FOOD STANDARDS AGENCY

UNIVERSITY OF LEEDS

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## THE ANALYSIS OF URINE SAMPLES FOR BIOMARKERS OF EXPOSURE TO FUSARIUM MYCOTOXINS

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**Abbreviations**

| | |
|---|---|
| AUC | area under curve |
| CI | confidence interval |
| CV | coefficient of variation |
| DON | deoxynivalenol |
| F | female |
| FB1 | fumonisin B1 |
| IAC | immunoaffinity chromatography |
| IS | internal standard |
| LC-MS | liquid chromatography – mass spectrometry |
| M | male |
| n/a | not applicable |
| n/d | not detected |
| NDNS | National Diet and Nutrition Survey |
| QC | quality control |
| SD | standard deviation |
| TDI | tolerable daily intake |
| WHO | World Health Organisation |

## EXECUTIVE SUMMARY

Deoxynivalenol (DON) and fumonisin (FB) are two of the most frequently occurring *Fusarium* mycotoxins in cereals. DON, a trichothecene mycotoxin produced by *F. graminearum* and *F. culmorum*, causes feed refusal, decreased weight gain, gastroenteritis and immune dysfunction in animals. FB's are a family of mycotoxins produced by *F. verticilliodes and F. proliferatum* that cause a range of diseases in different animals including cancer and neural tube defects. Current *Fusaria* mycotoxin exposure estimates are of limited precision as they are based on measurements in foodstuffs combined with food consumption data. Biomarkers may provide an improved approach to exposure assessment. In **Phase 1** of the study, the prevalence and level of these two important *Fusarium* mycotoxins in urine was assessed as putative exposure biomarkers by utilising the 2000-2001 UK adult National Diet and Nutrition Survey data on food intake and urine collected. **Phase 2** of the study provided a more detailed assessment and validation of the deoxynivalenol biomarker, by the recruitment of UK adults who kept a personal food diary, and provided urine and food samples during a 12 day survey.

**Phase 1: The analysis of urine samples for biomarkers of exposure to deoxynivalenol (DON) and fumonisins (FB).** The 2000-2001 UK adult National Diet and Nutrition Survey provides 7-day food diary data on consumption of cereal-based commodities, food items which are potentially contaminated with *Fusaria* mycotoxins. In addition 24 hour urine samples were available from the survey. This resource was used to compare levels of urinary DON and FB with cereal intake.

**DON:** Urinary DON was assessed using LC-MS following immunoaffinity purification. To adjust for recovery through the extraction a [$^{13}$C] isotope-labelled DON was spiked into all urines. The detection limit was **0.1ng DON/ml of urine** in a 5ml sample. One hundred subjects were identified from low (female 38/male 62), medium (female 50/male 50) and high (female 54/male 46) cereal consumption groups translating respectively to 107g cereal/day; range 88-125g/day; 179g/day; range 162-195, and 300g/day; range 276-325g/day.

All 300 subjects had detectable DON in their urine sample. The mean level increased from low to medium to high cereal consumption groups: low, 7.24ng DON per mg creatinine (95%CI 6.23-8.17); medium, mean 9.21ng/mg (8.08-10.49ng/mg) and high 10.91ng/mg (95%CI 9.49-12.43ng/mg). **Cereal intake was significantly associated with urinary DON ($p<0.001$); those with high intake had a mean level 50% higher than those with low intake.**

In multivariable analysis wholemeal bread ($p<0.001$), "other" bread ($p<0.001$), white bread ($p=0.002$), buns and cakes ($p=0.051$) were significantly associated with urinary DON, with the strongest associations being found for wholemeal bread and "other" bread. In categorical analysis wholemeal bread was strongly associated with the level of urinary DON. However, in the statistical model only 11% of the variance in urinary DON was explained by the variation in types of cereal consumed, probably due to the heterogeneity of DON contamination of cereals among other factors.

**FB**: A method was developed to measure urinary FB involving extraction on a Waters Oasis MAX column followed by LC-MS analysis. This method had a limit of detection of **0.02ng**/ml in 5ml of urine. In urine samples from 50 individuals identified from the NDNS as having the highest maize consumption (49.3g/day (range 35.5 – 99.9g/day)) no urinary FB was detected. **Given the current analytical limit of detection it is unlikely that urinary FB will be a useful biomarker of exposure in the UK population.** It is possible that this assay may be used in regions with higher FB exposure.

**Phase Two: The further development and understanding of urinary biomarkers for exposure to deoxynivalenol.** A two week study was undertaken in which the temporal variation of urinary DON was assessed in 35 individuals. Food diaries were kept throughout the study, and five consecutive first morning urines collected in both week one and week two. Urinary measures of DON were compared to cereal intake over the study period. In **week one** the temporal variation in urinary DON was assessed for all individuals during their normal dietary practice. In **week two**, the study group was divided such that group one (n=25) took part in a partial intervention and group two (n=10) took part in a full intervention. The **partial intervention** restricted all major sources of dietary DON except bread for four consecutive days. A duplicate portion of each bread sample consumed was provided during this four day period such that the total intake of DON from this single major source could be established. This component of the study examined for the first time the relationship between intake of DON and the urinary measure. The availability of four consecutive measures of DON intake and urinary DON further allows assessment of the temporal nature of these measurements. The **full intervention** restricted all major sources of dietary DON for four consecutive days. This component of the study examined the kinetics of clearance of DON.

During the consumption of the normal diet DON was detected in 198/210 (94%) of urine samples; mean 11.6ng/ml (range nd – 78.2ng/ml). Urinary DON level was significantly associated with cereal intake ($p<0.001$). For the partial intervention, DON was detected in 94/98 (96%) of these samples; mean 6.3ng/ml (range nd – 34.0ng/ml). For the full intervention DON was detected in 17/40 (43%) of the samples; mean 0.7ng/ml (range nd – 3.2ng/ml). **Urinary DON was significantly associated ($p<0.001$) with DON intake during the intervention phase, and the variation in the mean urinary measure over four days was well explained by mean DON intake (adjusted $R^2 = 0.83$)**. The transfer of DON to the urine was estimated to be 72.3%, and the mean DON intake during the normal phase was estimated to be 298ng/kg bw/day. However, 6 of 35 individuals were predicted to exceed the recommended tolerable daily intake of 1000ng/kg bw/day on at least one day. Based on the levels of urinary DON obtained in Phase One and the more accurate measure of the percent transfer of DON into urine, **the mean estimated DON intake for Phase One was 222ng/kg bw/day (n=300)**; with 2/300 individuals predicted to exceed the recommended TDI. Again it should be noted that these individuals were a stratified sample based on cereal consumption and no samples were taken from individuals in the highest decile of cereal intake in the NDNS survey.

The urinary assay includes an overnight incubation with β-glucuronidase as it was previously suggested that DON-glucuronide was the major DON metabolite following ingestion. The ratio of DON to DON glucuronide may provide a phenotypic marker of susceptibility to DON toxicity. For a subset of samples (n=100) the urinary assay was repeated but without β-glucuronidase, such that only 'free' DON was measured. This allowed an assessment of the ratio of free DON to the DON-glucuronide. On average the DON-glucuronide represented 90% of total DON. The ratio was independent of total DON concentration, but did vary by individual.

Overall these data indicate that the urinary DON assay was sensitive enough to measure DON exposure at the individual level in the UK population. DON exposure was frequent and for some UK adults DON intake was estimated to exceed the recommended TDI on at least one day. Cereal consumption and in particular bread significantly contributes to the levels of urinary DON observed in UK adults. Urinary DON was significantly associated with DON intake and the variation in urinary DON was well explained by DON intake.

**Urinary DON provides a useful biomarker with which to assess DON exposure.**

**General Background**

Fungal growth can lead to the production of highly potent toxins known as mycotoxins. There are many naturally occurring mycotoxins with five of major agricultural importance: aflatoxins, deoxynivalenol (DON), fumonisins (FB), zearalenone (ZON), and ochratoxin A (Miller, 1995). *Fusarium* mycotoxins include DON, FB and ZON and occur primarily on the cereal crops wheat, maize (corn) and barley. DON, a trichothecene mycotoxin produced by *F. graminearum* and *F. culmorum*, (WHO 1990) causes feed refusal, decreased weight gain, gastroenteritis, cardiotoxicity and teratogenicity in animals (Prelusky 1997; Rotter *et al.,* 1996). It results in a variety of immunological effects *in vitro* and *in vivo* (Bondy and Pestka, 2000; Meky *et al*., 2001). FB's are a family of mycotoxins produced by different *Fusarium* fungi, *F. verticilliodes and F. proliferatum* (IARC 2002) that cause diseases in a variety of animals with the organ specificity being species dependent (Turner et al., 1999). An IARC Working Group recently decided that although there is sufficient evidence of carcinogenicity of FB1 in animals, as yet there is inadequate evidence in humans (IARC 2002).

A recent scientific cooperation (SCOOP) task assessed the frequency of foods, predominantly cereal-based, that were contaminated by 16 *Fusarium* mycotoxins in EU member states. A total of 44,670 food samples were analysed, including eleven countries providing assessment for DON, and nine for FB and ZON. *Fusarium* mycotoxins were predominantly associated with wheat and maize-based foods. DON was the most frequently observed *Fusarium* mycotoxin, with 57% of all samples being positive, whilst fumonisins were detected in 46% of all samples.

For the UK, average dietary intakes for DON were estimated based on food consumption and levels of contamination in foodstuffs. For adults these levels were 176ng/kg(bw)/day and 142ng/kg bw/day, for males and females respectively. In terms of food commodities, the majority of exposure came from bread, breakfast cereals, corn-meal and snack foods. Higher mean intakes were estimated for infants and young children (range 400-500ng/kg bw/day). Although overall these estimated exposures are slightly or just below the recommended TDI of 1000ng/kg bw/day, they only represent mean levels and the contamination level in food was highly variable such that estimates of exposure may be 10 to 20-fold higher in individuals consuming the most highly contaminated foods. In the EU in general, lower intakes were estimated for FB compared to DON. FBs were found in 57% of maize from the UK, but as this was the only data source no intake estimates were made. However, maize is one of the major sources of FB and the mean level of contamination in the UK was ~800μg/kg representing the higher end of the range.

Given the uncertainties in the assessment of mycotoxin exposure from dietary data, biomarkers have been suggested to offer a better approach to measure human exposure. Biomarkers potentially provide data both at the individual level and establish the prevalence and range of exposure in a population (Turner et al., 1999; Wild and Turner 2002; Meky et al., 2003). For a number of years the Molecular Epidemiology Unit has followed a strategy to develop biomarkers of exposure for mycotoxins, namely aflatoxins (Wild and Turner 2002), DON (Meky et al., 2003) and FB (Turner et al., 1999; Nikiema et al., 2004). There are published data for putative urinary biomarkers for DON (Meky et al., 2003) and FB (Shetty and Bhat 1998).

Phase 1 of this study focused on understanding the level and distribution of DON and FB in urines from UK adults previously collected as part of the UK adult NDNS. Phase 2 focused on the further validation of the urinary measure for DON as a biomarker of exposure.

## PHASE ONE: The analysis of urine samples for biomarkers of exposure to deoxynivalenol (DON) and fumonisins (FB)

### Background

Current estimates of human exposure to mycotoxins produced by *Fusaria spp.* are of limited precision as they are based on measurements of mycotoxins in foodstuffs combined with food consumption data. The 2000-2001 UK adult National Diet and Nutrition Survey (NDNS) provides data on consumption of cereal-based commodities potentially contaminated with mycotoxins. The combination of these data with biomarkers offers an opportunity to improve the basis for exposure assessment for DON and FB in relation to cereal intake in the UK adult population. The data would provide an improved understanding of the prevalence and level of *Fusarium* mycotoxin exposure in the UK and assist the Food Standards Agency in developing regulatory policy for mycotoxin contamination of cereals.

**PHASE 1: Objective 01 - *Select 300 urine samples from UK adult NDNS.*** Fusarium mycotoxins predominantly contaminate cereals including wheat, maize and barley. In order to understand the range of exposure within the UK population it was necessary to establish a stratified sample of individuals with different levels of cereal intake (for DON measurement) plus an additional group of 25 subjects with high and 25 subjects with medium cereal intake (for fumonisin measurement), based on the adult NDNS 2001 for whom urine samples were available.

