JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Subscriber access provided by Library, Special Collections and Museums, University of Aberdeen

Food Safety and Toxicology

Multi-mycotoxin exposure assessment in UK children using urinary biomarkers – a pilot survey.

Silvia W. Gratz, Valerie Currie, Gary Duncan, and Diane Jackson

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.9b03964 • Publication Date (Web): 12 Dec 2019

Downloaded from pubs.acs.org on December 16, 2019

Just Accepted

"Just Accepted" manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides "Just Accepted" as a service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. "Just Accepted" manuscripts appear in full in PDF format accompanied by an HTML abstract. "Just Accepted" manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are citable by the Digital Object Identifier (DOI®). "Just Accepted" is an optional service offered to authors. Therefore, the "Just Accepted" Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the "Just Accepted" Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these "Just Accepted" manuscripts.

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Published by American Chemical Society. Copyright © American Chemical Society. However, no copyright claim is made to original U.S. Government works, or works produced by employees of any Commonwealth realm Crown government in the course of their duties.

Multi-mycotoxin exposure assessment in UK children using urinary biomarkers – a pilot survey.

Silvia W Gratz*, Valerie Currie, Gary Duncan, Diane Jackson

Rowett Institute, University of Aberdeen, Foresterhill, AB25 2ZD, Aberdeen, UK

*Corresponding author: Tel: +44(0)1224438675, FAX: +44(0)1224438700, Email: s.gratz@abdn.ac.uk

1 **Abstract**

2 Cereal foods are commonly contaminated with multiple mycotoxins resulting in
3 frequent human mycotoxin exposure. Children are at risk of high-level exposure
4 due to their high cereal intake relative to body weight. Hence this study aims to
5 assess multi-mycotoxin exposure in UK children using urinary biomarkers. Spot
6 urines (n=21) were analysed for multi-mycotoxins (deoxynivalenol, DON;
7 nivalenol, NIV; ochratoxin A, OTA; zearalenone, ZEN; α- zearalenol, α-ZEL; β-
8 zearalenol, β-ZEL; T-2 toxin, T-2; HT-2 toxin, HT-2; aflatoxin B_1 and M_1, AFB_1,
9 AFM_1) using liquid chromatography-coupled tandem mass spectrometry. Urine
10 samples frequently contained DON (13.10±12.69 ng/mL), NIV (0.36±0.16 ng/mL),
11 OTA (0.05±0.02 ng/mL) and ZEN (0.09±0.07 ng/mL). Some samples (1-3)
12 contained T-2, HT-2, α-ZEL and β-ZEL, but not aflatoxins. Dietary mycotoxin
13 estimation showed that children were frequently exposed to levels exceeding the
14 tolerable daily intake (52% and 95% of cases for DON and OTA). This
15 demonstrates that UK children are exposed to multiple mycotoxins through their
16 habitual diet.

17

18 **Keywords**: trichothecenes, deoxynivalenol, ochratoxin, zearalenone, tolerable daily
19 intake, co-exposure, diet

20 **Introduction**

21 Mycotoxins are toxic fungal secondary metabolites which are important contaminants in
22 cereal-based foods and nuts. Mycotoxins have been demonstrated to have a range of
23 potent toxicities including carcinogenicity (aflatoxins, ochratoxin), intestinal and
24 immunotoxicity (trichothecenes), and aflatoxins are linked to the development of
25 hepatocellular carcinoma in adults and impaired growth in children [(1,2)]. Based on
26 extensive evidence from toxicity studies, the WHO/FAO Joint expert committee on Food
27 (JECFA) and the European Food Safety Authority (EFSA) have determined tolerable
28 daily intakes (TDI) for several dietary mycotoxins including trichothecenes, ochratoxin
29 A, zearalenone, fumonisins and patulin and strict maximum permitted levels for
30 mycotoxins are set for agricultural commodities and foods by regulatory agencies around
31 the world [(3-5)]. To estimate human dietary exposure to mycotoxins both food analysis and
32 biomarker studies are frequently used [(6-12)]. It is difficult to estimate mycotoxin exposure
33 through food analysis due to the complex nature of the human diet with numerous food
34 constituents contributing to exposure to several mycotoxins. Furthermore, modified
35 mycotoxins are often overlooked during dietary mycotoxin exposure assessment [(13)].
36 Urinary biomarker studies have been successfully used to estimate total dietary exposure
37 to multiple mycotoxins including trichothecenes, zearalenone and aflatoxins (recent
38 examples include [11,14-21]). These biomonitoring studies have reported variable levels of
39 exposure in European adults, with TDI exceedances for DON reported in 13 - 48% of
40 adults [(14,20,22,23)].

41 Mycotoxin exposure in children has been reported in Africa (recent examples
42 include [24,25]), but in Europe reports are less common despite children being identified as
43 an at-risk group for higher exposure due to their low body weight and high relative
44 consumption of cereal-based foods [(26)].

45 Especially co-exposure to multiple mycotoxins in this vulnerable population group is
46 understudied [27,28]. The aim of the current study was therefore to determine the urinary
47 biomarker levels for multiple mycotoxins in UK children and to estimate their total
48 dietary mycotoxin exposure in relation to the tolerable daily intakes for each mycotoxin.
49

50 **Materials and Methods**

51 *Study design*

52 21 healthy children (12 boys, 9 girls, aged 2-6 years) provided spot samples of early
53 morning urine. All children followed their normal habitual diet. Anthropometric details
54 of participating children are summarised in Table 1. This study was approved by the
55 Rowett Institute's Ethics Review Panel following favourable consideration by the
56 Grampian Research Ethics Committee (Reference 01/0306).

57 *Urine analysis*

58 This study focusses on six regulated mycotoxins deoxynivalenol (DON), ochratoxin A
59 (OTA), zearalenone (ZEN), T-2 and HT-2 toxin (T-2, HT-2) and aflatoxin B_1 (AFB_1) as
60 well as their important metabolites de-epoxy deoxynivalenol (DOM-1), nivalenol (NIV),
61 α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) and aflatoxin M_1 (AFM_1). Mycotoxin
62 reference standards for all mycotoxin as well as stable isotope labelled internal standards
63 in acetonitrile ($^{13}C_{15}$ DON, $^{13}C_{22}$ HT-2, $^{13}C_{18}$ ZEN, $^{13}C_{20}$ OTA, $^{13}C_{17}$ AFB_1) were
64 purchased from Romer Labs (Tulln, Austria). Urine samples from the Rowett
65 biorepository were used in this study. Samples were collected in 2004 and stored at -20
66 ºC prior to analysis. Urine samples were analysed for multiple mycotoxins adapting a
67 digestion, extraction and LC-MS/MS method published previously [29]. In brief, 4 mL of
68 urine were spiked with a mixture of ^{13}C-labelled internal standards (5 ng/mL $^{13}C_{15}$ DON,

69 $^{13}C_{22}$ HT-2; 1.25 ng/mL $^{13}C_{18}$ ZEN, $^{13}C_{20}$ OTA, $^{13}C_{17}$ AFB_1) and adjusted to pH 6.8.
70 Samples were then digested over night with β-glucuronidase (Sigma-Aldrich, Ltd., Pool,
71 UK; 23,000U in 1 mL 75 mM KH_2PO_4 buffer at pH 6.8), diluted with 16 mL PBS, the
72 pH adjusted to 7.2 and purified and enriched using immunoaffinity columns (IAC, Myco-
73 6in1, Vicam V100000176, Biocheck, St Asaph, UK). The binding capacities for the
74 Myco-6in1 IAC are quoted by the manufacturer as 300 ng aflatoxins, 1250 ng DON/NIV,
75 800 ng FBs, 100 ng OTA, 500 ng T-2/HT-2, 350 ng ZEN. Samples were eluted with
76 methanol, evaporated to dryness and reconstituted in 250 µL of 10% ethanol, resulting in
77 a 16-fold concentration of urinary mycotoxins prior to LC-MS/MS analysis.

