuals, as listed in Table 6. Comparing detection rates between the two biological sample types yielded moderate associations ($0.4 < K \leq 0.6$) for the individual mycotoxins AFB2, AFG1, AFG2, AFM1, DAS, NEO, Ecrn, Esn, FB1, T2, βZEL, and ZEN, as well as the groups *Aflatoxins*, *Diacetoxyscirpenol*, *Fumonisins*, and *T2 & HT2* (Table 6). All of the individual mycotoxins that scored highly on Cohen's K were also found to have > 80% agreement between serum and urine detection rates. Spearman's test found significant positive correlations ($0.18 \leq \rho \leq 0.58$) for almost all individual and grouped mycotoxins (Table 6). Most notably excepted was OTA and the *Ochratoxins* group, though OTα ($\rho = 0.31$) was correlated. Other individual mycotoxins for which significant correlations were not found included CIT, Eco, Ekn, Em, Emn, Etn, and DON3Glu.

Total detected amounts of the grouped mycotoxins were found by Spearman's test to be significantly correlated between blood and urine samples for the groups *Aflatoxins* (Spearman's rho (ρ) = 0.32), *Fumonisins* (ρ = 0.32), *Ochratoxins* (ρ = 0.43), and *Type B Trichothecenes* (ρ = 0.66) (**Supplementary File S5**). Moderate and substantial agreements on incidence of levels above the median were calculated by Cohen's kappa for the groups *Type B Trichothecenes* (K = 0.57) and *Zearalenone* (K = 0.67), respectively (**Supplementary File S5**).

4. Discussion

Mycotoxin contamination is a ubiquitous problem, which is confirmed in the present study by three different methods. Accurate assessments of the contamination level are vital to understanding how dietary mycotoxins may affect individual health. Unfortunately, the three different measurements illustrate the relative outcome discrepancies of these measures, either individually or taken together, for determining dietary mycotoxin exposure in the individual.

4.1. Demographic variables and mycotoxin exposure

The significant differences identified between grouped mycotoxin exposure levels in different countries may be influenced by a wide range of factors. Distinct dietary habits may be investigated to determine if specific regional foods are vectors for mycotoxin exposure. Additionally, this may indicate differences in the presence of mycotoxigenic fungi and subsequent mycotoxin expression due to environmental characteristics (Van Der Fels-Klerx et al., 2012). The colder climate of Norway also affects local agricultural production. The import of agricultural products extends the supply chain, which potentially increases the risk of mycotoxin contamination (Magan and Aldred, 2007). Interestingly, biological sampling (serum and urine) did not differentiate Norwegians, leading to two possible hypotheses: either the distribution of mycotoxins in FoodEx1 standard classifications of food items was not accurately described prior to assessing dietary mycotoxin levels, or the mycotoxins were not consumed on the days when biological samples were taken. The significantly higher total mycotoxin levels of Belgian urine and French serum samples may be affected by

Table 5
Statistical comparisons between repeated mycotoxin exposure measurements taken one month apart, either from 24-hour dietary recall (24-HDR) surveys, or in pooled 24-hour urine collections. 24-HDR was compared to 24-hour urine from the same day; each timepoint was compared separately.