### Outputs

**A. DON** In 2000-2001 the adult NDNS collected 24-hour urine samples from 1724 individuals aged 19-64 years of age from across the UK. Each individual completed a detailed seven-day weighed food diary (Office for National Statistics 2005). Based on the dietary records, food groups were identified that provided the major sources of cereal in the UK diet; these included breads (wholemeal, white, soft grain, other), breakfast cereals (high-fibre and other), pasta, pizza, fruit pies, biscuits and buns/cakes. Other potential sources of DON were predicted to provide a smaller contribution and were excluded from the process of selecting individuals for inclusion in the current study. The contribution of cereal from each food group included was determined for each individual over the seven-day period and then divided by seven to create a 'total cereal intake' in grams per day. The total cereal intake value was used to rank individuals, and subsequently the 1724 individuals were divided into deciles. The lowest and highest deciles were excluded to avoid potential outliers. Three groups were created representing low ($2^{nd}$/$3^{rd}$ decile), medium ($5^{th}$/$6^{th}$ decile) and high ($9^{th}$ decile) cereal intake. The range of intake within each decile is not evenly distributed, and thus overall the average cereal intake for all 1724 individuals is close to the average of this sampling frame. From each of these three groups 100 individuals were selected on the basis of urine sample availability and that the urine sample had been collected during the period that data in the seven-day diary was provided. Roughly equal numbers of males and female samples were selected for each cereal intake group. From the seven day food diaries the average cereal intake for each individual were generated for each person. The intake groups were classified as **low** (mean 107g/day; range 88-125g/day), **medium (**mean 179g/day; range 162-195), and **high** (mean 300g/day; range 276-325g/day).

**B. Fumonisin** Selection criteria for FB analysis were amended following a meeting between the University of Leeds and the FSA on the $1^{st}$ of June 2006 (see appendix A for detail). The selection of subjects for FB analysis successfully established 50 individuals with the highest maize intake. Urine samples were obtained from the NDNS 2001 collection for each of these subjects.

**PHASE 1: Objective 02 - *Evaluate the need for an accelerated storage study for FB based on literature review and consultation with external experts***.

**Output**
FB tends to be stable to heating and processing, there are no reports of losses in cryo-preserved solutions (Visconti et al., 1994). Following literature surveys and advice from other groups working with these mycotoxins the recommendation was that no additional storage trial was warranted. This was agreed with the FSA.

**PHASE 1: Objective 03 - *Completion of training for research assistant in extraction process and LC-MS analysis***

**Output**
Joseph Rothwell was appointed in the summer of 2005. He had experience in HPLC, and was subsequently trained in the other aspects of the methodology. The Standard Operating Procedures for *IAC Extraction of DON Metabolites* and *Analysis of IAC-Extracted DON Metabolites by LC-MS* were provided at the 19/10/2005 meeting. The analysis of urinary DON was a modified version of the method of Meky *et al*., (2003). Following the commercial availability of an internal standard using $^{13}C$ isotope-labelled DON discussions were held with the FSA and an agreement was made to further develop the assay from that reported on 19/10/2005. A more robust assay was therefore developed in which test samples and LC-MS standards were spiked with the internal standard and test samples quantified by reference to a response ratio calibration curve generated by Quanlynx software. Using complete internal standardisation where quantification was based on a response ratio, any variation in extraction and LC-MS performance was accounted for. In addition the sensitivity of this assay was improved from 0.4ng DON/ml urine to 0.1ng/ml.

**Quality Control (QC):** For QC purposes one urine sample that had previously been identified to have detectable DON was used. Sixty aliquots (4ml) of this urine were frozen alongside the test samples. Prior to initiation of the main study 12 aliquots of this QC urine were assessed over 4 days. The mean level and 95% confidence interval (CI) are presented to indicate the reliability of the measure. For the QC the mean level was 14.9ng DON/ml urine (95%CI: 14.5, 15.4ng/ml; CV 5.5%). Two QC samples were run with each batch of test samples. The assay approach is detailed in the following two sections.

**A. Extraction procedure:** Following ingestion of DON the urine will contain a DON-glucuronide as well as the parent compound DON. The DON-glucuronide is not extracted by immunoaffinity columns and therefore this DON conjugate undergoes enzymic cleavage using the enzyme β-glucuronidase to release DON prior to extraction. In addition a fixed quantity of an internal standard is added at the beginning to adjust for recovery through the extraction process. In brief, each batch of 20 NDNS samples and 2 QCs were removed from storage at -80°C, allowed to thaw, and the samples centrifuged (2000g; 15 min; 4°C). Aliquots (4ml) were removed, transferred to 15ml tubes, and $^{13}C$-DON internal standard added (Biopure, Tulln, Austria) to give a final concentration of 5ng/ml. Each sample was adjusted to pH 6.8, β-glucuronidase added in 1ml $KPO_4$ (Type IX-A from *E. coli*; Sigma; 23,000 units), and the tubes immediately incubated in a shaking water bath for 18h at 37°C to release DON from the DON-glucuronide. After this period the samples were removed and again centrifuged (2000g; 15 min; 4°C), and the supernatant diluted to 16ml with PBS (pH 7.2). The diluted material was passed through DON test immunoaffinity columns (Vicam Ltd) as per manufacturer's instructions. DON was eluted from columns with methanol (4ml) and the extracts dried *in vacuo* using a Savant Speed Vac and reconstituted in 10% ethanol (250µl) for analysis.

**B. LC-MS analysis of extracted DON:** Extracts are analysed using a Waters 2795 separations module coupled to a Waters 995 photodiode array detector and a Micromass Quattro Micro triple quadropole mass spectrometer. Separation of DON was achieved using a Phenomonex Luna C18 column and a mobile phase sequence of 27min duration starting with 20% methanol, changing to a wash of 75% methanol after 10min and reverting to 20% methanol after 16min. Flow rate was 1 ml/min and injection volume was 25µl. One fifth of the eluent was directed into the desolvation chamber of the MS and the remainder pumped to waste. Selective ion recording was used to quantify DON by reference to $^{13}$C-DON internal standard. Two masses each of DON (m/z 297.2 and 319.2) and $^{13}$C-DON (m/z 333.2 and 334.2), see Figure 1A, were monitored for 0.25s each and summed to produce one total ion current peak each for analyte and internal standard (see Figure 1B).

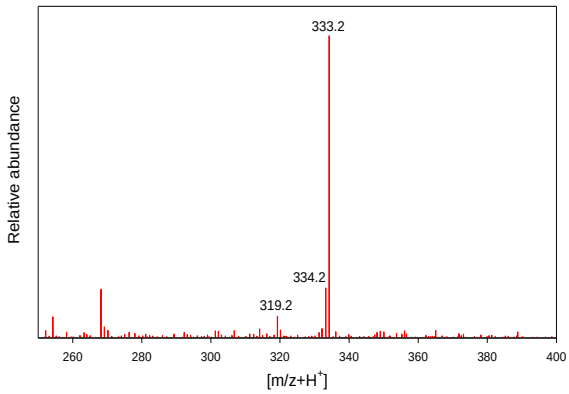



**Figure 1A: Mass spectrum of $^{13}$C-DON**

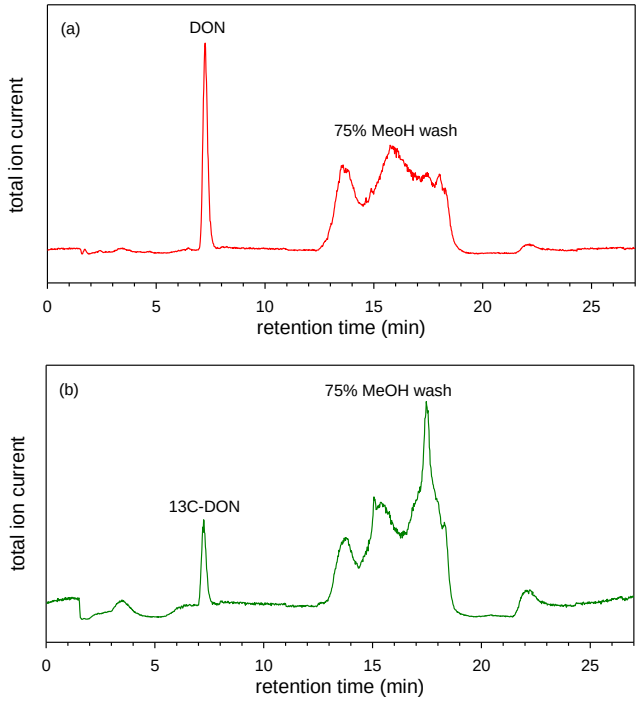



**Figure 1B: Total ion current traces of a urine sample (DON 13.1ng/ml) spiked with $^{13}$C-DON internal standard. Trace (a) generated by SIR of DON monitoring masses of 297.2 and 319.2; trace (b) generated by SIR of $^{13}$C-DON monitoring masses of 333.2 and 334.2. The retention time of both compounds was 7.4 min.**

External standards of concentrations 10, 20, 50, 100, and 200ng DON/ml spiked with internal standard (80ng/ml) were included at the start of each run and a further $^{13}C$-DON-only standard (80ng/ml) included at the end to monitor inter-run variation. Unknowns and QCs spiked with an equivalent concentration of internal standard were quantified by reference to a response-ratio calibration curve generated by Quanlynx software.

**PHASE 1: Objective 04 - *Analysis of urine samples for DON and DON-glucuronide combined using LC-MS.*** Extract DON and DON metabolites from 300 urine samples from high, medium and low cereal consumers. Enrich urinary extracts with immunoaffinity chromatography and determine mycotoxin level using LC-MS.

**Output**

The selected samples were randomised and analysed blinded in 15 batches of 20 samples each plus two pre-determined QC samples in each batch. This analytical approach was applied to a stratified sample of 300 individuals with three different levels of cereal intake (**low intake** mean 107g/day; range 88-125g/day; **medium intake** mean 179g/day; range 162-195, and **high intake** mean 300g/day; range 276-325g/day). Initially the DON levels were determined as ng DON/ml urine (Table 1) and were then adjusted for urinary creatinine measured by the Bayer Method with an ADVIA Chemistry Systems 1650 instrument (Dept. of Clinical Biochemistry, Leeds General Infirmary). The creatinine adjustment is a standard procedure to account for individual variability in urinary output. All 300 samples analysed had detectable urinary DON (mean 11.1ng/mg creatinine; range 0.6 – 48.2ng/mg). The distribution of the data by cereal intake group is presented in Figure 2. It is notable that the study mean is close to the mean of the medium intake group.

**Table 1: Urinary DON in comparison to high, medium and low cereal intake**

| Group | Cereal Intake g/day (range) | DON ng/ml Mean (range) | DON ng/mg Mean (range) | DON ng/mg GM (95%CI) |
|---|---|---|---|---|
| **Low** | **107 (88-125)** | **5.4 (0.1-19.8)** | **9.7 (0.6-43.9)** | **7.2 (6.2-8.2)** |
| **Medium** | **179 (162-195)** | **7.8 (0.4-56.4)** | **11.3 (1.1-45.0)** | **9.2 (8.1-10.5)** |
| **High** | **300 (276-325)** | **9.3 (2.1-44.6)** | **12.4 (2.1-48.1)** | **10.9 (9.5-12.4)** |

N.B. For statistical purposes natural log transformed data are used when data are not normally distributed. GM (geometric means) represent back transformed means of natural log transformed data.

**Quality control**

As mentioned above, fifteen batches of samples were run with two QC samples per batch. The mean level of DON in the QCs was 14.2ng/ml (95%CI 13.8-14.6ng/ml), and the CV for the complete set was 8.0%. There was no difference in the pre-test QC samples and study QC samples ($p>0.05$).

Twenty repeat samples from among the 300 DON analyses were repeated and a CV of the original and repeat data for each rerun obtained. The repeated data were in good agreement with the original set of data (mean CV 10.1% (95%CI 6.8-13.4%).

A series of ten PBS blank controls were also analyzed following concerns expressed by the FSA that some immunoaffinity columns might be contaminated by low amounts of DON. When these 10 blank samples were analysed we also included two urine QCs as previously described, and DON levels again in these latter samples were in good agreement with earlier data. When these two additional QCs were

included with the complete set of QCs for the study, the overall mean was 14.5ng/ml (95%CI 14.2-14.8ng/ml). The overall CV for the QCs was 7.6%. Two of the ten PBS samples were spiked with 13C-DON as internal standard. This allowed us to confirm that the recovery of 13C-DON from PBS blanks using the area under curve (AUC for two samples - 13, 410 and 17, 640) was similar to that for the two QC urines (AUC for two samples 13,330 and 14,910). The remaining eight extracted blanks contained PBS only. A trace peak corresponding to the elution time of DON was seen for all eight samples. However, the peak for each PBS blank was extremely small, and for every one of these blanks the AUC was below the cut off point for quantification, translating to a concentration below 0.1ng DON per ml PBS (the detection limit is 0.1ng/ml). Indeed if internal standard had been included in these samples the contribution of DON from the internal standard would have masked these trace peaks. If a mid-point between the detection limit and zero was used i.e. 0.05ng/ml and subtracted from the measured values reported, one of 300 samples would be classified as below the detection limit, and all but five others would have levels at least 10-fold higher than the detection limit (i.e. >1ng DON/ml urine).

