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Abstract

Cereal foods are commonly contaminated with multiple mycotoxins resulting in frequent human mycotoxin exposure. Children are at risk of high-level exposure due to their high cereal intake relative to body weight. Hence this study aims to assess multi-mycotoxin exposure in UK children using urinary biomarkers. Spot urines (n=21) were analysed for multi-mycotoxins (deoxynivalenol, DON; nivalenol, NIV; ochratoxin A, OTA; zearalenone, ZEN; α- zearalenol, α-ZEL; β-zearalenol, β-ZEL; T-2 toxin, T-2; HT-2 toxin, HT-2; aflatoxin B₁ and M₁, AFB₁, AFM₁) using liquid chromatography-coupled tandem mass spectrometry. Urine samples frequently contained DON (13.10±12.69 ng/mL), NIV (0.36±0.16 ng/mL), OTA (0.05±0.02 ng/mL) and ZEN (0.09±0.07 ng/mL). Some samples (1-3) contained T-2, HT-2, α-ZEL and β-ZEL, but not aflatoxins. Dietary mycotoxin estimation showed that children were frequently exposed to levels exceeding the tolerable daily intake (52% and 95% of cases for DON and OTA). This demonstrates that UK children are exposed to multiple mycotoxins through their habitual diet.

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

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

Mycotoxin exposure in children has been reported in Africa (recent examples include \(^{24,25}\)), but in Europe reports are less common despite children being identified as an at-risk group for higher exposure due to their low body weight and high relative consumption of cereal-based foods \(^{(26)}\).
Especially co-exposure to multiple mycotoxins in this vulnerable population group is understudied (27,28). The aim of the current study was therefore to determine the urinary biomarker levels for multiple mycotoxins in UK children and to estimate their total dietary mycotoxin exposure in relation to the tolerable daily intakes for each mycotoxin.

Materials and Methods

Study design

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

Urine analysis

This study focusses on six regulated mycotoxins deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEN), T-2 and HT-2 toxin (T-2, HT-2) and aflatoxin B₁ (AFB₁) as well as their important metabolites de-epoxy deoxynivalenol (DOM-1), nivalenol (NIV), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL) and aflatoxin M₁ (AFM₁). Mycotoxin reference standards for all mycotoxin as well as stable isotope labelled internal standards in acetonitrile (¹³C₁₅ DON, ¹³C₂₂ HT-2, ¹³C₁₈ ZEN, ¹³C₂₀ OTA, ¹³C₁₇ AFB₁) were purchased from Romer Labs (Tulln, Austria). Urine samples from the Rowett biorepository were used in this study. Samples were collected in 2004 and stored at -20 °C prior to analysis. Urine samples were analysed for multiple mycotoxins adapting a digestion, extraction and LC-MS/MS method published previously (29). In brief, 4 mL of urine were spiked with a mixture of ¹³C-labelled internal standards (5 ng/mL ¹³C₁₅ DON,
Samples were then digested overnight with β-glucuronidase (Sigma-Aldrich, Ltd., Pool, UK; 23,000U in 1 mL 75 mM KH₂PO₄ buffer at pH 6.8), diluted with 16 mL PBS, the pH adjusted to 7.2 and purified and enriched using immunoaffinity columns (IAC, Myco-6in1, Vicam V10000176, Biocheck, St Asaph, UK). The binding capacities for the Myco-6in1 IAC are quoted by the manufacturer as 300 ng aflatoxins, 1250 ng DON/NIV, 800 ng FBs, 100 ng OTA, 500 ng T-2/HT-2, 350 ng ZEN. Samples were eluted with methanol, evaporated to dryness and reconstituted in 250 µL of 10% ethanol, resulting in a 16-fold concentration of urinary mycotoxins prior to LC-MS/MS analysis.