Mycotoxins		2x 24-HDR, n = 595				2x 24hr Urine, n = 125				24-HDR vs 24hr Urine, n = 313			
		K	% agr	ρ	+ve (%)	K	% agr	ρ	+ve (%)	K	% agr	ρ	+ve (%)
Aflatoxins	**Group total**	0	99.8	0.69***	594 (99.8)	−0.01	59.2	0.08	9 (7.2)	0	33.4	0.12	103 (32.9)
	AFB1	0	99.5	0.73***	592 (99.5)	−0.05	88.0	−0.05	0 (0.0)	0	7.2	−0.09	20 (6.4)
	AFB2	−0.01	98.5	0.31***	586 (98.5)	0.11	81.6	0.12	3 (2.4)	0	16.8	0.09	47 (15.0)
	AFG1	−0.01	98.5	0.81***	586 (98.5)	0	98.4	n.a.	0 (0.0)	0	12.1	0.14*	26 (8.3)
	AFG2	−0.01	98.5	0.22***	586 (98.5)	0.18	89.6	0.18*	2 (1.6)	0	8.0	0.12*	23 (7.3)
	AFM1	0.38	94.8	0.25***	553 (92.9)	0.26	87.2	0.27*	4 (3.2)	0	17.0	0.11	35 (11.2)
***Alternaria* Toxins**	**Group total**	0.15	96.6	0.20***	573 (96.3)	0.13	72.8	0.12	7 (5.60)	−0.02	20.0	0	58 (18.5)
	Altenuene	0.05	93.1	0.23***	552 (92.8)	Not included				Not included			
	AOH	0.11	95.6	0.25***	567 (95.3)	0.10	90.4	0.10	1 (0.80)	0	7.3	0.08	14 (4.5)
	AME	0.10	95.3	0.14***	565 (95.0)	0.12	91.2	0.16	1 (0.80)	0	8.0	0.03	15 (4.8)
	Altertoxin I	0.17	92.1	0.20***	540 (90.8)	Not included				Not included			
	Tentoxin	0.06	91.3	0.27***	540 (90.8)	Not included				Not included			
	TA	0.09	95.1	0.03	564 (94.8)	0.13	80.0	0.14	4 (3.2)	0	15.9	0	42 (13.4)
Beauvericin		0.33	71.9	0.28*	94 (15.8)	Not included							
Citrinin		0	99.0	n.a.	0 (0.0)	−0.10	81.6	−0.10	0 (0.00)	−0.02	90.1	−0.01	1 (0.30)
Diacetoxyscirpenol	**Group total**	n.a.				0.04	87.2	0.06	3 (2.40)	n.a.			
	DAS	−0.02	96.5	0.06	570 (95.8)	−0.03	93.6	−0.03	0 (0.0)	0	9.8	0.09	24 (7.7)
	MAS	Not included				−0.02	95.2	n.a.	0 (0.0)	n.a.			
	NEO	Not included				0.30	93.6	0.15	2 (1.60)	n.a.			
Enniatins	**Group total**	0.25	65.0	0.69***	119 (20.0)	n.a.				n.a.			
	Enniatin A	0.25	65.0	0.99***	119 (20.0)	Not included				Not included			
	Enniatin A1	0.25	65.0	0.91***	119 (20.0)	Not included				Not included			
	Enniatin B	0.25	65.0	0.51***	119 (20.0)	Not included				Not included			
	EnB1	0.25	65.0	0.59***	119 (20.0)	0.22	64.0	0.21*	22 (17.6)	−0.14	50.0	−0.03	25 (8.0)
Ergot Alkaloids	**Group total**	0.17	59.0	0.54**	141 (23.7)	−0.03	52.0	0.08	16 (12.8)	−0.01	52.5	−0.02	65 (20.8)
	Eco	0.22	64.7	0.88***	100 (16.8)	−0.04	92.0	−0.04	0 (0.0)	0	61.7	−0.03	6 (1.9)
	Econ	0.24	67.1	0.71***	92 (15.5)	−0.08	84.8	−0.08	0 (0.0)	0	59.2	−0.05	5 (1.6)
	Ecr	0.17	59.0	0.28*	141 (23.7)	−0.03	92.8	−0.04	0 (0.0)	−0.02	49.2	−0.05	4 (1.3)
	Ecrn	0.24	67.1	0.71***	92 (15.5)	−0.06	89.6	−0.06	0 (0.0)	0.02	64.4	−0.05	11 (3.5)