78 *LC-MS/MS analysis*

79 The liquid chromatography separation of mycotoxins was performed on a Shimadzu
80 Nexera X2 LC system, using an Agilent Poroshell column (3 x 50 mm, 2.7 µm). The
81 linear gradient comprised of 10 mM ammonium acetate (Sigma-Aldrich, Ltd., Pool, UK;
82 solvent A) and methanol (solvent B). Starting conditions were 5% B, increasing to 95%
83 B over 10 minutes, a 30-second hold at 95% B, and then re-equilibrated at 5% B for 1.5
84 minutes. The injection volume was 10 µL, the column oven was set to 40 ºC and the flow
85 rate was 400 µL/min. The LC eluent was directed into a Shimadzu 8060 triple-quadrupole
86 MS. Mycotoxins were quantified using the multiple reaction monitoring (MRM)
87 technique. Standard solutions of 500 ng/mL were injected into a flow of solvent and their
88 transition values optimized. 8-point calibration curves (DON 1-500 ng/mL; NIV, HT-2
89 and T-2 0.2-100 ng/m; DOM-1, OTA, ZEN, α-ZEL, β-ZEL, AFB_1 0.1-50 ng/mL and
90 AFM_1 0.05-25 ng/mL) including internal standards (80 ng/mL $^{13}C_{15}$ DON, $^{13}C_{22}$ HT-2;
91 20 ng/mL $^{13}C_{18}$ ZEN, $^{13}C_{20}$ OTA, $^{13}C_{17}$ AFB_1) were used to quantify all analytes.

92 *Method validation*

93 Urine mycotoxin analysis is based on the method described [29]. Myco-6in1
94 immunoaffinity columns were used to monitor the specified mycotoxins DON, ZEN,
95 OTA, T-2, HT-2, AFB_1 and AFM_1 as well as additional closely related mycotoxins NIV,
96 DOM-1, α-ZEL and β-ZEL. The retention of DON, NIV and DOM-1 on DON-specific
97 IAC has been recently demonstrated [30]. LOD and LOQ were determined in solvents and
98 urine matrix by a signal to noise ratio of 10/1 and 3/1, respectively (Table 2, Figure 1).
99 Urine matrix effects (signal suppression/enhancement, SSE) were evaluated by
100 comparing the slopes of matrix matched standard curves (8 levels, in triplicates) with
101 solvent standard curves calculated as: matrix slope/solvent slope x 100. Matrix effects
102 were efficiently compensated by using IAC and stable isotope internal standards,
103 resulting in SSE ranging from 98-119% for all mycotoxins tested (Table 2). As no blank
104 urine samples could be obtained, recovery experiments were performed in PBS spiked
105 with a mycotoxin mixture at three different levels (DON 20.0, 5.0, 2.5 ng/mL; NIV, HT-2
106 and T-2 at 4.0, 1.0, 0.5 ng/ml; DOM-1, OTA, ZEN, α-ZEL, β-ZEL, AFB_1 and AFM_1 at
107 2.0, 0.5, 0.25 ng/mL) in triplicate on three different days under repeatability conditions
108 [31]. Recoveries were expressed as % of predicted final concentration (Table 2).
109 Recoveries and repeatability (RSDr) were within criteria established by EU Reg No
110 401/2006 [32]. All results were corrected for recovery and urinary mycotoxin
111 concentrations are expressed as ng/mL urine and ng/mg creatinine. Urinary creatinine
112 was analysed using alkaline picrate solution on an automated clinical analyser
113 (KONELAB 30, Labmedics, Stockport, UK). Four quality controls of freshly spiked PBS
114 (5.0 ng/mL DON; 1.0 ng/mL NIV, HT-2, T-2; and 0.5 ng/mL DOM-1, OTA, ZEN, α-
115 ZEL, β-ZEL, AFB_1, AFM_1) were included in the analysis.

116 *Estimation of daily urinary mycotoxin excretion*

117 Total 24-hour urinary creatinine excretion was calculated as described previously [29]

118 using a clinical calculator

119 (http://www.clinicalculator.com/english/nephrology/excrea/excrea.htm) on the basis of

120 anthropometric data (Table 1) as follows:

121 Creatinine excretion females (mg/d) = (22 – age/9) x BW

122 Creatinine excretion males (mg/d) = (28 – age/6) x BW

123 BW = body weight

124 Daily urinary mycotoxin excretion was calculated as:

125 Urinary mycotoxin (ng/mg creatinine) x Total 24-hour creatinine excretion (mg/d)

126 Urinary mycotoxin excretion is presented as ng/mL urine, ng/mg creatinine and µg/d in

127 table 3.

128 *Estimation of dietary mycotoxin exposure*

129 Dietary mycotoxin intake was calculated as:

130 Daily urinary mycotoxin excretion (µg/d)/CR

131 CR = urinary clearance rate for DON 72.3%/day [7], OTA 5% [33] and ZEN 9.4% [23].

132 Dietary mycotoxin exposure was then expressed as % of the tolerable daily intake (TDI):

133 %TDI = Daily mycotoxin intake (µg/d) / BW x 100 / TDI

134 TDI = Tolerable daily intake (µg/kg BW/d)

135 *Statistical analysis*

136 All results are presented as average concentration of 21 urine samples. Data for boys

137 (n=12) and girls (n=9) were analysed by using an independent t-test (SPSS version 24)

138 and no significant differences ($p>0.05$) were found for urinary mycotoxin excretion or

139 dietary exposure between boys and girls for any mycotoxin tested. Hence, all results are
140 presented as average for all children.
141