**LC-MS/MS analysis**

The liquid chromatography separation of mycotoxins was performed on a Shimadzu Nexera X2 LC system, using an Agilent Poroshell column (3 x 50 mm, 2.7 µm). The linear gradient comprised of 10 mM ammonium acetate (Sigma-Aldrich, Ltd., Pool, UK; solvent A) and methanol (solvent B). Starting conditions were 5% B, increasing to 95% B over 10 minutes, a 30-second hold at 95% B, and then re-equilibrated at 5% B for 1.5 minutes. The injection volume was 10 µL, the column oven was set to 40 °C and the flow rate was 400 µL/min. The LC eluent was directed into a Shimadzu 8060 triple-quadrupole MS. Mycotoxins were quantified using the multiple reaction monitoring (MRM) technique. Standard solutions of 500 ng/mL were injected into a flow of solvent and their transition values optimized. 8-point calibration curves (DON 1-500 ng/mL; NIV, HT-2 and T-2 0.2-100 ng/m; DOM-1, OTA, ZEN, α-ZEL, β-ZEL, AFB₁ 0.1-50 ng/mL and AFM₁ 0.05-25 ng/mL) including internal standards (80 ng/mL ¹³C₁₅ DON, ¹³C₂₂ HT-2; 20 ng/mL ¹³C₁₈ ZEN, ¹³C₂₀ OTA, ¹³C₁₇ AFB₁) were used to quantify all analytes.
Method validation

Urine mycotoxin analysis is based on the method described \(^{(29)}\). Myco-6in1 immunoaffinity columns were used to monitor the specified mycotoxins DON, ZEN, OTA, T-2, HT-2, AFB\(_1\) and AFM\(_1\) as well as additional closely related mycotoxins NIV, DOM-1, \(\alpha\)-ZEL and \(\beta\)-ZEL. The retention of DON, NIV and DOM-1 on DON-specific IAC has been recently demonstrated \(^{(30)}\). LOD and LOQ were determined in solvents and urine matrix by a signal to noise ratio of 10/1 and 3/1, respectively (Table 2, Figure 1).

Urine matrix effects (signal suppression/enhancement, SSE) were evaluated by comparing the slopes of matrix matched standard curves (8 levels, in triplicates) with solvent standard curves calculated as: matrix slope/solvent slope x 100. Matrix effects were efficiently compensated by using IAC and stable isotope internal standards, resulting in SSE ranging from 98-119% for all mycotoxins tested (Table 2). As no blank urine samples could be obtained, recovery experiments were performed in PBS spiked with a mycotoxin mixture at three different levels (DON 20.0, 5.0, 2.5 ng/mL; NIV, HT-2 and T-2 at 4.0, 1.0, 0.5 ng/ml; DOM-1, OTA, ZEN, \(\alpha\)-ZEL, \(\beta\)-ZEL, AFB\(_1\) and AFM\(_1\) at 2.0, 0.5, 0.25 ng/mL) in triplicate on three different days under repeatability conditions \(^{(31)}\). Recoveries were expressed as % of predicted final concentration (Table 2).

Recoveries and repeatability (RSDr) were within criteria established by EU Reg No 401/2006 \(^{(32)}\). All results were corrected for recovery and urinary mycotoxin concentrations are expressed as ng/mL urine and ng/mg creatinine. Urinary creatinine was analysed using alkaline picrate solution on an automated clinical analyser (KONELAB 30, Labmedics, Stockport, UK). Four quality controls of freshly spiked PBS (5.0 ng/mL DON; 1.0 ng/mL NIV, HT-2, T-2; and 0.5 ng/mL DOM-1, OTA, ZEN, \(\alpha\)-ZEL, \(\beta\)-ZEL, AFB\(_1\), AFM\(_1\)) were included in the analysis.
Estimation of daily urinary mycotoxin excretion

Total 24-hour urinary creatinine excretion was calculated as described previously \(^{(29)}\) using a clinical calculator (http://www.clinicalcalculator.com/english/nephrology/excrea/excrea.htm) on the basis of anthropometric data (Table 1) as follows:

\[
\text{Creatinine excretion females (mg/d)} = (22 - \text{age}/9) \times \text{BW} \\
\text{Creatinine excretion males (mg/d)} = (28 - \text{age}/6) \times \text{BW}
\]

\(\text{BW} = \text{body weight}\)

Daily urinary mycotoxin excretion was calculated as:

\[
\text{Urinary mycotoxin (ng/mg creatinine)} \times \text{Total 24-hour creatinine excretion (mg/d)}
\]

Urinary mycotoxin excretion is presented as ng/mL urine, ng/mg creatinine and µg/d in table 3.