	Ek	0.29	81.7	0.22	36 (6.1)	−0.02	94.4	−0.03	0 (0.0)	−0.05	78.4	−0.04	2 (0.6)
	Ekn	0.29	81.7	0.22	36 (6.1)	0.07	88.8	0.06	1 (0.8)	−0.05	78.6	−0.04	2 (0.6)
	Em	0.23	67.9	0.72***	80 (13.4)	−0.02	95.2	−0.02	0 (0.0)	0	69.3	−0.02	4 (1.3)
	Emn	0.24	70.3	0.74***	72 (12.1)	−0.03	92.8	−0.03	0 (0.0)	0.05	67.4	−0.03	3 (1.0)
	Es	0.22	64.7	0.77***	100 (16.8)	0.30	93.6	0.29**	2 (1.6)	0	62.1	−0.03	5 (1.6)
	Esn	0.24	67.1	0.70***	92 (15.5)	0.52	96.0	0.50***	3 (2.4)	0.10	65.6	−0.05	14 (4.5)
	Et	0.16	59.8	0.51***	118 (19.8)	−0.02	94.4	−0.03	0 (0.0)	0	56.1	−0.05	6 (1.9)
	Etn	0.24	70.3	0.74***	72 (12.1)	−0.03	93.6	−0.03	0 (0.0)	0.09	68.8	−0.03	4 (1.3)
Fumonisins	**Group total**	0.13	76.1	0.12	426 (71.6)	0	61.6	0.08	8 (6.4)	−0.01	34.3	0.08	70 (22.4)
	FB1	0.13	75.6	−0.01	422 (70.9)	−0.05	72.8	−0.06	2 (1.6)	0.01	72.3	0.06	45 (14.4)
	FB2	0.13	75.6	0.05	422 (70.9)	0.09	80.0	0.06	3 (2.4)	−0.01	80.2	0.02	31 (9.9)
	FB3	0.15	70.3	0.31	47 (7.9)	0	98.4	n.a.	0 (0.0)	0.02	69.2	−0.02	1 (0.3)
Ochratoxins	**Group total**	n.a.				0.37	70.4	0.44***	26 (20.8)	n.a.			
	OTA	0	100	0.09*	595 (100.0)	0.46	76.0	0.53***	26 (20.8)	0	33.2	−0.02	103 (32.9)
	OTα	Not included				−0.13	73.6	−0.14	0 (0.0)	n.a.			
PAT		0.19	98.7	0.01	586 (98.5)	Not detected				Not detected			
ROQ-C		Not included				0.44	94.4	0.50***	3 (2.40)	Not detected			
STE		0.27	90.9	0.83***	13 (2.20)	0.12	86.4	0.14	2 (1.60)	−0.04	81.3	−0.03	2 (0.60)
T2 & HT2	**Group total**	0.18	72.3	0.10*	384 (64.5)	0.39	83.2	0.18*	10 (8.0)	0.01	27.4	0.08	61 (19.5)
	HT2	0.09	85.5	0.10*	500 (84.0)	−0.03	95.2	−0.02	0 (0.0)	0	12.1	0.12*	11 (3.5)
	T2	0.13	85.9	0.08	500 (84.0)	0.39	87.2	0.37***	7 (5.60)	0	23.8	0.09	46 (14.7)
	T2OH3	Not included				0	98.4	n.a.	0 (0.0)	n.a.			
	T2OH4	Not included				−0.02	96.0	−0.02	0 (0.0)	n.a.			
Type B Trichothecenes	**Group total**	0	99.7	0.36***	593 (99.7)	0.12	59.2	0.12	19 (15.2)	0	37.7	0.06	116 (37.1)
	3AcDON	−0.04	92.4	0.22***	550 (92.4)	−0.03	92.0	−0.03	0 (0.0)	0	8.0	0.03	16 (5.1)
	15AcDON	−0.04	92.4	0.22***	550 (92.4)	0.26	87.2	0.26	4 (3.20)	0.01	15.3	0.03	35 (11.2)
	DOM	Not included				0.03	86.4	0.03	1 (0.80)	n.a.			
	DON	0	99.2	0.17***	590 (99.2)	0.25	78.4	0.25	8 (6.4)	0	17.3	0.16*	54 (17.3)
	DON3Glu	0.28	84.0	0.53**	28 (4.7)	−0.04	92.0	−0.04	0 (0.0)	−0.02	83.0	−0.05	2 (0.6)
	F-X	0.38	82.4	0.54*	50 (8.4)	Not detected				n.a.			
	NIV	0.17	94.8	0.20***	560 (94.1)	−0.01	96.8	−0.01	0 (0.0)	0	3.2	0.01	6 (1.9)