142 **Results and Discussion**

143 *Prevalence of mycotoxins in urine*

144 In this study spot urine samples from 21 children were analysed for 11 mycotoxins. Of
145 these mycotoxins, DON and ZEN were detected in all urine samples. OTA and NIV were
146 also frequently detected (95 and 81% of all samples, respectively), while α-ZEL, β-ZEL,
147 DOM-1, T-2 and HT-2 were only present in 1-3 samples (Table 3). AFB_1 and AFM_1 were
148 not detectable in any of the samples. Urinary DON was highly prevalent (100% of
149 samples, mean 39.7 ng/mg creatinine or 13.1 ng/mL) and two samples also contained
150 DOM-1, the microbial metabolite of DON, at low levels (Table 3). Both prevalence and
151 urinary concentration of DON are comparable to a recent UK-based pediatric study [(16)]
152 which found 100% of urine samples from 40 children aged 3-9 to be contaminated with
153 DON at a mean concentration of 41.6 ng/mg creatinine. Similarly, a large study in 155
154 Belgian children [(27)] and a small study in 16 Spanish children [(28)] report DON and DON-
155 glucuronide, the major urinary metabolite, to occur in 100% and 56% of urine samples,
156 respectively. Urinary DON+DON-glucuronide was reported at 83.1 ng/mg creatinine or
157 74.2 ng/mL urine (Belgium) and 27.8 ng/mg creatinine (Spain). DON was also frequently
158 detected in adults from Austria (96% of 27 samples, mean 19.5 ng/mg creatinine, [22]), Italy
159 (96% of 52 samples, mean 11.9 ng/mL, [34]), Portugal (78% of 94 samples, median 4.0
160 ng/mg creatinine, [35]) and pregnant women from Croatia (98% of 40 samples, 93.7 ng/mg
161 creatinine, [20]) and less frequently in Nigerian children (18% of 120 samples, mean 2.4
162 ng/mL, [36]) and adults from Cameroon (42% of 175 samples, mean 5.9 ng/mL, [37]). DON-
163 glucuronides are the main urinary DON metabolites detected [(9)], and our method of urine

164 analysis including an enzymatic β-glucuronidase pre-digestion detects the sum of free
165 and glucuronidated DON in the same analysis. DOM-1 was detectable in two urine
166 samples (9% prevalence, at approximately 1% of the urinary DON concentration) which
167 is lower than our previous studies in UK adults (20 – 40% prevalence of DOM-1, [14,29]).
168 DOM-1 is a microbial metabolite of DON produced by the human gut microbiota of some
169 individuals, but not others, and our previous work found the frequency of DOM-1
170 production to range between 10-20% in faecal microbiota from adults [(14,29,39)]. To date,
171 no work has been published on the activity of children's microbiota towards mycotoxin
172 degradation, but profiles of children's microbiota resemble that of adults from an early
173 age [(40)]. A recent study in UK children did not detect any urinary DOM-1 [(16)], and the
174 Spanish study reports DOM-1 in 1/16 samples [(28)]. In contrast, the Belgian cohort reports
175 DOM-1 in 17% of samples at very high concentrations (101 ng DOM-1 glucuronide/mg
176 creatinine), which even exceeded the detected sum of DON and DON-glucuronide [(27)]. In
177 adults, DOM-1 has been detected in 96% of Spanish adults (DOM-1 at 8.9 ng/mg
178 creatinine, [38]), 28% of Portuguese adults spot urine (26 ng/mg creatinine, [35]), but not in
179 Austrian [(22)] or Italian subjects [(34)]. In addition to DON and DOM-1, DON-3-suflate has
180 been identified as a novel urinary DON metabolite in humans [(41)] with an excretion rate
181 of 4% of dietary DON. DON-3-sulfate was not analysed in the current study and could
182 further increase the estimate of dietary DON.

183 Nivalenol was detectable in 81% of urine samples in the current study, but at very
184 low levels (mean 1.1 ng/mg creatinine, Table 3). NIV was not detectable in children from
185 Spain [(28)], but the Spanish study reports urinary NIV in 18.7% of young adults and 18.2%
186 of adults at mean concentrations of 13.3 and 16.7 ng/mg creatinine, respectively. Other
187 studies did not determine NIV in children's urine. Our current method uses IAC cleanup
188 and enrichment which facilitates a low detection limit (LOQ 0.125 ng/mL) which is

189 superior to methods which report NIV in children's urine below LOQ (e.g. LOQ 1 ng/mL,
190 [28]). NIV excretion in urine is reported in adults in Africa [(10,37,42)] but reports in children
191 are rare [(36)] and further studies are needed to assess dietary exposure. Type A
192 trichothecenes T-2 and HT-2 were each detected once (0.03 and 6.1 ng/mg creatinine,
193 respectively; Table 3), but not in the same urine sample. This low incidence of T-2 and
194 HT-2 is comparable to the Spanish cohort (HT-2 in 1/16 sample at 12.6 ng/mg creatinine,
195 [28]) and no T-2 or HT-2 were found in Belgian children [(27)], Nigerian children [(43)] or adults
196 from Cameroon [(37)]. OTA was detected in 95% of samples in the current study at low
197 levels (mean concentration of 0.15 ng/mg creatinine) which is higher than the Belgian
198 cohort of children (51% of samples contained OTA at 0.08 ng/mg creatinine, [27]). In adults,
199 studies report a wide range of urinary OTA concentrations (0.006 ng/mg creatinine, [35];
200 0.019 ng/mg creatinine, [38]; 0.15 ng/mg creatinine, [21]).

201 Zearalenone and its metabolites are potent xenoestrogens and exposure to these
202 compounds is of great concern, especially in girls. Alternariol is another estrogenic
203 mycotoxin with potential synergistic effects to ZEN [(44)]. In the current study, ZEN was
204 detected in all urine samples at mean levels of 0.3 ng/mg creatinine (Table 3), with no
205 significant difference between boys and girls. The hepatic metabolites α-ZEL and β-ZEL
206 were detected less frequently (3/21 and 2/21 samples, respectively), but at higher mean
207 levels compared to ZEN (0.5 and 0.6 ng/mg creatinine, respectively; Table 3). α-ZEL and
208 β-ZEL only co-occurred in samples which were also contaminated with ZEN at ratios of
209 66-82% for α-ZEL and 77-116% for β-ZEL. Prevalence of urinary ZEN is higher in our
210 study (100% of samples) compared to a study in 163 US girls aged 9-10 (55%, [45]) but the
211 mean urinary concentration is lower (0.1 ng/mL urine in the current study compared to
212 1.3 ng/mL in US girls. Two European studies [(27,28)] and one study from Nigeria [(43)] report
213 ZEN and ZEL as not detectable in children's urine whereas another study from Nigeria

214 reports ZEN in 82% and α- and β-ZEL in 4 and 6% of urine samples from children and
215 adults [36].

216 AFB_1 and AFM_1 were not detected in any urine samples from children in this
217 study, which is in agreement with other studies in European cohorts [27]. Urinary aflatoxin
218 excretion reflects recent, acute dietary exposure, whereas aflatoxin-lysin adducts in
219 plasma are validated biomarkers for chronic aflatoxin exposure [46]. Aflatoxins are
220 frequently detected in cohorts of children and adults in Africa (14 – 72% of samples
221 positive for urinary AFM_1 [36,43]) where aflatoxin exposure is linked to impaired growth
222 [47]. However, strict regulations for aflatoxins in food in Europe and low consumption of
223 high-risk foods such as peanuts lead to a negligible exposure to aflatoxins in children and
224 adults.