Estimation of dietary mycotoxin exposure

Dietary mycotoxin intake was calculated as:

\[
\text{Daily urinary mycotoxin excretion (µg/d)/CR}
\]

\(\text{CR} = \text{urinary clearance rate for DON 72.3%/day}^{(7)} \), OTA 5\% \(^{(33)}\) and ZEN 9.4\% \(^{(23)}\).

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

\[
\%\text{TDI} = \frac{\text{Daily mycotoxin intake (µg/d)}}{\text{BW}} \times 100 / \text{TDI}
\]

\(\text{TDI} = \text{Tolerable daily intake (µg/kg BW/d)}\)

Statistical analysis

All results are presented as average concentration of 21 urine samples. Data for boys (n=12) and girls (n=9) were analysed by using an independent t-test (SPSS version 24) and no significant differences (p>0.05) were found for urinary mycotoxin excretion or
Results and Discussion

Prevalence of mycotoxins in urine

In this study spot urine samples from 21 children were analysed for 11 mycotoxins. Of these mycotoxins, DON and ZEN were detected in all urine samples. OTA and NIV were also frequently detected (95 and 81% of all samples, respectively), while α-ZEL, β-ZEL, DOM-1, T-2 and HT-2 were only present in 1-3 samples (Table 3). AFB$_1$ and AFM$_1$ were not detectable in any of the samples. Urinary DON was highly prevalent (100% of samples, mean 39.7 ng/mg creatinine or 13.1 ng/mL) and two samples also contained DOM-1, the microbial metabolite of DON, at low levels (Table 3). Both prevalence and urinary concentration of DON are comparable to a recent UK-based pediatric study (16) which found 100% of urine samples from 40 children aged 3-9 to be contaminated with DON at a mean concentration of 41.6 ng/mg creatinine. Similarly, a large study in 155 Belgian children (27) and a small study in 16 Spanish children (28) report DON and DON-glucuronide, the major urinary metabolite, to occur in 100% and 56% of urine samples, respectively. Urinary DON+DON-glucuronide was reported at 83.1 ng/mg creatinine or 74.2 ng/mL urine (Belgium) and 27.8 ng/mg creatinine (Spain). DON was also frequently detected in adults from Austria (96% of 27 samples, mean 19.5 ng/mg creatinine, 22), Italy (96% of 52 samples, mean 11.9 ng/mL, 34), Portugal (78% of 94 samples, median 4.0 ng/mg creatinine, 35) and pregnant women from Croatia (98% of 40 samples, 93.7 ng/mg creatinine, 20) and less frequently in Nigerian children (18% of 120 samples, mean 2.4 ng/mL, 36) and adults from Cameroon (42% of 175 samples, mean 5.9 ng/mL, 37). DON-glucuronides are the main urinary DON metabolites detected (9), and our method of urine
analysis including an enzymatic β-glucuronidase pre-digestion detects the sum of free and glucuronidated DON in the same analysis. DOM-1 was detectable in two urine samples (9% prevalence, at approximately 1% of the urinary DON concentration) which is lower than our previous studies in UK adults (20 – 40% prevalence of DOM-1, \(^{14,29}\)).

DOM-1 is a microbial metabolite of DON produced by the human gut microbiota of some individuals, but not others, and our previous work found the frequency of DOM-1 production to range between 10-20% in faecal microbiota from adults \(^{14,29,39}\). To date, no work has been published on the activity of children’s microbiota towards mycotoxin degradation, but profiles of children’s microbiota resemble that of adults from an early age \(^{40}\). A recent study in UK children did not detect any urinary DOM-1 \(^{16}\), and the Spanish study reports DOM-1 in 1/16 samples \(^{28}\). In contrast, the Belgian cohort reports DOM-1 in 17% of samples at very high concentrations (101 ng DOM-1 glucuronide/mg creatinine), which even exceeded the detected sum of DON and DON-glucuronide \(^{27}\). In adults, DOM-1 has been detected in 96% of Spanish adults (DOM-1 at 8.9 ng/mg creatinine, \(^{38}\), 28% of Portuguese adults spot urine (26 ng/mg creatinine, \(^{35}\)), but not in Austrian \(^{22}\) or Italian subjects \(^{34}\). In addition to DON and DOM-1, DON-3-sulfate has been identified as a novel urinary DON metabolite in humans \(^{41}\) with an excretion rate of 4% of dietary DON. DON-3-sulfate was not analysed in the current study and could further increase the estimate of dietary DON.