(*continued on next page*)

Table 5 (*continued*)

Mycotoxins		2x 24-HDR, n = 595				2x 24hr Urine, n = 125				24-HDR vs 24hr Urine, n = 313			
		K	% agr	ρ	+ve (%)	K	% agr	ρ	+ve (%)	K	% agr	ρ	+ve (%)
VER		Not included				0	98.4	0	0 (0.0)	n.a.			
Zearalenone	**Group total**	0	99.5	0.32	592 (99.5)	0.02	69.6	0.02	5 (4.0)	0	19.8	0	60 (19.2)
	αZEL	0.19	94.1	0.81	5 (0.8)	0.18	89.6	0.18	2 (1.6)	0	89.1	−0.02	1 (0.3)
	βZEL	0.19	94.1	0.18	5 (0.8)	−0.04	80.0	−0.04	1 (0.8)	−0.04	84.3	−0.02	1 (0.3)
	ZAN	−0.01	98.3	n.a.	0 (0.0)	−0.01	97.6	−0.01	0 (0.0)	−0.01	98.4	−0.01	0 (0.0)
	ZEN	0	99.2	−0.02	590 (99.2)	0	96.8	0	0 (0.0)	0	2.2	0.05	6 (1.9)

Legend: The percentage of recurring positive or recurring negative detections (% agreement) and Cohen's kappa (K) describe agreement between time points in terms of positive/negative screening. Counting non-detections as zero, Spearman's rho (ρ) was calculated. Significant correlations are denoted * for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$. The number of individuals (%) of intersecting positive detections was also counted (+ve). Mycotoxin abbreviations are as follows: 3-acetyl deoxynivalenol (3AcDON); 15-acetyl deoxynivalenol (15AcDON); aflatoxin B1 (AFB1); aflatoxin B2 (AFB2); aflatoxin G1 (AFG1); aflatoxin G2 (AFG2); aflatoxin M1 (AFM1); alpha zearalenol (αZEL); alternariol (AOH); alternariol methyl ether (AME); beta zearalenol (βZEL); citrinin (CIT); creatinine (CTN); deepoxydeoxynivalenol (DOM); deoxynivalenol (DON); deoxynivalenol-3-glucoside (DON3Glu); diacetoxyscirpenol (DAS); dihydroergotamine (DHEt); enniatin B1 (EnB1); ergocornine (Eco); ergocristine (Ecr); ergokryptine (Ek); ergometrine (Em); ergosine (Es); ergotamine (Et); and their respective epimers (Econ; Ecrn; Ekn; Emn; Esn; and Etn; respectively); fumonisin B1 (FB1); fumonisin B2 (FB2); fumonisin B3 (FB3); fusarenone X (F-X); HT-2 toxin (HT2); methylergometrine (MeEm); neosolaniol (NEO); nivalenol (NIV); ochratoxin A (OTA); ochratoxin α (OTα); patulin (PAT); roquefortine C (ROQ-C); sterigmatocystin (STE); T-2 toxin (T2); T-2 toxin tetraol (T2OH4); T-2 toxin triol (T2OH3); verrucarol (VER); zearalanone (ZAN); zearalenone (ZEN).

short-term fluctuations in actual mycotoxin consumption, or even uncharacterized cultural differences in food preparation. The manifold variables affecting differential contamination of consumed foods, as compared to the single median EFSA value taken to represent all five European countries, remain to be fully elucidated, as well as factors affecting mycotoxin uptake, metabolism, and excretion (*i.e.*, mycotoxicokinetics).

4.2. Comparison of external and internal mycotoxin exposure estimates

In the present study, dissimilar results were obtained between 24-HDR and biological sampling (Gerding et al., 2014; Ritieni et al., 2010). The primary explanation for this is the difference between the cross-sectional measurements, both spatially and temporally. EFSA contamination data (24-HDR) in raw agricultural commodities are regional and seasonal, while biological samples illustrate heterogeneity of distribution across individuals and over the time course of ingestion, metabolism, and excretion. This supports the contrast in a number of co-exposed mycotoxins detected between dietary mycotoxin exposure by 24-HDR and biological fluids (Fig. 2). On average, the study population was found to have one third as many different mycotoxins co-detected in biological samples, as compared to the number of mycotoxins calculated by 24-HDR.

The estimation of dietary exposure by means of 24-HDR relied on mycotoxin occurrence data from EFSA, which are regionally representative and contemporary to the sampling period. This instrument was validated in assessments of both nutrients and pesticide contaminants (Crispim et al., 2011; van Klaveren et al., 2012). Nutrients are regularly produced within food crops, and pesticides are relatively uniformly applied to crops, but also rapidly degrade (Darko and Akoto, 2008). By contrast, mycotoxin contamination is heterogeneously distributed, yet are reportedly very stable, and degradation often requires extremes of temperature and pressure (De Ruyck et al., 2015; Milani, 2013; Milani and Maleki, 2014; Schaafsma and Hooker, 2007). The mechanisms of production and distribution of these 3 classes of food-borne molecules differ substantially. As a consequence, tools for assessing food-distribution should be considerably tailored in order to describe any one of them accurately.

Additionally, some mycotoxins are metabolized into different forms as they pass from the blood to the urine, further adding the complexity that must be targeted in order to survey a population effectively (Nathanail et al., 2015). For 12 individual mycotoxins (AFB2, AFG1, AFG2, AFM1, DAS, NEO, Ecrn, Esn, FB1, FB2, T2 & ZEN) detection rates between blood and urine agreed well according to both Cohen's K and percentage agreement (Table 6, serum vs. mean 24hr urine). Further, almost all mycotoxins were significantly positively associated as shown by the Spearman's rho. This serves as a confirmation that the variable distribution of mycotoxin contaminants in the food supply can be described by surveys at the level of the individual. Observing significant correlations across almost all mycotoxins, when comparing serum to the 2 urinary measurements, with all three samples taken weeks apart, also strongly supports chronic exposure of the study population. Further, observing significant correlations in individuals between detectable levels of mycotoxins in their blood and urine implies that it is possible to identify and validate exposure in a consistent way between two different types of measurements.

Nevertheless, the quantitative levels of mycotoxin contamination described by biological sampling did not correlate well with the 24-HDR data. Mycotoxin exposure is represented homogenously over 24 h. However, mycotoxins are only transiently present in each biological fluid, and residency periods vary wildly (Yang et al., 2015; Reddy and Bhoola, 2010). This temporal heterogeneity may serve to partly explain the discrepancy in detection rates between 24-HDR and biological samples. In order to detect a mycotoxin in a biological fluid sample, that fluid must be sampled at a specific time when the mycotoxin has been consumed. In order to correlate with a 24-HDR, it would be necessary to ensure biological sampling takes place at the correct time after consumption of the dietary contaminant.