225 *Estimation of dietary mycotoxin exposure*

226 Children were exposed to substantial amounts of DON through their diet (average 26.5
227 µg/d, Table 4). When dietary exposure was compared to the tolerable daily intake for
228 DON, the TDI was exceeded in 52% of children (Figure 2). Average DON exposure in
229 all 21 children was 136% of TDI and the proportion of TDI exceedances for DON in
230 children in this study is much higher than in adults where we previously found 7% of TDI
231 exceedances in 15 subjects [14]. Our results are in line with a recent study [16] reporting up
232 to 63% of UK children exceeding the TDI for DON, and this high frequency of TDI
233 exceedances in children is of great concern. Similarly, cohorts in other countries also
234 report higher frequency of TDI exceedances in children (22% Spain, [28]; 69% Belgium,
235 [27]) than adults (4% Spain [28]; 29% Belgium, [27]; 10% Portugal, [35]; 40% Italy, [34]; 33%
236 Austria, [22]; 48% Croatia, [20]). Exposure assessment through urinary OTA biomarkers has
237 been performed in adults [34,35,38]. In adults TDI exceedances for OTA are reported

238 frequently (14% of subjects median 27% of TDI from Portugal, [35]; 94% of subjects mean
239 818% of TDI from Italy, [34]; and 96% of subjects mean 185% of TDI from Spain, [38]).
240 Vidal et al. [(38)] also state that exposure estimate from urinary biomarkers greatly exceed
241 estimates from dietary approaches. In UK adults, dietary OTA exposure was estimated at
242 average 1.5 ng/kg bw/d based on plasma levels or 0.9 ng/kg bw/d based on duplicate diet
243 analysis [(48)]. This exposure is significantly lower than our estimates of 74.1 ng/kg bw/d
244 (Table 4) in children based on urinary OTA excretion. Based on this exposure estimate,
245 95% of children in the current study exceeded the TDI for OTA (Figure 2). TDI
246 exceedances for ZEN were less common in children in the current study (5% of children)
247 and have not been reported in the literature. In adults, 24% of subjects exceeded the TDI
248 for ZEN in a recent study [(35)]. Further work is needed to better elucidate the exposure and
249 potential health risk associated with this mycotoxin in children. Dietary co-exposure to
250 several mycotoxins is highly likely as several mycotoxins are frequently detected in
251 important food commodities including cereal grains (trichothecenes, ZEN, OTA), corn
252 (ZEN and fumonisins) and dried fruits (OTA) [(1)]. Co-exposure to the mycotoxins DON,
253 OTA and ZEN was also evident in this study (Figure 3). Children exceeding the TDI for
254 DON or ZEN also exceeded the TDI for OTA and this co-exposure puts them at an even
255 greater risk of mycotoxin toxicity. Cereals and cereal based foods have been identified as
256 main contributors to mycotoxin exposure in the UK [(49)] and our study confirms the
257 prediction that children might be at high risk to exceed TDI. For carcinogenic mycotoxins
258 such as aflatoxins, no tolerable daily intakes are set and a benchmark dose is calculated
259 instead [(50)]. However, this does not apply to the current study as no aflatoxins were
260 detected in urine. Urinary biomarker analysis for mycotoxin exposure is an important
261 approach for estimating dietary exposure. Urinary DON is a well validated biomarker for
262 recent dietary exposure, whereas biomarkers for other mycotoxins such as ZEN and OTA

263 are less strong in predicting dietary exposure due to the low urinary excretion rate (9.4
264 and 5%, respectively) and complex metabolism in humans [18,35,51,52]. Hence, future
265 studies are needed to confirm the present finding of frequent and substantial TDI
266 exceedances for OTA in UK children.

267 In conclusion, our data clearly demonstrate that children are exposed to high
268 levels of some mycotoxins through their habitual diet and that maximum permitted levels
269 for mycotoxins in food do not fully protect them from exceeding the TDI. Regulators
270 need to consider further action to ensure consumer safety of all population groups to avoid
271 high exposure and potential toxic effects.

272 **Acknowledgements**

273 Authors would like to thank all volunteers for participating in this study. Ruth Slater is
274 acknowledged for her help with managing the urine sample collection and storage.

275 **Supporting Information**

276 LC-MS/MS chromatograms for DOM-1 quantifier ion and qualifier ion at LOQ are
277 presented in Supplemental Figure 1.

278

279 **References**

280 1. Wu F, Groopman JD, Pestka JJ. 2014. Public Health Impacts of Foodborne
281 Mycotoxins. Annu Rev Food Sci Technol. 5:351-372.
282 2. Pestka JJ. 2010. Deoxynivalenol: mechanisms of action, human exposure, and
283 toxicological relevance. Arch Toxicol. 84(9):663-679.
284 3. European Commission (EC) Regulation No 181/2006: Setting maximum levels for
285 certain contaminants in foodstuffs. Off J Eur Union. L364:5-24
286 4. WHO/FAO Joint Expert Committee on Food Additives (JECFA). 2001. Safety
287 evaluation of certain mycotoxins in food. FOA Food and Nutrition Paper 74.
288 http://www.fao.org/3/a-bc528e.pdf
289 5. Van Egmont HP. 2002. Worldwide regulations for mycotoxins. In: Mycotoxins and
290 Food Safety (Trucksess et al., Eds). Kluwer Academic Publishers.
291 6. Turner PC, Flannery B, Isitt C, Ali M, Pestka J. 2012. The role of biomarkers in
292 evaluating human health concerns from fungal contaminants in food. Nutr Res Rev.
293 25:162-179.
294 7. Turner PC, White KL, Burley VJ, Hopton RP, Rajendram A, Fisher J, Cade JE, Wild
295 CP. 2010. A comparison of deoxynivalenol intake and urinary deoxynivalenol in UK
296 adults. Biomarkers. 15(6):553-562.
297 8. Turner PC, Rothwell JA, White KL, Gong Y, Cade JE, Wild CP. 2008. Urinary
298 deoxynivalenol is correlated with cereal intake in individuals from the United Kingdom.
299 Environ Health Perspect. 116(1):21-25.
300 9. Warth B, Sulyok M, Berthiller F, Schuhmacher R, Fruhmann P, Hametner C, Adam
301 G, Frohlich J, Krska R. 2011. Direct quantification of deoxynivalenol glucuronide in
302 human urine as biomarker of exposure to the Fusarium mycotoxin deoxynivalenol. Anal
303 Bioanal Chem. 401:195-200.