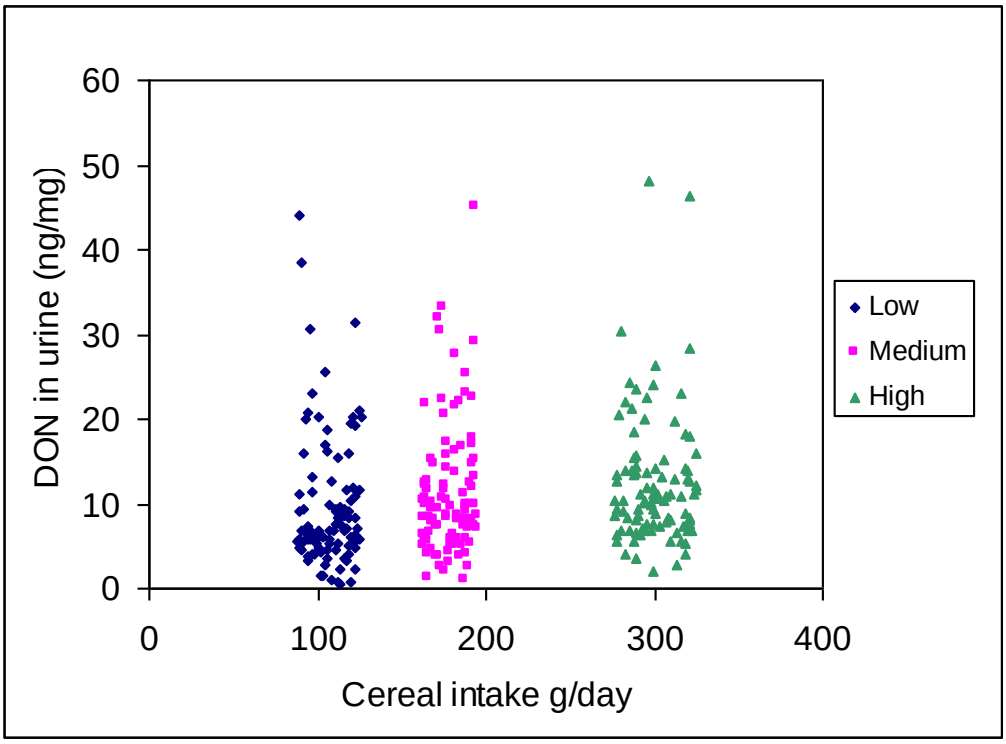



**Figure 2: Cereal intake versus DON (ng/mg creatinine) in urine for 300 subjects**
It is notable that whilst there was a significant positive trend in urinary DON levels from low to high cereal intake groups, within each intake group there is a large variation in DON exposure. This is an important part of the rationale in developing a biomarker of exposure, rather than relying on cereal intake alone as a surrogate marker.

**PHASE 1: Objective 5 – *Conduct statistical analysis of DON biomarker data with respect to the dietary survey***

Prior to statistical analysis urinary DON data (ng DON per mg creatinine) were natural log transformed. Data are back transformed and presented as geometric means with 95%CI (Table 1). The low cereal intake group had the lowest mean urinary level of DON 7.2ng/mg (95%CI 6.2-8.1), the medium intake had a higher level, 9.2ng/mg (8.1-10.5ng/mg), whilst the high cereal intake group had the highest level at 10.9ng/mg (95%CI 9.5-12.4ng/mg). Using regression analysis with

adjustment for sex, a significant difference in urinary DON remained between the low and medium intake group ($p<0.01$) between the medium and high intake group ($p=0.05$) and between the low and the high intake group ($p<0.001$).

In a second stage of statistical analysis the individual cereal items (pasta, pizza, white, wholemeal, soft grain and other bread, wholegrain and high fibre breakfast cereals, other breakfast cereals, biscuits, fruit pies, buns, cakes and pastries and other cereals) used to categorize subjects by cereal intake from the NDNS food diaries were individually compared with the log transformed levels of urinary DON. The purpose of this analysis was to examine the contribution of individual items to the variance in urinary DON levels.

Table 2 lists the mean weight eaten over the 7-day period of the food diaries from the NDNS for each of these categories of cereal among the 300 selected subjects. The highest intakes were of white bread, pasta, wholegrain and high fibre breakfast cereals.

**Table 2: Descriptive statistics of cereal intakes among 300 subjects**

| | Mean (g/week) | Std. Deviation |
|---|---|---|
| weight eaten of pasta | 182 | 255 |
| weight eaten of pizza | 69 | 157 |
| weight eaten of other | 39 | 85 |
| weight eaten of white bread | 428 | 330 |
| weight eaten of wholemeal bread | 122 | 213 |
| weight eaten of softgrain bread | 8 | 87 |
| weight eaten of other bread | 127 | 209 |
| weight eaten of whg & hf bf cereal | 144 | 231 |
| weight eaten of other bf cereals | 46 | 108 |
| weight eaten of biscuits | 76 | 113 |
| weight eaten of fruit pies | 21 | 68 |
| weight eaten of buns cakes pastries | 129 | 160 |
| weight eaten of sponge puds | 10 | 44 |

The Pearson correlation coefficients between the individual items and the urinary DON are given in Table 3. The strongest associations in this univariable analysis were between urinary DON and wholemeal bread and other bread, as well as buns, cakes and pastries.

**Table 3: Correlation between urinary DON and individual cereal groups**

| Cereal group | Pearson correlation coefficient (ln urinary DON) | P value |
|---|---|---|
| weight eaten of pasta | .055 | .172 |
| weight eaten of pizza | .022 | .349 |
| weight eaten of other | -.046 | .216 |
| weight eaten of white bread | .024 | .337 |
| weight eaten of wholemeal bread | .170 | .002 |
| weight eaten of softgrain bread | .005 | .469 |
| weight eaten of other bread | .150 | .005 |
| weight eaten of whg & hf bf cereal | .090 | .061 |
| weight eaten of other bf cereals | -.084 | .073 |
| weight eaten of biscuits | .036 | .267 |
| weight eaten of fruit pies | -.085 | .072 |
| weight eaten of buns cakes pastries | .126 | .014 |
| weight eaten of sponge puds | .059 | .154 |
| weight eaten of other | .006 | .456 |

Other factors examined by regression analysis included sex, age, timing of sample collection (the four "waves" of the NDNS survey), vegetarian (yes/no) and body mass index (BMI), see Table 4 for descriptive characteristics. Females had higher levels of DON (geometric mean 9.6, 95%CI: 8.7-10.6) than males (geometric mean 8.3, 95%CI: 7.3-9.4) though this failed to reach significance p=0.072. Vegetarians (n=18) had significantly (p=0.031) higher levels of urinary DON (geometric mean 12.7, 95%CI: 8.9-18.0) compared to non-vegetarians (geometric mean 8.8 95%CI: 8.1-9.5). Other factors were not associated with significant differences in urinary DON.

When the cereal variables were entered into a multivariable linear regression model model (Table 5) as predictors of urinary DON as the dependent variable they explained 11% of the variance (Table 5, $R^2$=0.108). When individual items were examined for their contribution to the model (Table 6) the most significant contributions were made by 1) wholemeal bread, 2) other bread, 3) white bread and 4) buns, cakes and pastries, in that order; all of these were statistically significant. These data indicate that consumption of bread in general, albeit with a stronger effect of wholemeal bread, made the most significant contribution to urinary DON levels in this study.

**Table 4. Descriptive data of selected individuals by cereal intake group**

| | Cereal Group | | | |
|---|---|---|---|---|
| | **Low** | **Medium** | **High** | **p value for trend** |
| **Cereal Intake *** | **107 (88 – 125)** | **179 (162 - 195)** | **300 (276 - 325)** | **<0.001** |
| **Sex – female** | **62** | **50** | **46** | **0.02** |
| **- male** | **38** | **50** | **54** | |
| **Age **** | **44.1 (19 – 64)** | **40.9 (19 - 64)** | **43.7 (19 - 64)** | **0.81** |
| **BMI #** | **27.4 (26.3 – 28.5)** | **26.8 (25.6 – 27.8)** | **27.1 (26.0 – 28.2)** | **0.73** |
| **Ethnicity ***** | **96 / 100** | **97 / 100** | **99 / 100** | **0.19** |
| **Vegetarians** | **4 / 100** | **6 / 100** | **8 / 100** | **0.23** |

*Cereal – cereal intake in g / day (Mean and range)
** Age (Mean and range)
*** Number of individuals that are white Caucasians
# Mean and 95%CI

**Table 5: Multivariable Model Summary Cereals [(b)]**

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1 | .329[(a)] | .108 | .064 | .67544 |

a Predictors: (Constant), weight eaten of other (e.g. corn flour or flour in other food items), weight eaten of other bread, weight eaten of pasta, weight eaten of biscuits, weight eaten of softgrain bread, weight eaten of other breakfast cereals, weight eaten of fruit pies, weight eaten of wholemeal bread, weight eaten of pizza, weight eaten of wholegrain & high fibre breakfast cereal, weight eaten of sponge puddings, weight eaten of buns cakes pastries, weight eaten of white bread
b Dependent Variable: ln DON

The R-squared value gives a measure of how well the variance in the urinary biomarker was explained by the model.

When sex, vegetarian status and BMI were additionally added to the multivariable model (Table 7), only sex contributed significantly to the model whilst the contribution of cereals was relatively unchanged by the inclusion of these additional variables.

**Table 6: Contribution of individual cereal components to urinary DON[a]**

| | Unstandardized Coefficients | | P-value |
|---|---|---|---|
| | **B** | **Std. Error** | |
| (Constant) | 1.806 | .111 | .000 |
| weight eaten of pasta | 1.463E-05 | .000 | .352 |
| weight eaten of pizza | .000 | .000 | .962 |
| weight eaten of other | 6.127E-06 | .000 | .896 |
| **weight eaten of white bread** | **3.274E-05** | **.000** | **.014** |
| **weight eaten of wholemeal bread** | **6.719E-05** | **.000** | **.001** |
| weight eaten of softgrain bread | 1.447E-05 | .000 | .752 |
| **weight eaten of other bread** | **6.213E-05** | **.000** | **.002** |
| weight eaten of whg & hf bf cereal | 1.786E-05 | .000 | .313 |
| weight eaten of other bf cereals | .000 | .000 | .170 |
| weight eaten of biscuits | 1.481E-05 | .000 | .680 |
| **weight eaten of fruit pies** | **.000** | **.000** | **.055** |
| **weight eaten of buns cakes pastries** | **5.439E-05** | **.000** | **.038** |
| weight eaten of sponge puds | 3.173E-05 | .000 | .734 |

a Dependent Variable: ln DON

**Table 7: Contribution of individual cereal and other components to urinary DON[a]**

| | Unstandardized Coefficients | | Sig. |
|---|---|---|---|
| | B | Std. Error | |
| (Constant) | 1.795 | .427 | .000 |
| **Sex** | **.235** | **.084** | **.005** |
| **weight eaten of white bread** | **4.240E-05** | **.000** | **.002** |
| **weight eaten of wholemeal bread** | **7.934E-05** | **.000** | **.000** |
| **weight eaten of other bread** | **7.128E-05** | **.000** | **.000** |
| **weight eaten of fruit pies** | **.000** | **.000** | **.068** |
| **weight eaten of buns cakes pastries** | **5.098E-05** | **.000** | **.051** |

a Dependent Variable: ln DON

14% of the variance was attributable to cereal intakes and to these additional variables (Table 8). It should be noted that no DON measurements were made in the foods consumed in this NDNS analysis and that the heterogeneity of contamination will in large parts explain the large variations in urinary DON by cereal consumption and the relatively weak explanatory power of individual food items when seeking to explain overall variance in urinary DON levels.