Unfortunately, the optimal time to take a single blood sample would not be uniform across various mycotoxins with varying rates of metabolism. Fortunately, continuous collection of urine samples is somewhat more accessible than multiple venipunctures per day and can be used to address this issue. In the present study, urine was collected over the same 24 h covered by the 24-HDR. However, urine collected early in the day would be the product of the previous night's dinner. Some mitigation against this confounding factor can be found in the study design selecting an age group that is expected to lead relatively regular lifestyles. For example, several type B trichothecenes are eliminated from the blood and urine within 6 h after ingestion (Vidal et al., 2018). Therefore, DON-contaminating foods eaten with dinner on the night, prior to recording a 24-HDR, may be excreted in the collected urine sample; the opposite situation is also possible with cereal-based products reported consumed late in the 24-HDR not being excreted until after the urine collection period. Another observation is the correlation of 4 mycotoxins (AFG1, AFG2, DON and HT2) between 24-HDR and urine when comparing single days' measurements (Table 5), while comparing the two-day means of each method did not yield significant positive correlations (Table 6). Hence, the true effect of this

Table 6
Statistical comparisons over intersecting populations between measurement types. The mean 24-HDR for each individual was compared to their mean 24-hour urine and to the serum measurement, where available.

Mycotoxins		Mean 24-HDR vs Serum, n = 268				Mean 24-HDR vs mean 24hr Urine, n = 188				Serum vs mean 24hr Urine, n = 147			
		K	% agr	ρ	+ve (%)	K	% agr	ρ	+ve (%)	K	% agr	ρ	+ve (%)
Aflatoxins	**Group total**	0.01	58	−0.11	154 (57)	0	51	−0.01	97 (52)	0.52	76	0.49***	60 (41)
	AFB1	0	15	0.01	39 (15)	0	11	−0.01	20 (11)	0.31	84	0.23***	8 (5)
	AFB2	0	27	−0.03	73 (27)	0	23	−0.01	44 (23)	0.44	80	0.43***	19 (13)
	AFG1	0	3	0.02	7 (3)	0	1	−0.07	2 (1)	0.49	99	0.49***	1 (1)
	AFG2	0	16	0.03	44 (16)	0	11	0.20	21 (11)	0.54	90	0.51***	11 (7)
	AFM1	0	22	0	54 (20)	0	20	0	32 (17)	0.56	84	0.57***	22 (15)
Alternaria Toxins	**Group total**	Not detected in serum				−0.01	28	0.06	54 (29)	Not detected in serum			
	AME					0	8	−0.08	14 (7)				
	AOH					0	8	0.03	14 (7)				
	TA					−0.01	20	0.09	38 (20)				
CIT		0.05	85	0.09	2 (1)	−0.04	84	−0.06	2 (1)	0.03	78	0	3 (2)
Diacetoxyscirpenol	**Group total**	0	31	0.01	84 (31)	0	19	0.05	38 (20)	0.40	76	0.52***	21 (14)
	DAS	0	23	0	61 (23)	0	13	0.05	25 (13)	0.43	80	0.51***	17 (12)
	NEO	Not included in 24-HDR dataset				Not included in 24-HDR dataset				0.41	93	0.40***	4 (3)