304 10. Warth B, Sulyok M, Fruhmann P, Mikula H, Berthiller F, Schuhmacher R,
305 Hametner C, Abia WA, Adam G, Frohlich J, Krska R. 2012. Development and
306 validation of a rapid multi-biomarker liquid chromatography/tandem mass spectrometry
307 method to assess human exposure to mycotoxins. Rapid Commun Mass Spectrom.
308 26:1533-40.
309 11. Warth B, Sulyok M, Krska R. 2013. LC-MS/MS-based multibiomarker approaches
310 for the assessment of human exposure to mycotoxins. Anal Bioanal Chem. 405:5687-
311 95.
312 12. Braun D, Ezekiel CN, Abia WA, Wisgrill L, Degen GH, Turner PC, Marko D,
313 Warth B. 2018. Monitoring early life mycotoxin exposures via LC-MS/MS breast milk
314 analysis. Anal Chem. 90:14569-14577.
315 13. Kovac M, Subaric D, Bulaic M, Kovac T, Sarkanj B. 2018. Yesterday masked,
316 today modified; what do mycotoxins bring next? Arh Hig Rada Toxsikol. 69:196-214.
317 14. Gratz SW, Richardson A, Duncan G, Holtrop G. 2014. Annual variation of dietary
318 deoxynivalenol exposure during years of different Fusarium prevalence: a pilot
319 biomonitoring study. Food Addit Contamin A. 31(9):1579-85.
320 15. Huybrechts B, Martins JC, Debongnie P, Uhlig S, Callebaut A. 2015. Fast and
321 sensitive LC-MS/MS method measuring human mycotoxin exposure using biomarkers in
322 urine. Arch Toxicol. 89(11):1993-2005.
323 16. Papageorgiou M, Wells L, Williams C, White K, De Santis B, Liu Y, Debegnach F,
324 Miano B, Moretti G, Greetham S, Brera C, Atkin SL, Hardie LJ, Sathyapalan T. 2018.
325 Assessment of Urinary Deoxynivalenol Biomarkers in UK Children and Adolescents.
326 Toxins. 10:50.
327 17. Papageorgiou M, Wells L, Williams C, White K, De Santis B, Liu Y, Debegnach F,
328 Miano B, Moretti G, Greetham S, Brera C, Atkin SL, Hardie LJ, Sathyapalan T. 2018.

329 Occurrence of deoxynivalenol in an elderly cohort in the UK: a biomonitoring approach.
330 Food Addit Contam A. 10:2032-2044.
331 18. Ali N, Degen GH. 2018. Urinary biomarkers of exposure to the mycoestrogen
332 zearalenone and its modified forms in German adults. Arch Toxicol. 92(8):2691-2700.
333 19. Wallin S, Hardie LJ, Kotova N, Lemming EW, Nalsen C, Ridefelt P, Turner PC,
334 White KLM, Olsen M. 2013. Biomonitoring study of deoxynivalenol exposure and
335 association with typical cereal consumption in Swedish adults. World Mycotox J.
336 6(4):439-448.
337 20. Sarkanj B, Warth B, Uhlig S, Abia WA, Sulyok M, Klapec T, Krska R, Banjari I.
338 2013. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from
339 Croatia. Food Chem Toxicol. 62:231-237.
340 21. Klapec T, Sarkanj B, Banjari I, Strelec I. 2012. Urinary ochratoxin A and ochratoxin
341 alpha in pregnant women. Food Chem Toxicol. 50:4487-4492.
342 22. Warth B, Sulyok M, Fruhmann P, Berthiller F, Schuhmacher R, Hametner C, Adam
343 G, Frohlich J, Krska R. 2012. Assessment of human deoxynivalenol exposure using an
344 LC-MS/MS based biomarker method. Toxicol Lett. 211:85-90.
345 23. Warth B, Sulyok M, Berthiller F, Schuhmacher R, Krska R. 2013. New insights into
346 the human metabolism of the Fusarium mycotoxins deoxynivelonol and zearalenone.
347 Tox Lett. 220:88-94.
348 24. Ojuri OT, Ezekiel CN, Eskola MK, Sarkanj B, Babalola AD, Sulyok M, Hajslova J,
349 Elliott CT, Krska R. 2019. Mycotoxin co-exposure in infants and young children
350 consuming household- and industrially-processed complementary foods in Nigeria and
351 risk management advice. Food Control. 98:312-322.
352 25. Ojuri OT, Ezekiel CN, Sulyok M, Exeokoli OT, Oyedele OA, Ayeni KI, Eskola
353 MK, Sarkanj B, Hajslova J, Adeleke RA, Nwangburuka CC, Elliott CT, Krska R. 2018.

354 Assessing the mycotoxicological risk from consumption of complementary foods by
355 infants and young children in Nigeria. Food Chem Toxicol. 121:37-50.
356 26. Schothorst RC, van Egmond HP. 2004. Report from SCOOP task 3.2.10 Collection
357 of occurrence data of Fusarium toxins in food and assessment of dietary intake by the
358 population of EU member states: Subtask: trichothecenes. Toxicol Lett. 153(1):133-143.
359 27. Heyndrickx E, Sioen I, Huybrechts B, Callebaut A, De Henauw S, De Saeger S. 2015.
360 Human biomonitoring of multiple mycotoxins in the Belgian population: results of the
361 BIOMYCO study. Envrion Internat. 84:82-89.
362 28. Rodriguez-Carrasco Y, Molto JC, Manes J, Berrada H. 2014. Exposure assessment
363 approach through mycotoxin/creatinine ratio evaluation in urine by GC-MS/MS. Food
364 Chem Toxicol. 72:69-75.
365 29. Gratz SW, Duncan G, Richardson AJ. 2013. The human fecal microbiota metabolizes
366 deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary
367 deepoxy-deoxynivalenol. Appl Environ Microbiol. 79(6):1821-1825.
368 30. Uhlig S, Stanic A, Hussain F, Miles CO. 2017. Selectivity of commercial
369 immunoaffinity columns for modified forms of the mycotoxin 4-deoxynivalenol DON. J
370 Chromatography B. 1061-1062:322-326.
371 31. European Commission (EC) Decision 2002/657/EC implementing Council directive
372 96/23/EC concerning the performance of analytical methods and the interpretation of
373 results. Off J Eur Comm. L221:8-36.
374 32. European Commission (EC) Regulation No 401/2006: Laying down the methods of
375 sampling and analysis for the official control of the levels of mycotoxins in foodstuff.
376 Off J Eur Union. L70:12-34.