**Table 8: Multivariable Model Summary Cereal Age, BMI, Sex [(b)]**

| Model | R | R Square | *Adjusted R Square* | Std. Error of the Estimate |
|---|---|---|---|---|
| 1 | .380[(a)] | .144 | *.093* | .66508 |

a Predictors: (Constant), weight eaten of other, weight eaten of other bread, weight eaten of pasta, weight eaten of biscuits, weight eaten of other, weight eaten of softgrain bread, BMI calculated from dvweight/dvmetres squared, weight eaten of other bf cereals, sex, weight eaten of fruit pies, weight eaten of pizza, weight eaten of wholemeal bread, Can I check, are you vegetarian or vegan, weight eaten of sponge puds, weight eaten of whg & hf bf cereal, weight eaten of buns cakes pastries, weight eaten of white bread
b Dependent Variable: ln DON

The R-squared value gives a measure of how well the variance in the urinary biomarker was explained by the model.

Additional analysis involved assessment of urinary DON level by categorization of levels of food intake. All data was modelled by multivariable regression analysis including only those variables providing a significant contribution to the model, sex, BMI and age were however maintained in all models. For white bread quintiles of intake (n=60 in each) there was an increase of 31.0% in urinary DON (p=0.040) from those with low intake to those with high intake. See Figure 3a. For wholemeal bread 180 individuals had no intake and for the remainder tertiles of intake were used. There was an increase of 63.2% in urinary DON (p=0.000) from those with no intake to those with high intake (Figure 3b). Though no significant differences were apparent between different tertiles of intake. For 'other' bread 147 individuals had no intake and for the remainder tertiles of intake were used. There was an increase of 46.2% in urinary DON (p=0.000) from those with no intake to those with high intake (Figure 3c). When all bread consumption was combined and investigated as quintiles of intake there is a mean increase of 56.8% in urinary DON (p=0.000) from those with low intake to those with high intake (Figure 3d).

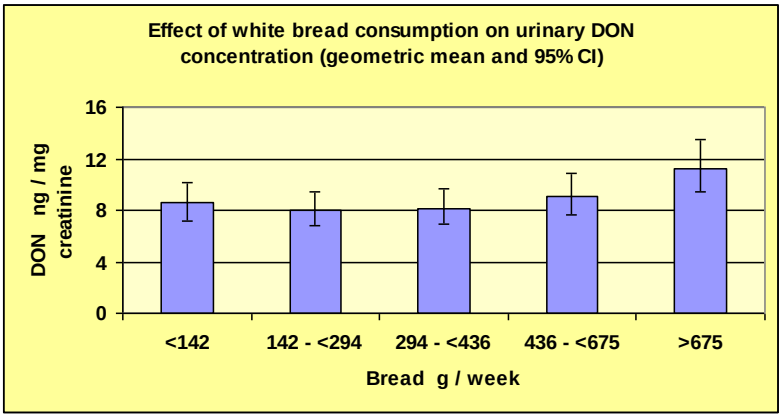



Figure 3a: Effect of white bread consumption on urinary DON

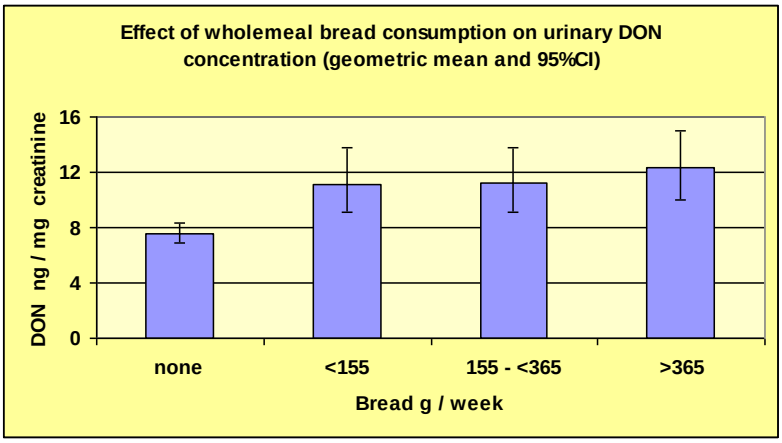



Figure 3b: Effect of wholemeal bread consumption on urinary DON

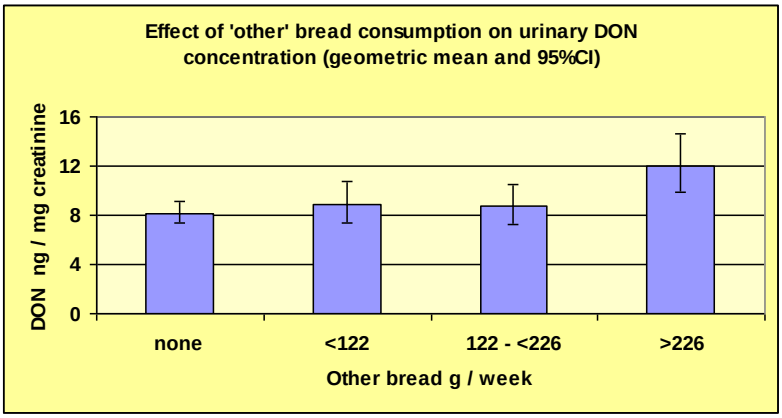



Figure 3c: Effect of other bread consumption on urinary DON

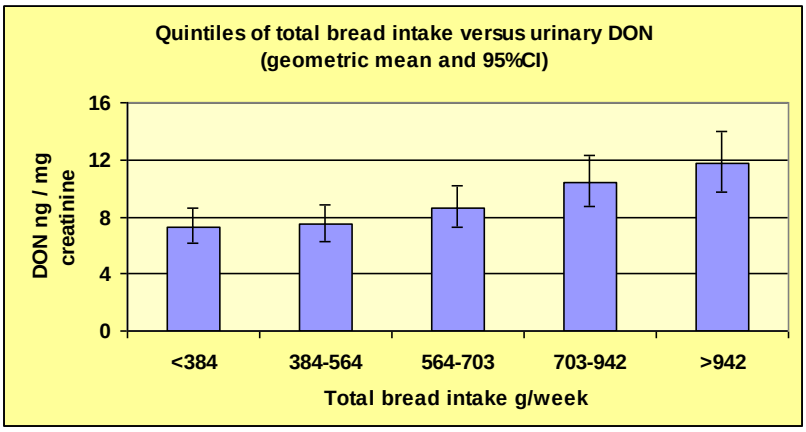



Figure 3d: Effect of total bread intake on urinary DON

Fruit pie consumption was reported by only 34 individuals and these 34 had 23.7% lower urinary DON levels (p=0.024) than those not consuming fruit pies (n=266) (Figure 4). When divided into non consumers of cakes etc. (n=109) and quartiles of consumers, there was a 24.6% increase in urinary DON albeit non significant (p=0.069) from those with no intake to those with high intake (Figure 5).

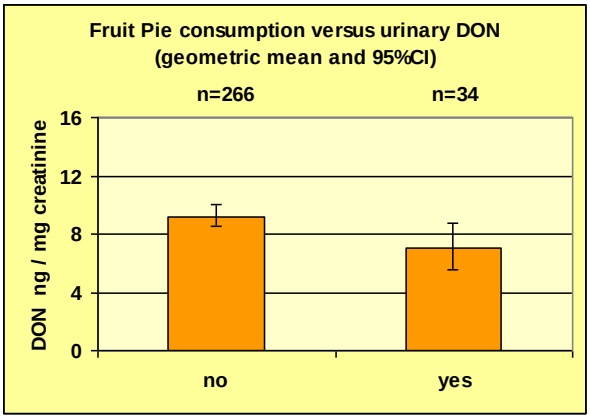



Figure 4: Fruit pie consumption and urinary DON

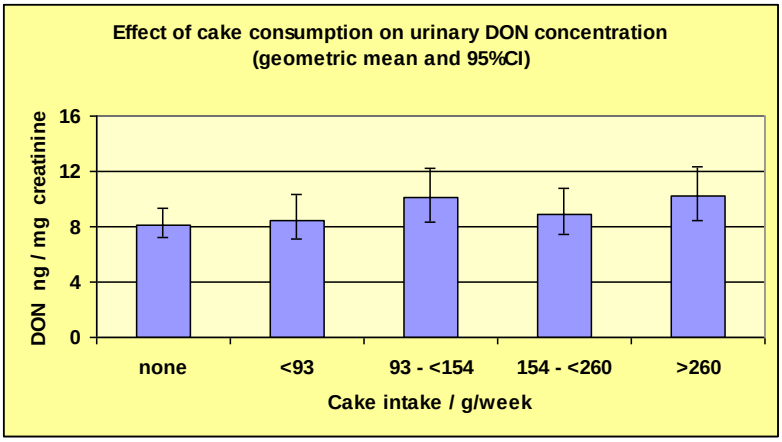



Figure 5: Cake consumption and urinary DON

When whole meal bread, other bread and cakes/biscuits etc intake was combined and categorized as non consumer (n=37) and quartiles of consumers there was an increase of 116.0% in urinary DON (p=0.0001) from those with no intake to those with high intake (Figure 6), whilst a 71% increase (p=0.01) was observed between those with low (<153g/day) compared to the highest consumers (>595g/day) .

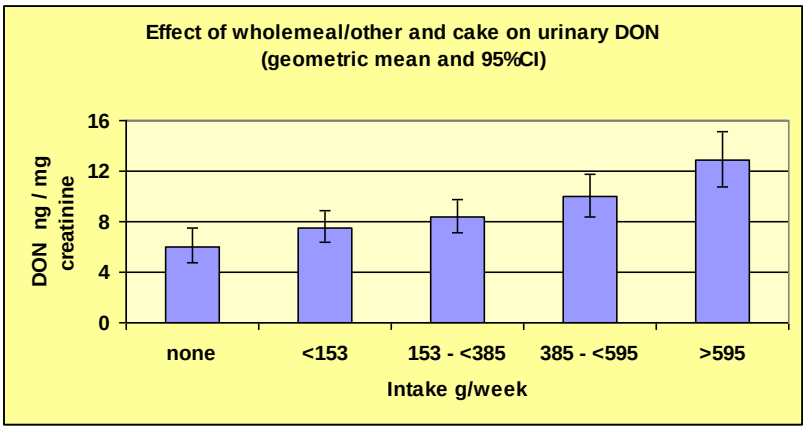



Figure 6: Wholemeal bread/other bread/'cake' consumption and urinary DON

**These data suggest that combined intake of whole meal, other bread and cakes make the major contribution to DON intake.**

**PHASE 1: Objective 06 - *Demonstrate fitness for purpose of urinary analysis of FB, and as necessary develop methodology using immunoaffinity and LC-MS***.

**Output**

Prior to the outset of the study the methodology for FB in urine had been explored in the Molecular Epidemiology Unit, based on the method of Shetty and Bhat (1998). This approach involved strong anion exchange enrichment (Varian Bond Elute SAX) of FB from urine, followed by derivatisation with o-phthaldialdehyde and quantification by HPLC with fluorescence detection. The detection limit was 1ng/ml, and based on the estimated UK intake for FB (SCOOP 2003) and the predicted transfer to urine of 1-2% of intake (reviewed by Turner et al., 1999) it was felt that this assay may not be sufficiently robust or sensitive to provide an accurate assessment of UK exposure to FB. There was no LC-MS methodology for measuring FB in urine. Thus this objective represented a method development step to improve sensitivity. Based on the limit of analytical detection reported by Newkirk et al., (1998) for FB, in maize, of 250pg, we estimated a possible level of sensitivity of 0.05ng/ml of urine. Initial work focused on the LC-MS method, and once this was established extraction processes were optimised.

**Detection**

For LC-MS analysis, separation was achieved using a 50 x 4.6mm, 5µm Luna C18 column on a Waters 2795 HPLC instrument. 25µl of the reconstituted sample, following extraction, is injected on to the column at a flow rate of 1ml/min. The mobile phase gradient is composed of (A) water:acetonitrile:formic acid 90:10:0.1, (B) water:acetonitrile:formic acid 10:90:0.1. Post column 1/5 of the HPLC flow is diverted into a triple quadrupole mass spectrometer (Micromass UK Ltd., Manchester, UK), which is tuned with cone voltage at 70V; capillary voltage at 3.3 kV; capillary temp at $140^{O}$C. Selective ion monitoring mode is set with 2 channel ion scan at (M+H)+ M/Z 722.4 (FB1). Authentic FB1 eluted at 7.5 minutes, and to quantify FB1 a standard curve was used. The LC-MS method has a limit of detection of 125pg FB1 pure standard, translating to a detection limit in 5ml urine of 0.20ng/ml.