Enniatin B1		Not detected in serum				−0.17	41	−0.20**	36 (19)	Not detected in serum			
Ergot Alkaloids	**Group total**	0.03	51	0.09	97 (36)	−0.08	53	−0.08	79 (42)	0.18	58	0.28***	58 (39)
	Eco	0.01	60	0	6 (2)	−0.06	55	−0.07	4 (2)	−0.03	90	−0.04	0 (0)
	Econ	−0.05	58	−0.04	11 (4)	−0.02	56	0	8 (4)	0.29	84	0.28***	7 (5)
	Ecr	0	48	0.01	10 (4)	−0.02	44	−0.05	7 (4)	0.29	92	0.30***	3 (2)
	Ecrn	−0.03	60	−0.03	7 (3)	0.02	59	0.02	8 (4)	0.44	93	0.43***	5 (3)
	Ek	0	1	−0.10	4 (1)	0	5	−0.05	9 (5)	0.27	97	0.27***	2 (1)
	Ekn	0.03	74	0.02	6 (2)	−0.11	66	−0.15	1 (1)	−0.01	93	−0.02	0 (0)
	Em	0.03	73	0.06	4 (1)	0.01	72	0.03	2 (1)	−0.02	96	−0.02	1 (1)
	Emn	0.02	74	0.03	5 (2)	0.01	71	0.04	3 (2)	0.13	93	0.12	0 (0)
	Es	−0.03	57	−0.05	11 (4)	0	58	0	4 (2)	0.21	88	0.25*	3 (2)
	Esn	0	61	0.03	11 (4)	0.08	61	0.13	10 (5)	0.49	92	0.49***	7 (5)
	Et	0.07	60	0.15*	10 (4)	−0.03	52	−0.02	3 (2)	0.28	91	0.27***	3 (2)
	Etn	−0.01	77	−0.03	0 (0)	0.09	73	0.13	5 (3)	0	97	n.a.	0 (0)
Fumonisins	**Group total**	0.01	43	0.03	113 (42)	0	55	0.04	76 (40)	0.40	71	0.39***	40 (27)
	FB1	0	32	0.04	75 (28)	0	29	0.03	46 (24)	0.47	79	0.46***	25 (17)
	FB2	0	19	−0.07	40 (15)	−0.01	22	0	32 (17)	0.22	78	0.24**	9 (6)
	FB3	0.05	79	0.07	4 (1)	0.05	80	0.07	0 (0)	0.38	96	0.39***	1 (1)
Ochratoxins	**Group total**	0.01	43	0.09	115 (43)	0	48	0.14	92 (49)	0.06	55	−0.05	24 (16)
	OTA	0	39	0.13*	104 (39)	0	40	0.13	76 (40)	0.05	56	−0.07	22 (15)
	OTα	Not included in 24-HDR dataset				Not included in 24-HDR dataset				0.14	86	0.31***	2 (1)
ROQ-C		Not included in 24-HDR dataset				Not included in 24-HDR dataset				0.14	86	0.31***	2 (1)
STE		0.12	81	0.13*	7 (3)	−0.04	4 3	−0.06	4 (2)	0.18	84	0.18*	4 (3)
T2 & HT2	**Group total**	0.01	38	0.07	100 (37)	0.02	31	0	58 (31)	0.57	82	0.53***	32 (22)
	HT2	0	15	0	37 (14)	0	9	−0.04	12 (6)	0.31	93	0.32***	3 (2)
	T2	0.01	27	0.05	68 (25)	0.01	24	0	41 (22)	0.53	82	0.55***	25 (17)
Type B	**Group total**	−0.01	42	−0.01	114 (43)	0	52	0.04	99 (53)	0.37	69	0.28***	38 (26)
Trichothecenes	**AcDON**	0	8	0.15*	22 (8)	0	23	−0.06	44 (23)	0.32	86	0.30***	7 (5)
	DOM	Not included in 24-HDR dataset				Not included in 24-HDR dataset				0.25	90	0.32**	3 (2)
	DON	0	14	−0.06	38 (14)	0	24	−0.05	46 (24)	0.39	80	0.37***	15 (10)
	DON3Glu	0.07	75	0.07	8 (3)	−0.05	78	−0.06	1 (1)	−0.05	90	−0.05	2 (1)
	F-X	0.05	75	0.06	5 (2)	Not detected in urine				Not detected in urine			
	NIV	−0.01	8	0.02	21 (8)	0	3	0.09	6 (3)	0.21	92	0.23*	2 (1)
Zearalenone	**Group total**	0.01	30	−0.03	83 (31)	0	28	0.03	54 (29)	0.30	70	0.29***	24 (16)
	αZEL	−0.04	82	−0.06	2 (1)	−0.08	85	−0.08	0 (0)	0.03	78	0.36***	8 (5)
	βZEL	0.12	82	0.14	6 (2)	−0.05	77	−0.06	0 (0)	0.43	80	0.23*	11 (7)
	ZAN	Not detected in serum				−0.02	96	−0.02	0 (0)	Not detected in serum			
	ZEN	−0.05	2	−0.05	5 (2)	0	4	−0.02	6 (3)	0.49	99	0.58***	2 (1)