377 33. Studer-Rohr I, Schlatter J, Dietrich DR. 2000. Kinetic parameters and
378 intraindividual fluctuations of ochratoxin A plasma levels in humans. Arch Toxicol.
379 74:499-510.
380 34. Solfrizzo M, Gambacorta L, Visconti A. 2014. Assessment of multi-mycotoxin
381 exposure in southern Italy by urinary multi-biomarker determination. Toxins. 6523-538.
382 35. Martins C, Vidal A, De Boevre M, De Saeger S, Nunes C, Torres D, Goios A,
383 Lopes C, Assuncao R, Alvito P. 2019. Exposure assessment of Portuguese population to
384 multiple mycotoxins: The human biomonitoring approach. Internat J Hygiene Envrion
385 Health. 222:913-925.
386 36. Sarkanj B, Ezekiel C, Turner PC, Abia WA, Rychlik M, Krska R, Sulyok M, Warth
387 B. 2018. Ultra-sensitive, stable isotope assisted quantification of multiple urinary
388 mycotoxin exposure biomarkers. Analyt Chim Acta. 1019:84-92.
389 37. Abia WA, Warth B, Sulyok M, Krska R, Tchana A, Njobeh PB, Turner PC,
390 Kouanfack C, Eyongetah M, Dutton M, Moundipa PF. 2013. Bio-monitoring of
391 mycotoxin exposure in Cameroon using a urinary multi-biomarker approach. Food
392 Chem Toxicol. 62:927-934.
393 38. Vidal A, Cano-Sancho G, Marin S, Ramos AJ, Sanchis V. 2016. Multidetection of
394 urinary ochratoxin A, deoxynivalenol and its metabolites: pilot time-course study and
395 risk assessment in Catalonia, Spain. World Mycotox J. 9(4):597-612.
396 39. Gratz SW, Dinesh R, Yoshinari T, Holtrop G, Richardson AJ, Duncan G, MacDonald
397 S, Lloyd A, Tarbin J. 2017. Masked trichothecene and zearalenone mycotoxins withstand
398 digestion and absorption in the upper GI tract but are efficiently hydrolyzed by human
399 gut microbiota *in vitro.* Mol Nutr Food Res. 61(4):1-10.
400 40. Milani C, Duranti S, Bottacini F, Casey E, Turroni F, Mahony J, Belzer C, Delgado
401 Palacio S, Arboleya Montes S, Mancabelli L, Lugli GA, Rodriguez JM, Bode L, de Vos

402 W, Gueimonde M, Margolles A, van Sinderen D, Ventura M. 2017. The first microbial
403 colonizers of the human gut: Composition, Activities, and health implications of the
404 infant gut microbiota. Microbiol Molec Biol Rev. 81(4):e00036-17.
405 41. Warth B, Del Favero G, Wiesenberger G, Puntscher H, Woelflingseder L,
406 Fruhmann P, Sarkanj B, Krska R, Schuhmacher R, Adam G, Marko D. 2016.
407 Identification of a novel human deoxynivalenol metabolite enhancing proliferation of
408 intestinal and urinary bladder cells. Nature Sci Report 6:33854.
409 42. Shephard SG, Burger G-M, Gambacorta L, Gong YY, Krska R, Rheeder JP,
410 Solfrizzo M, Srey C, Sulyok M, Visconti A, Warth B, Van de Westhuizen L. 2013.
411 Multiple mycotoxin exposure determined by urinary biomarkers in rural subsistence
412 farmers in the former Transkei, South Africa. Food Chem Toxicol. 62:217-225.
413 43. Ezekiel CN, Warth B, Ogara IM, Abia WA, Ezekiel VC, Atehnkeng J, Sulyok M,
414 Turner PC, Tayo GO, Krska R, Bandyopadhyay R. 2014. Mycotoxin exposure in rural
415 residents in northern Nigeria: A pilot study using multi-urinary biomarkers. Envrionm
416 Internat. 66:138-145.
417 44. Vejdovszky K, Hahn K, Braun D, Warth B, Marko D. 2016. Synergistic estrogenic
418 effects of Fusarium and Alternaria mycotoxins in vitro. Arch Toxicol. 91:1447-1460.
419 45. Bandera EV, Chandrana U, Buckley B, Lin Y, Isukapalli S, Marshall I, King M,
420 Zarbl H. 2011. Urinary mycoestrogens, body size and breast development in New
421 Jersey girls. Sci Tot Environ. 409(24):5221-5227.
422 46. Turner PC, Van de Westhuizen L, Da Costa AN. 2012. Biomarkers of Exposure:
423 Mycotoxins – Aflatoxin, Deoxynivalenol and Fumonisins. In: Biomarkers and Human
424 Biomonitoring: Volume 2. Knudsen L & Merlo DF (Eds). Royal Society of Chemistry,
425 eISBN 978-1-84973-354-0.

426 47. IARC working group report no 9. 2015. Mycotoxin control in low- and middle-
427 income countries. Wild CP, Miller JD, Groopman JD (Eds). WHO Press, Geneva,
428 Switzerland.
429 48. Gilbert J, Brereton P, MacDonald S. 2001. Assessment of dietary exposure to
430 ochratoxin A in the UK using a duplicate diet approach and analysis of urine and
431 plasma samples. Food Addit Contam A. 18(120):1088-1093.
432 49. Turner PC, Burley VJ, Rothwell JA, White KLM, Cade JE, Wild CP. 2008.
433 Deoxynivalenol: Rationale for development and application of a urinary biomarker.
434 Food Addit Contam A. 25(7):864-871.
435 50. Herrera M, Bervis N, Carraminana JJ, Juan T, Herrera A, Arino A, Loran S. 2019.
436 Occurrence and exposure assessment of aflatoxins and deoxynivalenol in cereal-based
437 baby foods for infants. Toxins. 11(3):150.
438 51. Degen GH. 2016. Are we ready to estimate daily ochratoxin A intake based on
439 urinary concentrations? Envrion Internat. 97:254-255.
440 52. Vidal A, Mengelers M, Yang S, De Saeger S, De Boevre M. 2018. Mycotoxin
441 biomarkers of exposure: A comprehensive review. Comprehensive Rev Food Sci Food
442 Safety. 17:1127-1155.
443

444 **Funding**

445 The Rowett Institute receives funding from the Scottish Government, Rural and
446 Environment Science and Analytical Services (RESAS).

Figure captions

Figure 1. LC-MS/MS chromatograms for the quantification of 11 mycotoxin in urine matrix. Mycotoxin concentrations in urine: DON 3.13 ng/mL; NIV, HT-2 and T-2 0.63 ng/mL; DOM-1, OTA, ZEN, α-ZEL, β-ZEL, AFB_1 0.31 ng/mL; AFM_1 0.16 ng/mL.

Figure 2. Estimated dietary mycotoxin exposure in children. Data are calculated as DON equivalents (sum of DON+DOM-1) and ZEN equivalents (sum of ZEN+α-ZEL+β-ZEL) and all calculations are based on 21 urine samples. Results are calculated as % of TDI for each toxin and grouped in five TDI categories (0-25%; 25-50%; 50-75%; 75-100%, >100%). Pie charts summarise the proportion of children in each TDI bracket. Abbreviations: deoxynivalenol (DON); de-epoxy deoxynivalenol (DOM-1); ochratoxin A (OTA); zearalenone (ZEN); α-zearalenol (α-ZEL); β-zearalenol, (β-ZEL); tolerable daily intake (TDI).