**Extraction**

A range of extraction processes were investigated using 5ml urine samples spiked with FB1. These comprised four Waters Oasis Cartridges (MAX, MCX, WCX and WAX), the Varian Bond-Elute SAX cartridge (described above) and the Vicam FumoniTest immunoaffinity column (IAC). In a set of triplicate extractions for each column type the Waters Oasis MAX yielded the highest FB1 peak area from a spiked urine (FB1 5ng/ml), followed by the SAX cartridge. The others extracted FB1 in the order Oasis WAX > Vicam IAC > Oasis WCX > Oasis MCX. Therefore the Waters Oasis MAX column was subsequently used to perform a test extraction of urine spiked at 1ng/ml and 5ng/ml using the following procedure.

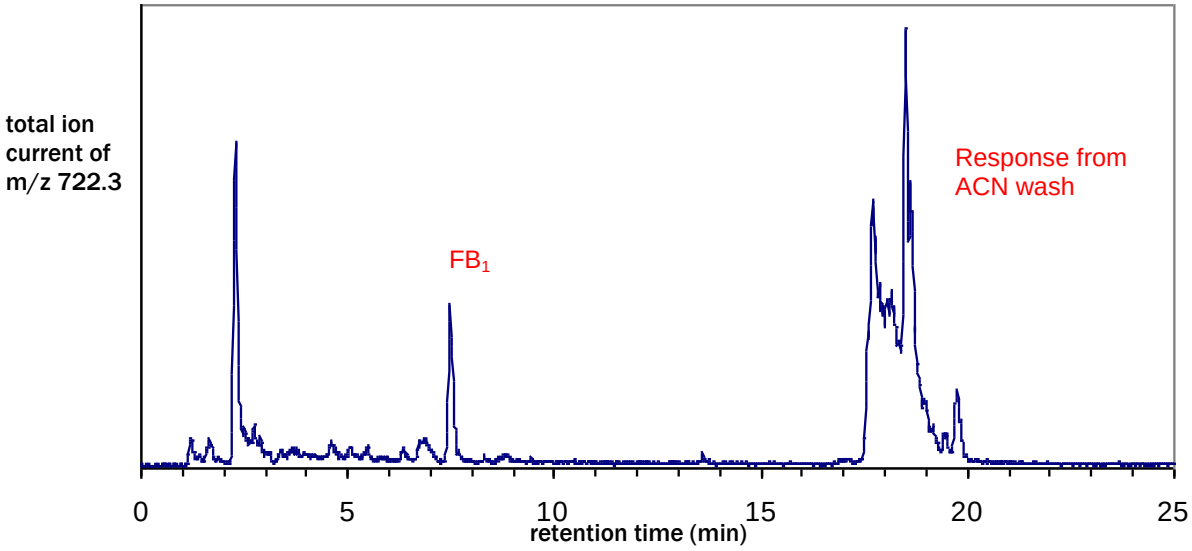
**Fumonisin extraction using Waters Oasis MAX cartridges.**

Spiked urine samples (5mls) were adjusted to pH 6.0 and diluted 1:1 in water and then passed through a MAX cartridge that had been pre-activated by passage of 2ml of MeOH:water (1:1). Following the passage of the diluted urine the cartridge was washed with 2ml of 5% ammonium hydroxide in water, followed by 2ml of MeOH. Finally fumonisin was eluted with 2ml of 2% formic acid in MeOH. All solvent passages were conducted using gravity flow. Each sample was extracted in duplicate and a control urine sample (without spike) was also included. Extracted samples were dried *in vacuo* and reconstituted in 200ul MeOH:water (1:1) and quantified by LC-MS as previously described.

An example of the detection of FB1 in spiked human urine is given in Figure 7. The first peak is the solvent front and was seen for all injections, FB1 eluted at 7.5 minutes. As shown in Table 7, recoveries were high and reproducible for these spiked samples. No difference in FB1 recovery was found when FB1 was extracted in triplicate from spiked urine (5ng/ml) containing no boric acid, 1g/L boric acid, or 5g/L boric acid.

Based on these data and predicted FB intake and non-human primate urinary FB excretion of around 1.2% of the dose (Shephard et al., 1995), we estimate that urinary levels in the UK are likely to be in the order of 0.06-0.84ng/ml, and therefore sensitivity of the assay would be sufficient to detect exposure in at least the predicted high exposure group. If the range of urinary FB levels for a given intake group were to be as varied as we have determined for DON (**Objective 04** of this report), it is possible that there would be sufficient analytical sensitivity to detect $FB_1$ in some of the urines from all selected groups.

**Figure 7: Selective ion recording trace of a urine sample spiked with $FB_1$ (5 ng/ml in urine)**




Internal standardisation was not used in the above trial since the internal standard (deuterated $FB_1$) contributes 6-7% to 'normal' $FB_1$ and thus may interfere with detection at the probable low levels of fumonisin.

**Table 7: Recoveries of FB from non-spiked and spiked urine using Waters Oasis MAX cartridges**

| Spike concentration (ng/ml) | FB detected (ng/ml) | Recovery (%) |
|---|---|---|
| 0 | nd* | - |
| 1.00 | 0.93 | 93 |
| 1.00 | 0.98 | 98 |
| 5.00 | 5.20 | 104 |
| 5.00 | 5.46 | 109 |

*A small peak was identified corresponding to $FB_1$ in the non-spiked urine, though this was below the limit of detection

**PHASE 1: Objective 07 - *Analysis of urine samples for FB in high cereal consumers***

**Output**

This objective was altered such that 50 urine samples from high maize consumers only were analyzed, instead of the original plan of 25 from the medium and 25 from the high cereal intake group; selection as described in Appendix A. Urines were extracted using Oasis Max cartridges and quantified using LC-MS, as described above. FB was not detected in urine samples from this predicted high risk group. After running the samples a first time, the LC-MS was cleaned and this led to a marked improvement in the limit of sensitivity to 0.02ng/ml of urine. Re-analyses of the samples did not reveal any positives. The lack of detection at this level of sensitivity may reflect lower exposures than predicted by SCOOP (2003), or that the proportion of fumonisin excreted in human urine was lower than predicted from work in non-human primates (reviewed by Turner et al., 1999).

**Summary Phase One**

**Deoxynivalenol**

We have provided a protocol describing the sampling of the 300 urines in which to analyse DON. One hundred individuals from each of the $2^{nd}/3^{rd}$ deciles (low intake), $5^{th}/6^{th}$ deciles (medium intake) and $9^{th}$ decile (high intake) were selected (overall: 142 females and 158 males). Following the treatment of urine with β-glucuronidase, DON was isolated using IAC columns and the levels of DON quantified by LC-MS.

The originally proposed LC-MS methodology was substantially improved during the study due to the incorporation of 13C-DON as an internal standard. The mean level of QC samples run prior to the main analysis was 14.9ng DON/ml (95%CI 14.5-15.4ng/ml; CV 5.5%). Two QCs were analyzed alongside each batch of 20 test samples (15 batches in total). The mean level of the QCs was 14.2ng/ml (95%CI 13.8-14.6; CV 8.0%). There were no differences in the pre-test QC samples and study QC samples ($p>0.05$).

All 300 urine samples had detectable urinary DON (mean 11.1ng/mg creatinine; range 0.6 – 48.2ng/mg). Twenty samples from among the 300 were repeated and a CV of the original and repeat data for each re-analysed sample obtained. The data from the repeated analysis were in good agreement with the original set of data (mean CV 10.1% (95%CI 6.8-13.4%).

Cereal intake was significantly associated with the level of urinary DON ($p<0.001$) with those in the high intake group having a geometric mean 50.1% higher than those in the low intake group. In multivariable (MV) analysis looking at the amounts of specific foods associated with urinary DON level, wholemeal bread ($p<0.001$), 'other' bread ($p<0.001$), white bread ($p=0.002$), buns and cakes ($p=0.051$) were most predictive. In categorical analysis wholemeal bread consumption was again most strongly associated with the level of urinary DON, probably reflecting the distribution of DON contamination in the wheat grain. Consumption of fruit pies was negatively associated with the level of urinary DON, though numbers within this group were small. In the MV model, sex was also significantly associated with urinary DON levels with females having a geometric mean level 16.1% higher than males ($p=0.005$). We are unaware of any supporting data that this represents any physiological difference in DON pharmacokinetics by sex.

A crude estimate of DON intake was made based on the concentration of DON in urine. This estimate was based on studies derived from a rat model (Meky et al., 2003) and assumes (a) excretion of 1.5 litres of urine per day, (b) 30% of the ingested DON being excreted in the urine, (c) the urinary DON all originating from DON intake in the previous 24 hours.

DON intake ng/kg bw = (urinary DON (ng/ml) x 100/30 x 1500mls) / body weight (kg)

Accordingly, for each individual the intake was calculated based on the assumption above and the individual urinary DON concentration and body weight. For the 300 individuals the mean intake was calculated as 494ng/kg bw/day; around 2-3 fold higher than that estimated by the 2003 SCOOP report for UK adults (176ng/kg bw/day and 142ng/kg bw/day, for males and females respectively) using mean levels in food and estimates of intake. The maximum level predicted was 3,624ng/kg bw/day and an estimated 10% of individuals were predicted to exceed the recommended TDI of 1000ng/kg bw/day (SCF, 2002). Please note that this estimate of intake was subsequently refined (see page 42) based on the more detailed understanding of the relationship between intake and urinary excretion developed in **Phase Two.**

**Fumonisins**

Initial studies to measure urinary FB involved refining the extraction approach prior to LC-MS quantification. A range of extraction processes, including a variety of ion exchange technologies and an immunoaffinity cartridge were tested. Recoveries were most reliable for the Waters Oasis MAX (93-109% recovery of spike samples (1-5ng/ml). 50 urine samples were then selected from the predicted highest intake of FB based on maize consumption. No samples were above the detection limit of 0.02ng/ml. Given a 0.5% transfer and 1.5 litre of urine per day the detection limit would be predicted at 6µg/day. Assuming the average weight of 70Kg and a transfer of around 2% to urine, an intake of 86ng/kg bw/day was predicted to be detectable. Given the analytical limits and the observation of no detectable fumonisins in the top 50 maize consumers, urinary FB may not be a useful exposure biomarker of intake within the UK population.

## PHASE TWO
## The further development and understanding of urinary biomarkers for exposure to deoxynivalenol

### BACKGROUND

Mycotoxins are highly potent secondary fungal metabolites that frequently contaminate cereal crops throughout the world. A key requirement in the undertaking of epidemiological investigations to understand the potential health risk posed by mycotoxins is exposure assessment. Current estimates of human exposure to mycotoxins produced by *Fusaria spp.* are of limited precision as they are based on measurements of mycotoxins in foodstuffs combined with food consumption data. In Phase 1 of the project, highly robust assays to assess urinary deoxynivalenol (DON) and urinary fumonisin (FB) as biomarkers of exposure were developed. The assay for urinary DON incorporates immunoaffinity enrichment and LC-MS detection. The recent commercial availability of a [$^{13}$C] isotope-labelled DON as an internal standard, allows adjustment for variation in both extraction and LC-MS performance (Turner et al., 2008a). The availability of urine samples from the 2000-2001 UK adult National Diet and Nutrition Survey (NDNS) allowed us to assess the utility of this biomarker against known consumption of cereal-based commodities based on an average weighed dietary record over seven days. This analytical approach was applied to a stratified sample of 300 individuals with three different levels of average cereal intake (low intake mean 107g/day, range 88-125g/day; medium intake mean 179g/day, range 162-195; and high intake mean 300g/day, range 276-325g/day). DON was detected in 296/300 urine samples (mean 9.0ng/mg creatinine; range 0.6–48.2ng/mg) and the level was significantly ($p<0.001$) associated with average cereal intake (Turner et al., 2008b). A more detailed analysis of individual food items consumed revealed a strong association between bread consumption ($p<0.001$) and levels of urinary DON. However, none of the food items alone or in combination could fully explain the variation in the level of urinary DON. In addition, the combined cereal intake on the day of urine collection and the previous 24 hours was strongly associated with the urinary measure, and these data somewhat better explained the variation in urinary DON compared to average cereal intake (Turner et al., 2009). However, the known heterogeneity of DON in food is likely to restrict the complete characterisation of exposure based on questionnaire information. To date urine sampling protocols have involved collection at only one time point, thus the intra-individual variation in this urinary biomarker remains unknown. In addition the pharmacokinetics of DON excretion in humans has not been determined and thus the contribution from DON intake over several days to a given urinary measure is also unknown. The current study addressed two of the outstanding questions related to fully validating the urinary measure as a reliable exposure marker in order to conduct informative epidemiological studies.