Nivalenol was detectable in 81% of urine samples in the current study, but at very low levels (mean 1.1 ng/mg creatinine, Table 3). NIV was not detectable in children from Spain \(^{28}\), but the Spanish study reports urinary NIV in 18.7% of young adults and 18.2% of adults at mean concentrations of 13.3 and 16.7 ng/mg creatinine, respectively. Other studies did not determine NIV in children’s urine. Our current method uses IAC cleanup and enrichment which facilitates a low detection limit (LOQ 0.125 ng/mL) which is
superior to methods which report NIV in children’s urine below LOQ (e.g. LOQ 1 ng/mL, 
28). NIV excretion in urine is reported in adults in Africa (10,37,42) but reports in children 
are rare (36) and further studies are needed to assess dietary exposure. Type A 
trichothecenes T-2 and HT-2 were each detected once (0.03 and 6.1 ng/mg creatinine, 
respectively; Table 3), but not in the same urine sample. This low incidence of T-2 and 
HT-2 is comparable to the Spanish cohort (HT-2 in 1/16 sample at 12.6 ng/mg creatinine, 
28) and no T-2 or HT-2 were found in Belgian children (27), Nigerian children (43) or adults 
from Cameroon (37). OTA was detected in 95% of samples in the current study at low 
levels (mean concentration of 0.15 ng/mg creatinine) which is higher than the Belgian 
cohort of children (51% of samples contained OTA at 0.08 ng/mg creatinine, 27). In adults, 
studies report a wide range of urinary OTA concentrations (0.006 ng/mg creatinine, 35; 
0.019 ng/mg creatinine, 38; 0.15 ng/mg creatinine, 21).

Zearalenone and its metabolites are potent xenoestrogens and exposure to these 
compounds is of great concern, especially in girls. Alternariol is another estrogenic 
mycotoxin with potential synergistic effects to ZEN (44). In the current study, ZEN was 
detected in all urine samples at mean levels of 0.3 ng/mg creatinine (Table 3), with no 
significant difference between boys and girls. The hepatic metabolites α-ZEL and β-ZEL 
were detected less frequently (3/21 and 2/21 samples, respectively), but at higher mean 
levels compared to ZEN (0.5 and 0.6 ng/mg creatinine, respectively; Table 3). α-ZEL and 
β-ZEL only co-occurred in samples which were also contaminated with ZEN at ratios of 
66-82% for α-ZEL and 77-116% for β-ZEL. Prevalence of urinary ZEN is higher in our 
study (100% of samples) compared to a study in 163 US girls aged 9-10 (55%, 45) but the 
mean urinary concentration is lower (0.1 ng/mL urine in the current study compared to 
1.3 ng/mL in US girls. Two European studies (27,28) and one study from Nigeria (43) report 
ZEN and ZEL as not detectable in children’s urine whereas another study from Nigeria
reports ZEN in 82% and $\alpha$- and $\beta$-ZEL in 4 and 6% of urine samples from children and adults\(^{(36)}\).

AFB\(_1\) and AFM\(_1\) were not detected in any urine samples from children in this study, which is in agreement with other studies in European cohorts\(^{(27)}\). Urinary aflatoxin excretion reflects recent, acute dietary exposure, whereas aflatoxin-lysine adducts in plasma are validated biomarkers for chronic aflatoxin exposure\(^{(46)}\). Aflatoxins are frequently detected in cohorts of children and adults in Africa (14 – 72% of samples positive for urinary AFM\(_1\)\(^{36,43}\)) where aflatoxin exposure is linked to impaired growth\(^{(47)}\). However, strict regulations for aflatoxins in food in Europe and low consumption of high-risk foods such as peanuts lead to a negligible exposure to aflatoxins in children and adults.