Figure 3. Co-exposure to DON, OTA and ZEN in children. Data are calculated as DON equivalents (sum of DON+DOM-1) and ZEN equivalents (sum of ZEN+α-ZEL+β-ZEL). Results are presented as percentage of TDI for each mycotoxin in each individual child. Abbreviations: deoxynivalenol (DON); de-epoxy deoxynivalenol (DOM-1); ochratoxin A (OTA); zearalenone (ZEN); α-zearalenol (α-ZEL); β-zearalenol, (β-ZEL); tolerable daily intake (TDI).

Tables

Table 1. Anthropometric Details of Participating Children.

Gender	**Boys, n = 12**	**Girls, n = 9**
Age (years)	4.6 ± 1.3	4.6 ± 1.0
Body weight (kg)	18.4 ± 2.8	20.3 ± 6.9
BMI (kg/m^2)	15.9 ± 1.3	17.3 ± 2.6
Urinary creatinine (mg/d)	501.2 ± 72.7	435.7 ± 146.1

All data are expressed as average ± SD.

Table 2. Method Performance Parameters of the LC-MS/MS Method.

Compound	RT	m/z precursor ion	Polarity	m/z product ions	Relative response ratio	LOD/LOQ urine (ng/mL)	Matrix effect (SSE%±RSD)	Recovery (%±RSDr)
DON	2.9	355.25[M+CH_2COO]$^-$	-ve	295.2/265.3	67.9	0.156/0.328	97.8±6.1	111.8±6.8
DOM-1	3.8	339.25[M+CH_2COO]$^-$	-ve	59.1/249.2	42.2	0.063/0.158	103.2±3.3	94.8±14.7
NIV	2.2	371.20[M+CH_2COO]$^-$	-ve	281.2/311.2	66.7	0.066/0.125	105.5±0.6	113.0±10.8
OTA	6.9	404.15[M+H]$^+$	+ve	239.1/221.1	43.1	0.003/0.006	106.7±2.2	101.6±13.7
ZEN	8.1	317.20[M-H]$^-$	-ve	175.3/131.2	59.6	0.016/0.033	112.2±2.5	91.2±13.0
α-ZEL	7.9	319.20[M-H]$^-$	-ve	275.3/160.3	50.0	0.033/0.063	106.6±4.2	95.4±12.8
β-ZEL	7.4	319.20[M-H]$^-$	-ve	275.3/160.3	51.5	0.033/0.063	109.6±4.1	94.3±14.3
HT-2	7.2	442.30[M+NH_4]$^+$	+ve	263.3/215.3	70.9	0.013/0.031	106.4±3.1	126.8±13.2
T-2	7.8	484.20[M+NH_4]$^+$	+ve	215.2/305.2	99.2	0.006/0.013	119.0±1.7	91.8±8.9
AFB_1	6.1	313.10[M+H]$^+$	+ve	285.1/241.0	84.3	0.003/0.006	112.9±8.9	100.9±10.0
AFM_1	5.3	329.10[M+H]$^+$	+ve	273.2/229.2	51.0	0.003/0.008	108.9±6.4	91.0±15.6
DON $^{13}C_{15}$	2.9	370.20[M+CH_2COO]$^-$	-ve					
OTA $^{13}C_{20}$	6.9	424.20[M+H]$^+$	+ve					
ZEN $^{13}C_{18}$	8.1	335.20[M-H]$^-$	-ve					
HT-2 $^{13}C_{22}$	7.2	464.30[M+NH_4]$^+$	+ve					
AFB_1 $^{13}C_{17}$	6.1	330.20[M+H]$^+$	+ve					

RT=retention time. LOD=Limit of detection, LOQ=Limit of quantification, SSE=Signal suppression/enhancement. For all analytes the retention time shifts between standards and samples were ≤ 0.2% and the relative response ratios quantifier/qualifier ion were within the target range [31,32]. LOD/LOQ levels were determined using 8-point calibration curves prepared in urine matrix. All LOD/LOQ levels are expressed as ng/mL urine, taking into account the 16-fold concentration of urine during processing. Matrix effects (SSE) were obtained by comparing the slopes obtained from matrix-matched standard curves with slopes from solvent standard curves. Recovery was determined in PBS spiked at 3 levels in triplicates in 3 repeat experiments. DOM-1 transitions (qualifier and quantifier ion) are shown in Supplemental Figure 1.

Table 3. Urinary Excretion of Multiple Mycotoxins in 21 UK Children.

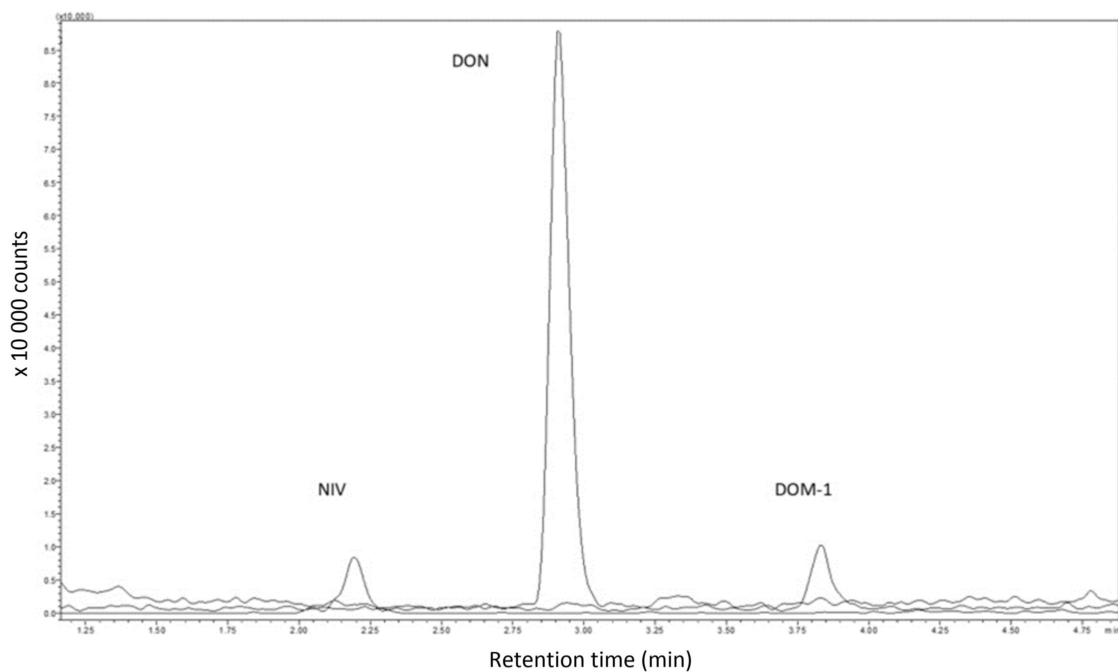
Mycotoxin	**Number (%) positive samples**	**Mean of positive samples (range) ng/mL urine**	**Mean of positive samples (range) ng/mg creatinine**	**Mean of positive samples (range) μg/d**
DON	21 (100%)	13.10 (0.69-42.03)	39.68 (1.88-152.99)	19.13 (0.87-76.96)
DOM-1	2 (9%)	0.15 (0.15,0.15)	0.40 (0.25,0.56)	0.21 (0.14,0.28)
NIV	17 (81%)	0.36 (0.13-0.58)	1.13 (0.42-2.43)	0.54 (0.11-1.19)
HT-2	1 (5%)	1.77	6.13	2.73
T-2	1 (5%)	0.02	0.03	0.01
OTA	20 (95%)	0.05 (0.02-0.11)	0.15 (0.06-0.33)	0.07 (0.03-0.15)
ZEN	21 (100%)	0.09 (0.03-0.25)	0.28 (0.09-1.20)	0.14 (0.04-0.65)
α-ZEL	3 (14%)	0.18 (0.11-0.22)	0.50 (0.19-0.92)	0.26 (0.12-0.49)
β-ZEL	2 (9%)	0.16 (0.10,0.22)	0.63 (0.33,0.93)	0.37 (0.24,0.50)
AFB_1	0	<LOD	<LOD	<LOD
AFM_1	0	<LOD	<LOD	<LOD