### Key Questions

**1. What is the relationship at the individual level between DON intake and the urinary biomarker?**

**2. What are the pharmacokinetics of DON and DON-glucuronide excretion, and, what are the consequent temporal variations in this biomarker?**

### RESEARCH PLAN

This study was designed to assess urinary DON levels in 40 individuals over a two week time period (see Appendix B). Food diaries were kept throughout the study, and five consecutive first morning urines collected in both of week one and week two. Urinary measures of DON would be compared to cereal intake over the study period.

In **week one** the temporal variation in urinary DON would be assessed for all individuals during their normal dietary practice. In **week two**, the study group was divided such that group one took part in a partial intervention and group two took part in a full intervention. The rationale for the choice of food group restrictions during both interventions was based on observations reported in Phase 1. The **partial intervention** restricted all major sources of dietary DON except bread for four consecutive days. A duplicate portion of each bread sample consumed was provided during this four day period such that the total intake of DON from this single major source could be established. This component of the study examined for the first time the relationship between intake of DON and the urinary measure. The availability of four consecutive measures of DON intake and urinary DON further allows assessment of the temporal nature of these measurements. The **full intervention** restricted all major sources of dietary DON for four consecutive days. This component of the study examined the kinetics of clearance of DON. The urinary assay used in this study measured total urinary DON (free DON plus DON-glucuronide) following hydrolysis of the DON-glucuronide using β-glucuronidase. One hundred of these urine samples were additionally analysed without the hydrolysis step. This component of the study examined the ratio of free DON to total DON; a potential phenotypic measure of susceptibility to DON toxicity.

**PHASE 2: Objective 01 -** ***Obtain ethical approval to conduct the proposed study.***

**Output:** Ethical approval was obtained through the Leeds Teaching Hospitals NHS Trust Research Ethics Committee. Subsequently recruitment of participants began in September 2007.

**PHASE 2: Objective 02 -** ***Prepare (i) recruitment poster, (ii) information leaflet, (iii) informed consent form, (iv) volunteer questionnaire, (v) 12-day food diary***

**Output:** The above documentation was prepared in order to recruit and conduct the 12 day study.

**PHASE 2: Objective 03 -** ***Recruit 40 volunteers to take part in the study.***

**Output:** The heavy demands of the study, 12-day food diaries and 10 urine samples, created difficulty in recruitment of the specified number of volunteers. Thirty-five volunteers were recruited, and the study took place between the 19$^{th}$ and 30$^{th}$ November 2007. The quantitative comparison of intake versus biomarker level (partial intervention) was the priority over the kinetics of removal of DON (full intervention), and the kinetics required fewer observations. For these reasons it was decided to include 25 subjects in the former, whilst 10 subjects were included in the latter group.

**PHASE 2: Objective 04 –** ***Conduct two week study and collect diary, urine and bread samples.***

**Output:** All participants were asked to keep detailed **food diaries** throughout the period of the study (see Annex 1). A staff member (Dr V Burley) trained in nutritional epidemiology and experienced in food diary analysis went through each person's diary during the study period and again at the end of the study. All 35 completed diaries were received.

Of the 35 subjects who took part 33 gave ten **urine samples** [five in week one and five in week two] as requested, and 2 subjects gave 9 samples (both missing the last day only of week two). In addition seven subjects gave two x 24 hr urine samples

during the second week of the study, one subject gave 24hr urine samples for the first five days of the study and one subject gave 24hr urine samples throughout the 12 days of the study. These samples were not included in the FSA protocol but permit additional valuable comparisons of morning urines versus 24hr collection.

In the partial intervention participants provided a duplicate **food sample** of the bread consumed each day. Over the four days of intervention 118 bread samples were returned.

**PHASE 2: Objective 05 -** ***Obtain data from RHM Technology, Premier Foods, on the levels of DON contamination in bread samples***

**Output:** A total of 118 bread samples were obtained, though a number of samples were clearly identified from food diaries as being for the same loaf, and thus only 84 distinct samples were apparent. For two samples the package returned was poorly labelled and only 82 distinct samples were submitted to RHM Technology – Premier Foods, UK for analysis. The laboratories are accredited to UKAS 17025 standards. Accreditation by UKAS demonstrates competence, impartiality and performance capability of the laboratory. The internationally recognised standard for the competence of laboratories is ISO17025 and is the standard against which UKAS accredits laboratories. The method of analysis involved solvent extraction and GC/MS analysis. The detection limit was 5µg/kg. The mean recovery was 96% with a range of 85-103%, and was within the 70-110% acceptable limits established by Premier Foods (see Appendix C).

DON was detected in all 82/82 (100%) of samples; mean 74µg/kg; range 20 – 316µg/kg. These data were used to determine DON intake following coding of diaries (see objective 08)

**PHASE 2: Objective 06 -** ***Extract DON and DON-glucuronide combined (total DON) from all urine samples and measure DON level using LC-MS.***

**Analytical design and validation.**

i. **Standard Curve.** In Phase 1 of the study the standards used for the LC-MS were in the range 10-200ng/ml. This range has been extended to include 5ng/ml and 2ng/ml, providing an improvement in assay sensitivity.

ii. **Sample flow.** The immunoaffinity component of the analysis was labour intensive and time consuming. Vicam, who supplied the original narrow bore columns, placed on the market a modified 'wide-bore' DON IAC column with the same antibody but improved flow properties. Initial analysis revealed decreased preparation time without compromising the assay. The use of reduced urine volumes was additionally investigated. Twenty-two urine samples were tested to compare the original narrow bore columns with 4ml urine and the new wide bore columns with 1ml urine, see Figure 1a, regression analysis revealed an $R^2 > 0.99$. When the difference between each paired data sets was plotted against the mean (Bland-Altman plot), 21/22 (95%) of data points were within ± 2 standard deviations (Figure 1b); providing considerable confidence that the more rapid extraction with lower volume did not impact on the accuracy of the assay.

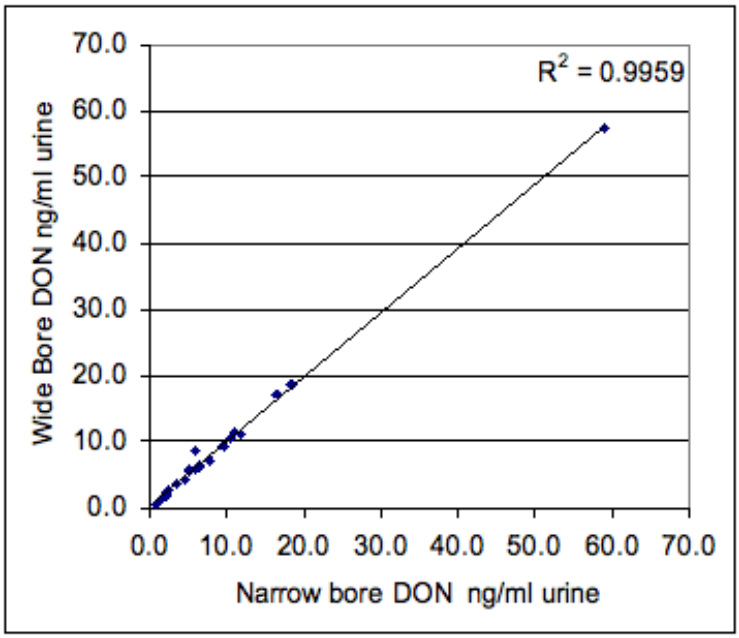



**Figure 1a. Wide bore versus narrow bore IAC comparison**

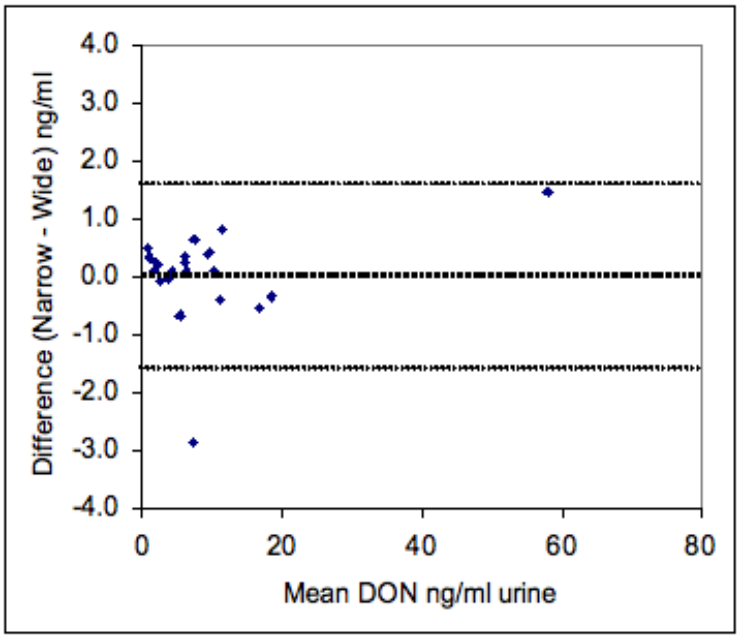



**Figure 1b. Bland-Altman plot comparing wide bore and narrow bore**

**Long Term storage.** In order to allow urine samples from biological banks to be analysed for DON it is important to determine the stability of DON during frozen storage. Twenty-two human urines analysed in November 2005 were re-analysed in September 2007 using the original narrow bore column IAC method. This comparison of samples analysed following 22 months of storage gave an $R^2$ value of 0.99, see Figure 2. for each pair of data the difference between the original and the repeat sample was plotted against the mean of each pair (see Figure 2b). The repeat data was on average 1.1ng/ml (10.7%) lower than the original data (mean 10.3ng/ml; range 0.7 – 61.3ng/ml), and 21 of 22 data points were within ± 2 standard deviations;

again indicative that urinary DON was stable over this period during cryo-preservation.

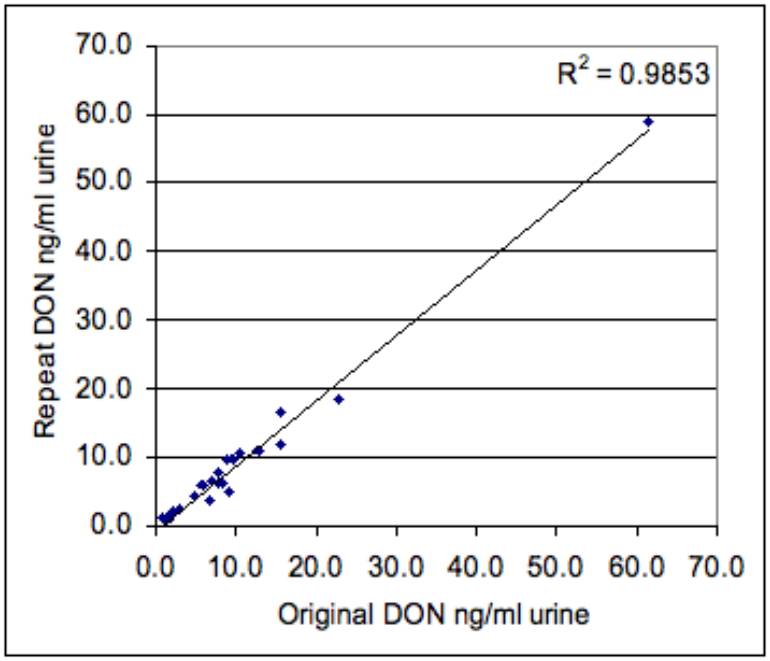



**Figure 2a. Long term storage of urinary DON**

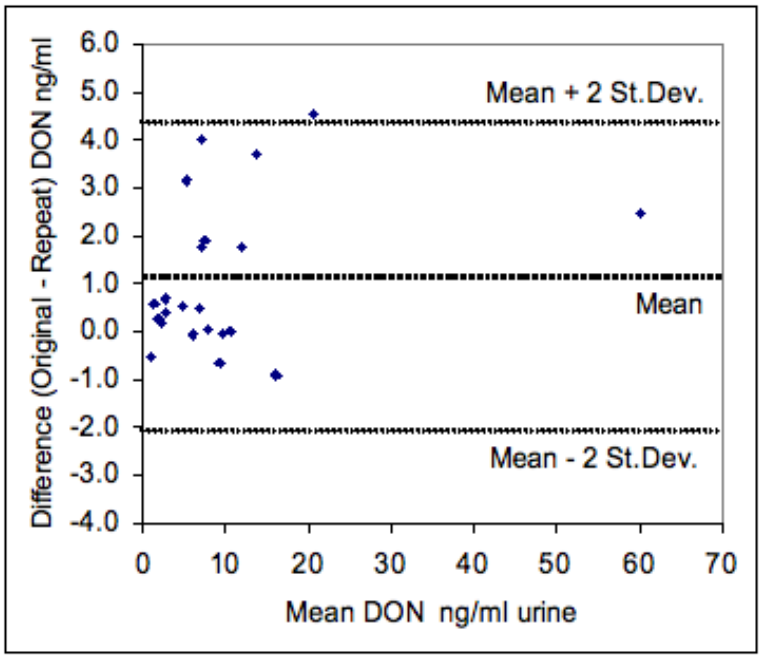



**Figure 2b. Bland-Altman plot comparing original with repeat**

iii. **Short term urine stability.** One concern of any biofluid collection process is the potential risk of analyte degradation from collection to initial processing. To address this concern for DON, duplicate portions of a single urine sample were either frozen within 5 minutes of collection, or stored at either 4°C or 20°C for up to 72 hours. After 4 hours the amount of DON measured in urine at 4°C and 20°C were within 1% of that measured on the sample immediately frozen. No significant degradation of the urine stored at 4°C was apparent with 72 hrs, and at 20°C DON was stable for 48 hours, with losses of ~10% by 72hrs, see Figure 3. In practice all urine samples were processed within 10-12 hrs of collection, these data provide considerable confidence that pre-storage loss of DON is negligible.