Legend: Cohen's kappa (K) +/- describes agreement between methods in terms of mycotoxin detection rate, and Spearman's rho (ρ) total describes associations after values below quantification were replaced with zero; significant correlations are denoted * for p ≤ 0.05; ** for p ≤ 0.01; *** for p ≤ 0.001. The number of individuals (%) of individuals who tested positive to both of each pair of measurement types is also listed (2x + ve). Mycotoxin abbreviations are as follows: 3-acetyl deoxynivalenol (3AcDON); 15-acetyl deoxynivalenol (15AcDON); aflatoxin B1 (AFB1); aflatoxin B2 (AFB2); aflatoxin G1 (AFG1); aflatoxin G2 (AFG2); aflatoxin M1 (AFM1); alpha zearalenol (αZEL); alternariol (AOH); alternariol methyl ether (AME); beta zearalenol (βZEL); citrinin (CIT); creatinine (CTN); deepoxydeoxynivalenol (DOM); deoxynivalenol (DON); deoxynivalenol-3-glucoside (DON3Glu); diacetoxyscirpenol (DAS); dihydroergotamine (DHEt); enniatin B1 (EnB1); ergocornine (Eco); ergocristine (Ecr); ergokryptine (Ek); ergometrine (Em); ergosine (Es); ergotamine (Et); and their respective epimers (Econ; Ecrn; Ekn; Emn; Esn; and Etn; respectively); fumonisin B1 (FB1); fumonisin B2 (FB2); fumonisin B3 (FB3); fusarenone X (F-X); HT-2 toxin (HT2); methylergometrine (MeEm); neosolaniol (NEO); nivalenol (NIV); ochratoxin A (OTA); ochratoxin α (OTα); patulin (PAT); roquefortine C (ROQ-C); sterigmatocystin (STE); T-2 toxin (T2); T-2 toxin tetraol (T2OH4); T-2 toxin triol (T2OH3); verrucarol (VER); zearalanone (ZAN); zearalenone (ZEN

confounding mechanism remains to be clearly elucidated.

Also, in comparing biological samples with 24-HDR exposures, another key variable is the rate of uptake. Mycotoxins vary wildly in rates of intestinal absorption, and these rates may be further affected by co-exposures with other mycotoxins, or even varying dietary composition (Gonzalez et al., 2013; Grenier and Oswald, 2011). Reviews report the complexity to assess the effects of dietary composition on mycotoxin absorption (Gonzalez et al., 2013; Grenier and Oswald, 2011). Hence, models to accurately predict the intestinally absorbed fraction of oral mycotoxin intake remain to be constructed. The development of such models has implications for assessments of risk associated with mycotoxin contamination (Mengelers et al., 2018).

Over time, it is also possible for some mycotoxins to accumulate in the blood, which is gradually renewed over the course of several weeks, in contrast to urine, which is generally excreted within hours of production. The associations observed between the presence of some mycotoxins in the blood and urine samples taken several weeks later supports assumptions regarding the regularity of diet, as well as contamination.

Finally, besides contaminants, the 24-HDR instrument is also evaluated in literature with regard to *e.g.*, sodium consumption (McLean et al., 2018). Interestingly, on consideration of 14 studies validating 24-HDR, this systematic review recommended urinary analyses remain the gold standard to assess individuals' intake of dietary substances. It was also suggested that up to seven samples taken on non-consecutive days should be considered, in order to obtain an assessment within reliability for clinical assessments. Unfortunately, only two 24-h urine collections were available in EFCOVAL, limiting our analyses to single time-point exposures rather than long-term exposures. In the present work, this recommendation was supported in the case of 24-h urine collections and also by 24-HDR assessments by the very low Cohen's K values calculated between detection rates at the two time points, despite high percentages of agreement. Hence, more time points should be investigated in order to find further evidence of either under-estimation from direct sampling or over-estimation from indirect sampling methods. A different meta-analysis of five studies also compared the 24-HDR to biomarker measurements, calculating attenuation factors for each measurement type to a separately calculated true usual intake for four factors *i.e.*, energy, protein, potassium, and sodium (Freedman et al., 2017). A picture emerges of considerable discrepancy between what is measured by either the 24-HDR or biological fluid sampling. This is in support of Freedman's recommendations that the 24-HDR is not ideal for assessing long-term exposures, where biomarkers were reportedly better correlated to true usual intake (Freedman et al., 2017).

4.3. Limitations and bias of the present study

The principal inaccuracy of a 24-HDR in this research is the assumption that mycotoxins are homogeneously distributed throughout each type of food, in all of Europe and in that year (Paoletti and Esbensen, 2015). Though the median recorded EFSA contamination level was uniformly applied to all intakes of a food item, it has never been reported that the incidence of any mycotoxin reaches 100% in European food products. Even in the hypothetical case of total incidence and universally homogenous distribution, variations in food preparation (washing, sectioning, mixing, etc.) could affect rates of availability and uptake. Further, interpersonal differences in judgment on whether a given food item is considered "too moldy to eat" may affect actual mycotoxin intake (*e.g.* moldy fruit) (Ouhibi et al., 2019).

The principal weakness of quantitative analysis on biological samples is the left-censoring of the dataset due to the minimum detectable concentrations of target analytes (Wei et al., 2018). An additional vulnerability in assaying biological samples is the possibility for target components to exist in metabolized or otherwise modified forms, thus escaping targeted analysis (Righetti et al., 2016). This is further confounded by the assumption that metabolic rates and pathways are uniform between individuals in the study population; the effects of genetic variation on the production of various metabolites remain poorly understood.