LOD = limit of detection

Table 4. Dietary Intake Estimates of Major Mycotoxins by 21 UK Children.

	Mean dietary intake (range) µg/kg bw/d	**Mean total dietary intake (range) µg/d**	**Mean % TDI (range)**
DON	1.36 (0.07-5.77)	26.49 (1.20-106.86)	135.62 (7.13-577.31)
ZEN	0.10 (0.02-0.70)	2.20 (0.40-17.34)	40.73 (8.4-278.7)
OTA	0.07 (0.00-0.18)	1.39 (0.05-2.94)	435.88 (19.50-1071.39)

Figure 1



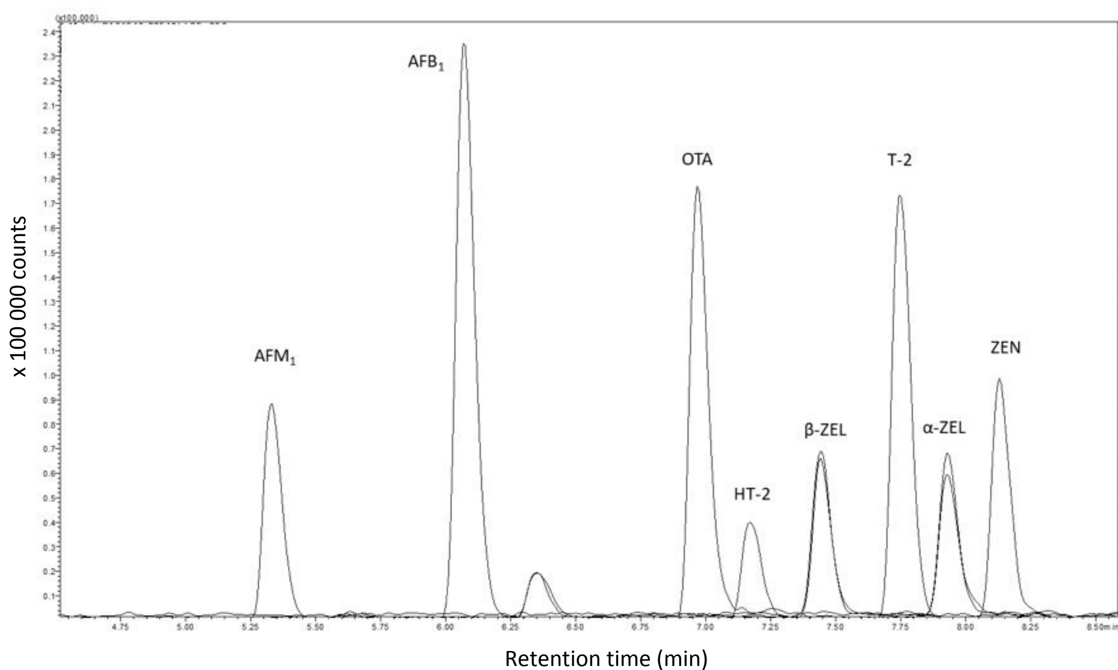


Figure 2

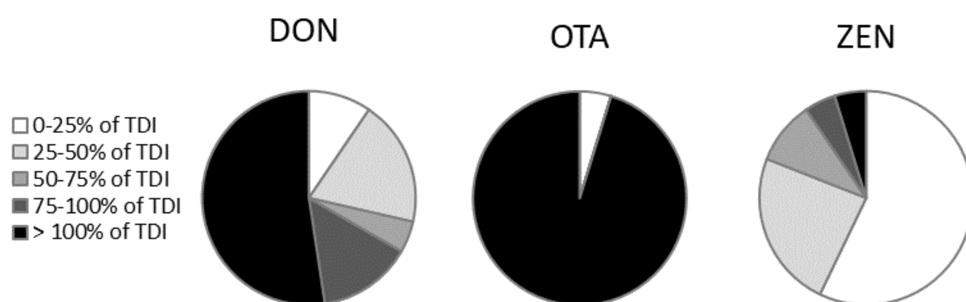
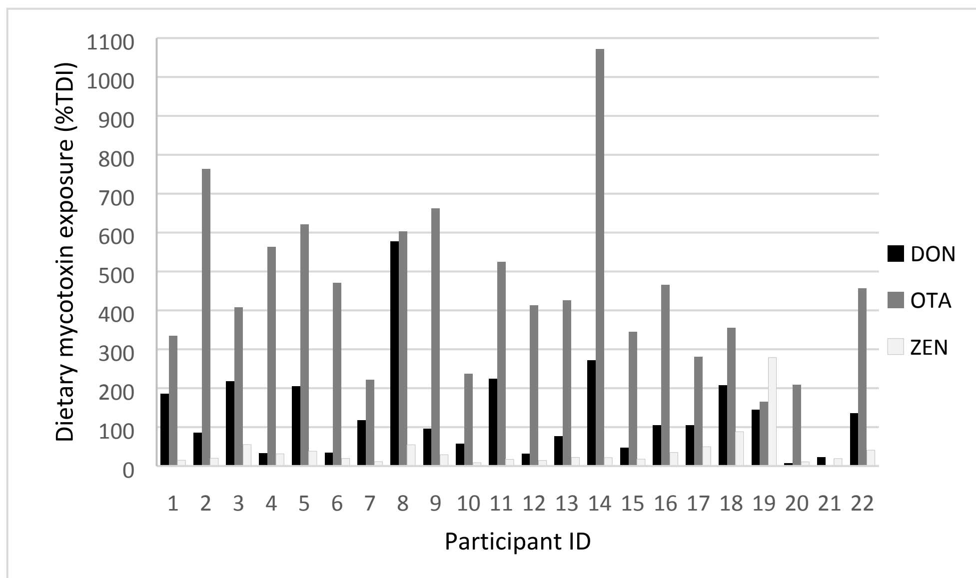


Figure 3



Graphic for table of contents