**Estimation of dietary mycotoxin exposure**

Children were exposed to substantial amounts of DON through their diet (average 26.5 $\mu$g/d, Table 4). When dietary exposure was compared to the tolerable daily intake for DON, the TDI was exceeded in 52% of children (Figure 2). Average DON exposure in all 21 children was 136% of TDI and the proportion of TDI exceedances for DON in children in this study is much higher than in adults where we previously found 7% of TDI exceedances in 15 subjects\(^{(14)}\). Our results are in line with a recent study\(^{(16)}\) reporting up to 63% of UK children exceeding the TDI for DON, and this high frequency of TDI exceedances in children is of great concern. Similarly, cohorts in other countries also report higher frequency of TDI exceedances in children (22% Spain, \(^{28}\); 69% Belgium, \(^{27}\)) than adults (4% Spain, \(^{28}\); 29% Belgium, \(^{27}\); 10% Portugal, \(^{35}\); 40% Italy, \(^{34}\); 33% Austria, \(^{22}\); 48% Croatia, \(^{20}\)). Exposure assessment through urinary OTA biomarkers has been performed in adults\(^{(34,35,38)}\). In adults TDI exceedances for OTA are reported
frequently (14% of subjects median 27% of TDI from Portugal, \textsuperscript{35}; 94% of subjects mean 818% of TDI from Italy, \textsuperscript{34}; and 96% of subjects mean 185% of TDI from Spain, \textsuperscript{38}).

Vidal et al. \textsuperscript{(38)} also state that exposure estimate from urinary biomarkers greatly exceed estimates from dietary approaches. In UK adults, dietary OTA exposure was estimated at average 1.5 ng/kg bw/d based on plasma levels or 0.9 ng/kg bw/d based on duplicate diet analysis \textsuperscript{(48)}. This exposure is significantly lower than our estimates of 74.1 ng/kg bw/d (Table 4) in children based on urinary OTA excretion. Based on this exposure estimate, 95% of children in the current study exceeded the TDI for OTA (Figure 2). TDI exceedances for ZEN were less common in children in the current study (5% of children) and have not been reported in the literature. In adults, 24% of subjects exceeded the TDI for ZEN in a recent study \textsuperscript{(35)}. Further work is needed to better elucidate the exposure and potential health risk associated with this mycotoxin in children. Dietary co-exposure to several mycotoxins is highly likely as several mycotoxins are frequently detected in important food commodities including cereal grains (trichothecenes, ZEN, OTA), corn (ZEN and fumonisins) and dried fruits (OTA) \textsuperscript{(1)}. Co-exposure to the mycotoxins DON, OTA and ZEN was also evident in this study (Figure 3). Children exceeding the TDI for DON or ZEN also exceeded the TDI for OTA and this co-exposure puts them at an even greater risk of mycotoxin toxicity. Cereals and cereal based foods have been identified as main contributors to mycotoxin exposure in the UK \textsuperscript{(49)} and our study confirms the prediction that children might be at high risk to exceed TDI. For carcinogenic mycotoxins such as aflatoxins, no tolerable daily intakes are set and a benchmark dose is calculated instead \textsuperscript{(50)}. However, this does not apply to the current study as no aflatoxins were detected in urine. Urinary biomarker analysis for mycotoxin exposure is an important approach for estimating dietary exposure. Urinary DON is a well validated biomarker for recent dietary exposure, whereas biomarkers for other mycotoxins such as ZEN and OTA
are less strong in predicting dietary exposure due to the low urinary excretion rate (9.4 and 5%, respectively) and complex metabolism in humans \(^{(18,35,51,52)}\). Hence, future studies are needed to confirm the present finding of frequent and substantial TDI exceedances for OTA in UK children.

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

Acknowledgements

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

Supporting Information

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


**Funding**

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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, AFB1 0.31 ng/mL; AFM1 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.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Boys, n = 12</th>
<th>Girls, n = 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>4.6 ± 1.3</td>
<td>4.6 ± 1.0</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>18.4 ± 2.8</td>
<td>20.3 ± 6.9</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>15.9 ± 1.3</td>
<td>17.3 ± 2.6</td>
</tr>
<tr>
<td>Urinary creatinine (mg/d)</td>
<td>501.2 ± 72.7</td>
<td>435.7 ± 146.1</td>
</tr>
</tbody>
</table>

All data are expressed as average ± SD.
Table 2. Method Performance Parameters of the LC-MS/MS Method.