Both the calculated dietary intake data as well as the measured urinary data are prone to bias due to the day of assessment if only one single measurement was available, and the individual ceased participation in the study prior to the second sampling. Long-term exposures require evaluation either by repeated cross-sectional assessments or with tools specifically designed for longitudinal assessment of dietary exposures, which may be partially achieved with food frequency questionnaires (not available in EFCOVAL).

After the 24-HDR, classification of consumed food items was done according to the FoodEx1 system, since deprecated in favor of FoodEx2 (revision 2) (EFSA, 2016).

Also, the biological samples obtained from the EFCOVAL Project were stored at −80 °C for over a decade. In the course of the present analysis, one cycle of thawing and freezing was required in order to aliquot large sample volumes. The stability of mycotoxins under these conditions has not been comprehensively addressed in the literature.

According to the acquired results and by broadcasting the limitations and bias of the present study, it is clear that in future research there is a strong need for more standardized and harmonized occurrence data in foods, covering multiple mycotoxin profiles.

5. Conclusion

According to the study results, the continuous monitoring of mycotoxin contamination in raw agricultural commodities remains to be prioritized. However, the use of biological sampling for surveys of dietary exposure to mycotoxins describes real dietary mycotoxin occurrence in higher resolution than calculations based on a database. Further, mycotoxin exposure measurements made in biological fluids seem to inform studies of single exposure better than chronic exposure. As such, mycotoxin exposure assessments of either the external method of dietary surveys or internal method of biological fluid sampling, illustrate different aspects of real-world mycotoxin exposure. Taken together, these measurements appear insufficient for holistically describing real exposure, due to many uncontrolled factors that lead to uncharacterized variance in the dataset. There is a growing interest in longitudinal monitoring of an accurately determined dietary contaminant intake, through periodic blood and urine collection over the course of absorption, metabolism, and excretion. Particularly, metabolomic profiling of dietary mycotoxin exposures could help not only with a comprehensive assessment of single exposures, but also with the identification of specifically chronic exposure biomarkers. Such detailed characterization would help inform population exposure assessments and aid in the interpretation of cross-sectional surveys. Considering the implicitly chronic nature of dietary exposure, as well as the variety of a modern diet, modelling real-world exposure requires a simultaneous assessment of multiple mycotoxins (Warth et al., 2013). Determining and monitoring mycotoxin presence informs assessments of food safety either by indirectly evaluating a population's probable external exposure or by directly quantifying internal exposure through biomarker analysis (Abrunhosa et al., 2016; Gerding et al., 2014; Heyndrickx et al., 2015; Wallin et al., 2015).

CRediT authorship contribution statement

Karl De Ruyck: Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Writing - review & editing. **Inge Huybrechts:** Conceptualization, Methodology, Data curation, Writing - original draft, Visualization, Investigation, Supervision, Writing - review & editing. **Shupeng Yang:** Data curation. **Davide Arcella:** Conceptualization, Methodology. **Liesel Claeys:** Conceptualization, Methodology, Data curation. **Souheila Abbeddou:** Conceptualization, Methodology. **Willem De Keyzer:** Conceptualization, Methodology. **Jeanne De Vries:** Conceptualization, Methodology. **Marga Ocke:** Conceptualization, Methodology. **Jiri Ruprich:** Conceptualization, Methodology. **Marthe De Boevre:** Conceptualization, Methodology, Writing - original draft, Supervision, Writing - review & editing. **Sarah De Saeger:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors acknowledge the funding agency, Flemish Agency for Innovation by Science and Technology (IWT), now the Agency for Innovation and Entrepreneurship (VLAIO), for the doctoral grant for strategic basic research (SB #141177). The Synapt G2-Si instrument was funded through the FWO Hercules infrastructure funding (Belgium) project № AUGE/13/13. The authors acknowledge the involvement of Masters students Lore De Crop, Mona Delagrange, Josephine Moerman, David Mathias Sialeu Monkam, and Manal Said, without whose work in data acquisition, this manuscript would not have been possible.

The data have been collected by the EFCOVAL investigators and have been made available for this publication by the EFCOVAL Consortium. Views expressed in this paper are those of the authors, and may not reflect those of EFCOVAL Participants. The European Community funding under the Sixth Framework Programme for the EFCOVAL Project is acknowledged (FOOD-CT-2006-022895).

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In accordance with journal recommendations, this manuscript was prepared with the assistance of the STROBE statement guidelines (von Elm et al., 2008). The authors declare they have no actual or potential competing financial interests.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105539.

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