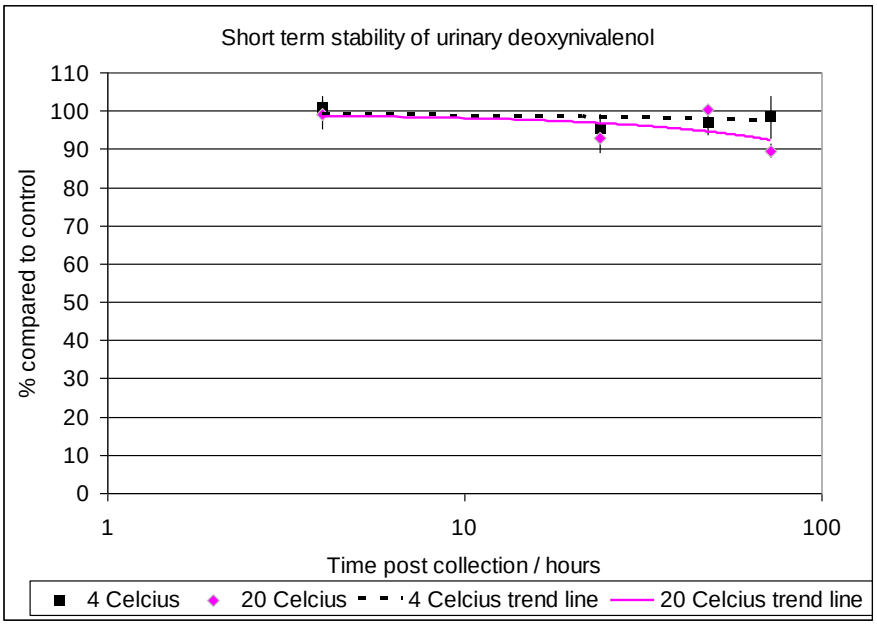



**Figure 3. Short Term stability of urinary DON**

**Outputs:** The detection limit for the assay was 0.5ng DON/ml of urine. Any non-detect was assigned a value of half the limit of detection i.e. 0.25ng/ml. All samples were analysed in batches of 20 with two quality controls (QC's) and two PBS blanks. The PBS samples gave rise to a signal corresponding to 0.8ng/ml; CV% of 42 samples was 5.8. The mean value of the two PBS samples within each run was subtracted from both the QCs and unknowns tested in that run. The QC's we produced in house from a urine sample with DON below the LOD, spiked to give 10ng/ml DON. For the QC the overall mean level of DON was 10.2ng/ml, CV% over the study [21 runs] was 8.3. Analysis of total urinary DON from 35 subjects in the study [348 samples] has been completed.

Of the 348 first morning samples tested DON was detected in 309 (approx 89%). All non-detectable (nd) samples (n=39) were assigned a value of half the detection limit. During the normal (no intervention) phase of the study 35 individuals provide six samples each (total n=210). DON was detected in 198/210 (94%) of these samples; overall normal phase study mean 11.6ng/ml (range nd – 78.2ng/ml, n=210). For the partial intervention, 23 individuals gave four samples and two individuals (code 14 and 15) gave three samples each (total n=98). DON was detected in 94/98 (96%) of these samples; overall mean 6.3ng/ml (range nd – 34.0ng/ml). For the full intervention, 10 individuals gave four samples each. DON was detected in 17/40 (43%) of the samples; overall mean 0.7ng/ml (range nd – 3.2ng/ml), see Table 1a.

**Table 1a: Summary data for urinary DON ng/ml urine**

| | Normal Diet | | | | Partial Intervention | | |
|---|---|---|---|---|---|---|---|
| **Code** | **Mean** | Low | High | | **Mean** | Low | High |
| 1 | **10.9** | 2.7 | 17.5 | 1 | **7.8** | 4.5 | 15.3 |
| 2 | **5.4** | nd | 17.9 | 2 | **15.8** | 4.8 | 34.0 |
| 3 | **14.9** | 1.5 | 53.7 | 3 | **1.8** | 0.6 | 3.0 |
| 4 | **3.8** | 2.0 | 7.6 | 4 | **1.9** | 1.1 | 3.1 |
| 5 | **8.0** | 4.0 | 17.8 | 5 | **5.0** | 0.8 | 8.7 |
| 6 | **2.9** | 0.8 | 6.0 | 6 | **2.7** | 0.7 | 8.2 |
| 9 | **7.0** | 2.0 | 15.2 | 9 | **4.1** | 2.0 | 6.0 |
| 10 | **4.0** | 1.2 | 7.6 | 10 | **3.0** | 0.8 | 5.2 |
| 12 | **5.8** | 3.3 | 7.2 | 12 | **3.6** | 1.5 | 7.4 |
| 13 | **27.3** | 5.0 | 40.5 | 13 | **1.3** | 0.7 | 1.6 |
| 14 | **1.8** | nd | 3.9 | 14 | **16.0** | 1.3 | 30.5 |
| 15 | **18.9** | 7.9 | 29.8 | 15 | **5.7** | 2.1 | 9.0 |
| 16 | **6.9** | 4.9 | 13.8 | 16 | **4.0** | 0.8 | 9.8 |
| 17 | **10.8** | nd | 49.5 | 17 | **0.6** | nd | 1.7 |
| 18 | **3.5** | 0.9 | 11.5 | 18 | **12.4** | 0.7 | 25.6 |
| 21 | **17.4** | 4.9 | 32.8 | 21 | **18.0** | 8.2 | 30.7 |
| 22 | **5.2** | 0.7 | 21.8 | 22 | **2.9** | 0.9 | 7.1 |
| 25 | **8.4** | 4.1 | 15.0 | 25 | **1.7** | nd | 3.5 |
| 26 | **4.4** | nd | 13.6 | 26 | **5.0** | 0.8 | 8.4 |
| 27 | **15.6** | 5.3 | 29.2 | 27 | **6.9** | 1.1 | 21 |
| 28 | **12.0** | 4.4 | 20.4 | 28 | **6.2** | 0.8 | 20.4 |
| 30 | **49.0** | 32.4 | 61.8 | 30 | **19.1** | 11.6 | 26.9 |
| 31 | **13.9** | 5.2 | 34.8 | 31 | **6.9** | 3.7 | 9.4 |
| 34 | **3.8** | 1.1 | 9.5 | 34 | **2.1** | 1.2 | 2.6 |
| 35 | **5.7** | 1.4 | 14.5 | 35 | **2.5** | 1.5 | 3.5 |
| | | | | | | | |
| **Total** | **10.7** | **nd** | **61.8** | | **6.3** | **nd** | **34.0** |
| | | | | | | | |
| | **Normal Diet** | | | | **Full Intervention** | | |
| | **Mean** | Low | High | | **Mean** | Low | High |
| 7 | **22.0** | 7.3 | 47.6 | 7 | **1.4** | nd | 3.2 |
| 8 | **27.3** | 4.9 | 40.4 | 8 | **1.2** | 0.7 | 1.6 |
| 11 | **2.4** | nd | 5.5 | 11 | **nd** | nd | 0.6 |
| 19 | **12.7** | 2.3 | 27.2 | 19 | **0.7** | nd | 1.3 |
| 20 | **12.5** | 2.3 | 29.2 | 20 | **0.5** | nd | 1.3 |
| 23 | **20.3** | 8.4 | 32.2 | 23 | **0.8** | nd | 1.6 |
| 24 | **13.2** | 1.3 | 22.0 | 24 | **0.7** | 0.5 | 1 |
| 29 | **10.0** | 2.7 | 21.5 | 29 | **nd** | nd | nd |
| 32 | **0.3** | nd | 0.8 | 32 | **nd** | nd | nd |
| 33 | **17.4** | 2.8 | 78.2 | 33 | **0.8** | nd | 2.3 |
| | | | | | | | |
| **Total** | **13.8** | **nd** | **78.2** | | **0.7** | **nd** | **3.2** |

The major disadvantages of spot or first morning void urine samples include the variability in the volume of urine and the concentrations of endogenous and exogenous chemicals from void to void. The most widely used method for adjusting for dilution is creatinine; adjustment that involves dividing the analyte concentration by the creatinine concentration. Many studies have documented that creatinine-

adjusted analyte concentrations serve as a good surrogate for size-related dose (reviewed by Barr et al., 2005). Urinary creatinine analysis was conducted for all samples using an in-house micro-titre plate assay modified from the alkaline-picrate method described by Varley (1967). Urinary creatinine (mg/ml urine) was used to adjust the DON concentration from ng/ml urine to ng/mg creatinine.

**Table 1b: Summary data for urinary DON ng/mg creatinine.**

| **Code** | **Normal Diet** | | | | **Partial Intervention** | | |
|---|---|---|---|---|---|---|---|
| | **Mean** | Low | High | | **Mean** | Low | High |
| 1 | **8.1** | 2.7 | 11.3 | 1 | **4.7** | 3.0 | 6.6 |
| 2 | **3.8** | nd | 8.8 | 2 | **12.4** | 5.9 | 18.1 |
| 3 | **13.9** | 2.2 | 47.9 | 3 | **2.8** | 0.7 | 5.2 |
| 4 | **9.3** | 2.9 | 22.1 | 4 | **4.2** | 1.2 | 5.7 |
| 5 | **9.0** | 2.7 | 17.0 | 5 | **6.8** | 1.2 | 11.6 |
| 6 | **3.0** | 0.8 | 6.2 | 6 | **3.6** | 1.0 | 10.5 |
| 9 | **11.7** | 4 | 23.5 | 9 | **8.6** | 3.1 | 16.0 |
| 10 | **3.2** | 1.3 | 5.2 | 10 | **2.6** | 0.4 | 6.2 |
| 12 | **6.4** | 2.5 | 9.4 | 12 | **4.1** | 1.5 | 7.1 |
| 13 | **42.3** | 6.1 | 70.7 | 13 | **2.3** | 1.2 | 3.8 |
| 14 | **1.0** | nd | 2.3 | 14 | **8.3** | 0.7 | 15.7 |
| 15 | **10.4** | 6.5 | 16.7 | 15 | **5.4** | 2.0 | 12.0 |
| 16 | **9.9** | 6.4 | 12.7 | 16 | **10.7** | 1.8 | 26.4 |
| 17 | **8.4** | nd | 38.2 | 17 | **0.8** | nd | 2.4 |
| 18 | **4.9** | 2.6 | 8.6 | 18 | **14.5** | 2.1 | 28.4 |
| 21 | **10.4** | 4.3 | 16.3 | 21 | **12.1** | 5.8 | 18.3 |
| 22 | **6.3** | 1.6 | 24.9 | 22 | **4.3** | 1.2 | 11.8 |
| 25 | **11.2** | 7.1 | 20.3 | 25 | **2.7** | nd | 4.7 |
| 26 | **4.4** | nd | 11.4 | 26 | **7.9** | 1.0 | 16.3 |
| 27 | **9.8** | 4.4 | 20.7 | 27 | **4.4** | 0.6 | 13.4 |
| 28 | **15.5** | 10.2 | 23.5 | 28 | **6.7** | 1.3 | 17.3 |
| 30 | **25.7** | 15.8 | 36.4 | 30 | **9.2** | 5.5 | 12.7 |
| 31 | **8.8** | 3.5 | 22.3 | 31 | **4.8** | 4.2 | 5.6 |
| 34 | **5.0** | 0.9 | 10.0 | 34 | **1.9** | 1.0 | 2.9 |
| 35 | **4.8** | 1.9 | 14.3 | 35 | **2.6** | 1.8 | 4.1 |
| | | | | | | | |
| **Total** | **9.9** | **nd** | **70.7** | | **5.9** | **nd** | **28.4** |
| | | | | | | | |
| | **Normal Diet** | | | | **Full Intervention** | | |
| | **Mean** | Low | High | | **Mean** | Low | High |
| 7 | **10.6** | 3.8 | 21.2 | 7 | **0.5** | nd | 1.2 |
| 8 | **17.5** | 6.0 | 32.2 | 8 | **0.7** | 0.1 | 1.8 |
| 11 | **3.3** | nd | 8.7 | 11 | **nd** | nd | 3.3 |
| 19 | **8.5** | 1.9 | 18.9 | 19 | **0.4** | nd | 1.0 |
| 20 | **11.1** | 2.9 | 41.2 | 20 | **0.6** | nd | 1.8 |
| 23 | **24.9** | 11.0 | 44.9 | 23 | **1.0** | nd | 2.6 |
| 24 | **7.8** | 0.3 | 13.2 | 24 | **0.6** | nd | 0.9 |
| 29 | **12.1** | 3.7 | 27.8 | 29 | **nd** | nd | 1.1 |
| 32 | **0.4** | 0.3 | 0.8 | 32 | **nd** | nd | 0.3 |
| 33 | **10.3** | nd | 22.6 | 33 | **0.4** | nd | 0.6 |
| | | | | | | | |
| **Total** | **10.6** | **nd** | **44.9** | | **0.5** | **nd** | **3.3** |