<table>
<thead>
<tr>
<th>Compound</th>
<th>RT</th>
<th>m/z precursor ion</th>
<th>Polarity</th>
<th>m/z product ions</th>
<th>Relative response ratio</th>
<th>LOD/LOQ urine (ng/mL)</th>
<th>Matrix effect (SSE%±RSD)</th>
<th>Recovery (%±RSDr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>2.9</td>
<td>355.25[M+CH₂COO]⁻</td>
<td>-ve</td>
<td>295.2/265.3</td>
<td>67.9</td>
<td>0.156/0.328</td>
<td>97.8±6.1</td>
<td>111.8±6.8</td>
</tr>
<tr>
<td>DOM-1</td>
<td>3.8</td>
<td>339.25[M+CH₂COO]⁻</td>
<td>-ve</td>
<td>59.1/249.2</td>
<td>42.2</td>
<td>0.063/0.158</td>
<td>103.2±3.3</td>
<td>94.8±14.7</td>
</tr>
<tr>
<td>NIV</td>
<td>2.2</td>
<td>371.20[M+CH₂COO]⁻</td>
<td>-ve</td>
<td>281.2/311.2</td>
<td>66.7</td>
<td>0.066/0.125</td>
<td>105.5±0.6</td>
<td>113.0±10.8</td>
</tr>
<tr>
<td>OTA</td>
<td>6.9</td>
<td>404.15[M+H]⁺</td>
<td>+ve</td>
<td>239.1/221.1</td>
<td>43.1</td>
<td>0.003/0.006</td>
<td>106.7±2.2</td>
<td>101.6±13.7</td>
</tr>
<tr>
<td>ZEN</td>
<td>8.1</td>
<td>317.20[M-H]⁻</td>
<td>-ve</td>
<td>175.3/131.2</td>
<td>59.6</td>
<td>0.016/0.033</td>
<td>112.2±2.5</td>
<td>91.2±13.0</td>
</tr>
<tr>
<td>α-ZEL</td>
<td>7.9</td>
<td>319.20[M-H]⁻</td>
<td>-ve</td>
<td>275.3/160.3</td>
<td>50.0</td>
<td>0.033/0.063</td>
<td>106.6±4.2</td>
<td>95.4±12.8</td>
</tr>
<tr>
<td>β-ZEL</td>
<td>7.4</td>
<td>319.20[M-H]⁻</td>
<td>-ve</td>
<td>275.3/160.3</td>
<td>51.5</td>
<td>0.033/0.063</td>
<td>109.6±4.1</td>
<td>94.3±14.3</td>
</tr>
<tr>
<td>HT-2</td>
<td>7.2</td>
<td>442.30[M+NH₄]⁺</td>
<td>+ve</td>
<td>263.3/215.3</td>
<td>70.9</td>
<td>0.013/0.031</td>
<td>106.4±3.1</td>
<td>126.8±13.2</td>
</tr>
<tr>
<td>T-2</td>
<td>7.8</td>
<td>484.20[M+NH₄]⁺</td>
<td>+ve</td>
<td>215.2/305.2</td>
<td>99.2</td>
<td>0.006/0.013</td>
<td>119.0±1.7</td>
<td>91.8±8.9</td>
</tr>
<tr>
<td>AFB₁</td>
<td>6.1</td>
<td>313.10[M+H]⁺</td>
<td>+ve</td>
<td>285.1/241.0</td>
<td>84.3</td>
<td>0.003/0.006</td>
<td>112.9±8.9</td>
<td>100.9±10.0</td>
</tr>
<tr>
<td>AFM₁</td>
<td>5.3</td>
<td>329.10[M+H]⁺</td>
<td>+ve</td>
<td>273.2/229.2</td>
<td>51.0</td>
<td>0.003/0.008</td>
<td>108.9±6.4</td>
<td>91.0±15.6</td>
</tr>
<tr>
<td>DON₁₃C₁₅</td>
<td>2.9</td>
<td>370.20[M+CH₂COO]⁻</td>
<td>-ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTA₁₃C₂₀</td>
<td>6.9</td>
<td>424.20[M+H]⁺</td>
<td>+ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZEN₁₃C₁₈</td>
<td>8.1</td>
<td>335.20[M-H]⁻</td>
<td>-ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HT-2₁₃C₂₂</td>
<td>7.2</td>
<td>464.30[M+NH₄]⁺</td>
<td>+ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB₁₁₃C₁₇</td>
<td>6.1</td>
<td>330.20[M+H]⁺</td>
<td>+ve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Mycotoxin</th>
<th>Number (%) positive samples</th>
<th>Mean of positive samples (range)</th>
<th>Mean of positive samples (range)</th>
<th>Mean of positive samples (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ng/mL urine</td>
<td>ng/mg creatinine</td>
<td>µg/d</td>
</tr>
<tr>
<td>DON</td>
<td>21 (100%)</td>
<td>13.10 (0.69-42.03)</td>
<td>39.68 (1.88-152.99)</td>
<td>19.13 (0.87-76.96)</td>
</tr>
<tr>
<td>DOM-1</td>
<td>2 (9%)</td>
<td>0.15 (0.15,0.15)</td>
<td>0.40 (0.25,0.56)</td>
<td>0.21 (0.14,0.28)</td>
</tr>
<tr>
<td>NIV</td>
<td>17 (81%)</td>
<td>0.36 (0.13-0.58)</td>
<td>1.13 (0.42-2.43)</td>
<td>0.54 (0.11-1.19)</td>
</tr>
<tr>
<td>HT-2</td>
<td>1 (5%)</td>
<td>1.77</td>
<td>6.13</td>
<td>2.73</td>
</tr>
<tr>
<td>T-2</td>
<td>1 (5%)</td>
<td>0.02</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>OTA</td>
<td>20 (95%)</td>
<td>0.05 (0.02-0.11)</td>
<td>0.15 (0.06-0.33)</td>
<td>0.07 (0.03-0.15)</td>
</tr>
<tr>
<td>ZEN</td>
<td>21 (100%)</td>
<td>0.09 (0.03-0.25)</td>
<td>0.28 (0.09-1.20)</td>
<td>0.14 (0.04-0.65)</td>
</tr>
<tr>
<td>α-ZEL</td>
<td>3 (14%)</td>
<td>0.18 (0.11-0.22)</td>
<td>0.50 (0.19-0.92)</td>
<td>0.26 (0.12-0.49)</td>
</tr>
<tr>
<td>β-ZEL</td>
<td>2 (9%)</td>
<td>0.16 (0.10,0.22)</td>
<td>0.63 (0.33,0.93)</td>
<td>0.37 (0.24,0.50)</td>
</tr>
<tr>
<td>AFB₁</td>
<td>0</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>AFM₁</td>
<td>0</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
<td>&lt;LOD</td>
</tr>
</tbody>
</table>

LOD = limit of detection
Table 4. Dietary Intake Estimates of Major Mycotoxins by 21 UK Children.

<table>
<thead>
<tr>
<th></th>
<th>Mean dietary intake (range) µg/kg bw/d</th>
<th>Mean total dietary intake (range) µg/d</th>
<th>Mean % TDI (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DON</td>
<td>1.36 (0.07-5.77)</td>
<td>26.49 (1.20-106.86)</td>
<td>135.62 (7.13-577.31)</td>
</tr>
<tr>
<td>ZEN</td>
<td>0.10 (0.02-0.70)</td>
<td>2.20 (0.40-17.34)</td>
<td>40.73 (8.4-278.7)</td>
</tr>
<tr>
<td>OTA</td>
<td>0.07 (0.00-0.18)</td>
<td>1.39 (0.05-2.94)</td>
<td>435.88 (19.50-1071.39)</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

DON

OTA

ZEN

- 0-25% of TDI
- 25-50% of TDI
- 50-75% of TDI
- 75-100% of TDI
- > 100% of TDI
Figure 3

[Bar chart showing dietary mycotoxin exposure (%TDI) for different participants with bars for DON, OTA, and ZEN]
Graphic for table of contents

**Dietary mycotoxin exposure in UK children frequently exceeds safe levels**

- **DON**
- **OTA**
- **ZEN**

Estimated dietary mycotoxin exposure as % TDI