For creatinine adjustment the urinary concentration of DON ng/ml urine was divided by the creatinine concentration in mg/ml urine to give DON ng/mg creatinine. All non detectable (assigned as 0.25ng DON /ml urine) samples, were treated in this way and then an average value for the 39 non detectable samples was used, to provide consistency in presentation. The mean of all samples was 7.9ng/mg (range nd – 70.7ng/mg). During the normal phase of the study the overall mean was 10.1ng/mg (range nd – 70.7ng/mg). For the partial intervention the overall mean was 5.9ng/mg (range nd – 28.4ng/mg), and for the full intervention the overall mean was 0.5ng/mg (range nd – 3.3ng/mg), see summary Table 1b.

For those individuals on the partial intervention, the mean level of urinary DON (ng/mg creatinine) for bread consumers was ~60% of that during the normal phase. For those individuals on the full intervention the reduction in mean level of urinary DON compared to the normal phase was 95%.

The levels of urinary DON reduced significantly ($p<0.001$) after 24 hours of the full intervention being introduced to about 15% of the previous days level and overall urinary DON levels were less than 10% of that during consumption of the normal diet. No urine was collected on days six and seven, and the normal diet was consumed on days 1-8, whilst the intervention diets were days 9-12. The variations in urinary DON level by day of collection for the study group as a whole are presented in Figure 6a (DON ng/ml) and Figure 6b (DON ng/mg). Light green was used to present data during the normal diet (n=35). Yellow was used to present data during the partial intervention i.e. bread only consumption, (n=25). Dark green was used to present data during the full intervention (n=10). Mean and 95%CI are presented for each day. No samples were collected on day 6 and 7. The axis for day includes a letter prefix e.g. N-1 is day 1 of urine collection, diet 'normal'; F-9 is day 9 of urine collection, diet 'full' intervention; P-9 is day 9 of urine collection, diet 'partial' intervention.

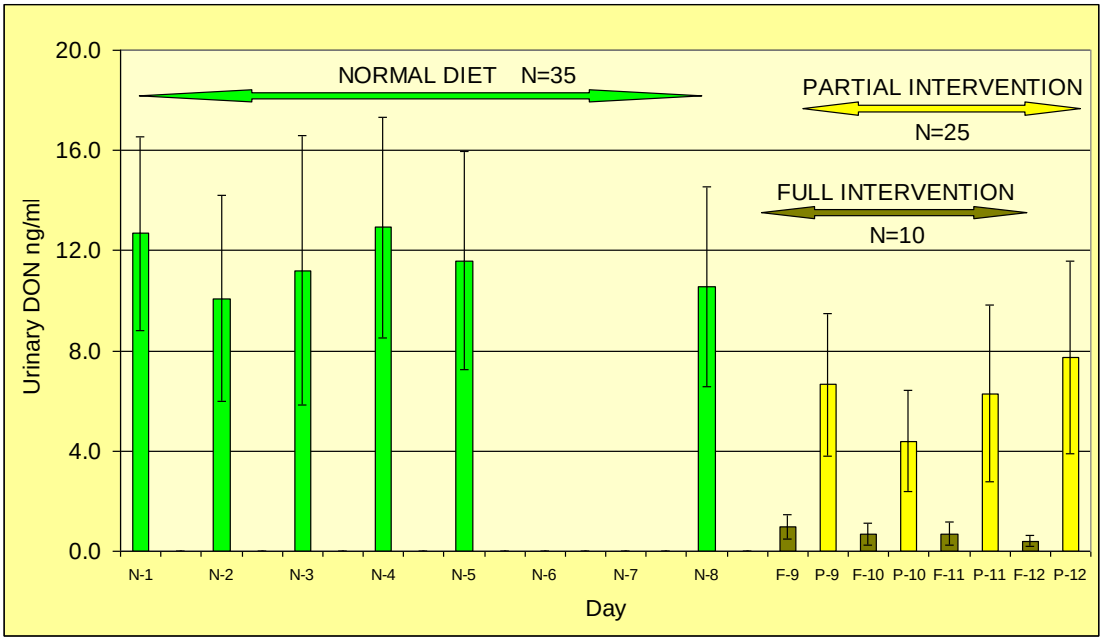



**Figure 6a. Variation in urinary DON (ng/ml) by day of collection (mean, 95%CI)**

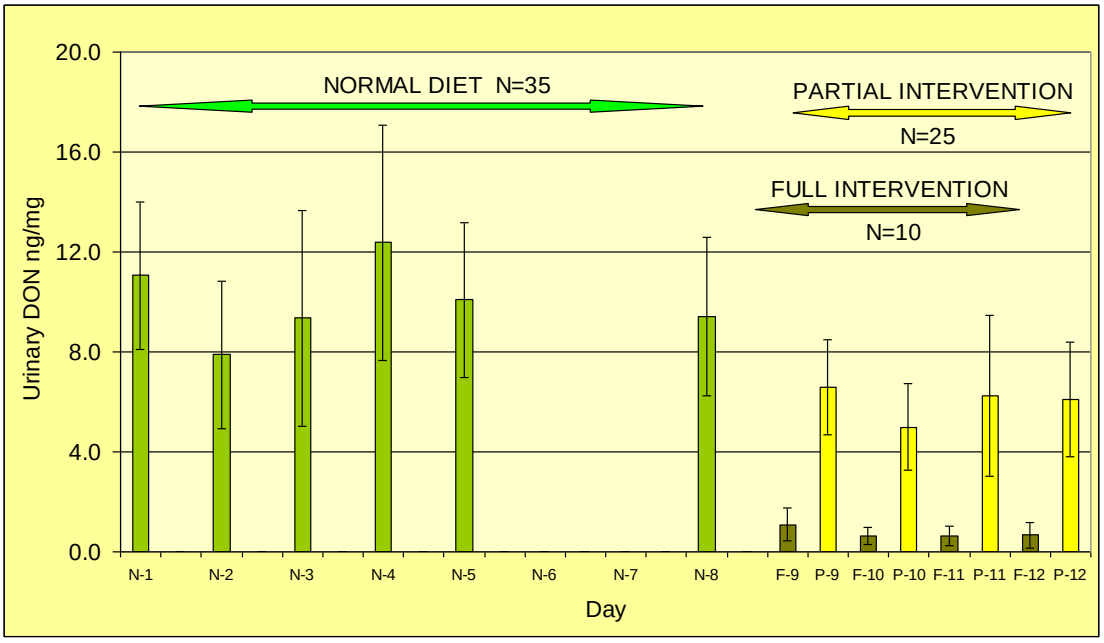



**Figure 6b. Variation in urinary DON (ng/mg) by day of collection (mean, 95%CI)**

There was no significant difference in the mean urinary output of DON by day within any dietary period of the study i.e. within the normal phase, within the partial intervention or within the full intervention, when considering urinary DON either as ng/ml or ng/mg. The mean daily DON levels are higher during the normal compared to both the partial and full intervention as detailed above.

**PHASE 2: Objective 07 - *Extract 'free' DON from the 100 urine samples with previously determined total DON > 5ng/ml, and measure using.***

In the original proposal all ten urine samples from 10 individuals within Group 2 (full intervention) were to be additionally analysed for the levels of free DON. The rationale being that this would allow assessment of the variation of the ratio of DON/DON glucuronide during the transition from normal to reduced DON exposure. However, the reduction in urinary levels following the full intervention proved to be rapid, such that DON was only detected in 17/40 (43%) of these samples, and the mean level of 0.7ng/ml was only just above the detection limit. Further detailed assessment within these samples would therefore not prove informative.

Previous analysis of six urine samples from Chinese women suggested a 1.2 – 2.8 fold increase in the level of detectable urinary DON following β-glucuronidase treatment to enzymically convert any DON-glucuronide to the parent compound DON (Meky et al., 2003). The larger data set in the present study may identify greater variability and thus, given a detection limit of 0.5ng/ml, it was decided to only assay samples where free DON could represent at least 10% of the total DON, i.e. assay samples where total DON was ≥ 5ng/ml. For one individual the highest level of total urinary DON was only 0.8ng/ml; therefore this persons samples were excluded from further analysis. Of the remaining 34 individuals, all had a detectable level of DON >5ng/ml on at least one occasion. Where it was possible, consecutive urine samples from a given individual were selected for this analysis. For 20 individuals at least three consecutive samples met this criterion, and of these 20 individuals, 15 had four consecutive samples.

**Output:** One hundred samples, from 34 individuals, 22 with 4 time points and 12 with 1 time point were selected for the measurement of free DON (DON detected in the absence of β-glucuronidase pre-treatment of urines).

**Table 2a. Percentage free DON in single sample collection from individuals.**

| ID | DON ng/ml | | % free DON | |
|---|---|---|---|---|
| | Total | Free | Calc | Est |
| **3** | **53.7** | **1.6** | **3.0** | **n/a** |
| **4** | 7.6 | n/d | n/a | <3.3 |
| **6** | 8.2 | n/d | n/a | <3.0 |
| **10** | **5.2** | **0.6** | **11.0** | **n/a** |
| **11** | **5.5** | **0.7** | **13.2** | **n/a** |
| **14** | **30.5** | **1.8** | **5.7** | **n/a** |
| **17** | **49.5** | **4.5** | **9.1** | **n/a** |
| **18** | 25.6 | n/d | n/a | <1.0 |
| **19** | **27.2** | **1.8** | **6.4** | **n/a** |
| **22** | **21.8** | **1.4** | **6.4** | **n/a** |
| **34** | 9.5 | n/d | n/a | <2.6 |
| **35** | **14.5** | **1.0** | **7.1** | **n/a** |

Total – assay conducted using β-glucuronidase to obtain total DON (previously analysed for selection purposes)
Free – assay conducted without β-glucuronidase to obtain free DON only
Calc – calculated percent free DON using measured total and free DON
Est – estimated percent free DON using measured total DON and limit of detection
n/d - none detected; n/a – not applicable
*Bold indicates free DON was detected – these data were used for Figures 7A and 7B below*

**Table 2b. Percentage free DON in multiple sample collection from individuals.**

| ID | DON ng/ml | | % free DON | | | ID | DON ng/ml | | % free DON | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Total | Free | Calc | Est | | | Total | Free | Calc | Est |
| **1** | **14.5** | **0.5** | **3.3** | **n/a** | | **21** | **30.7** | **2.0** | **6.6** | **n/a** |
| **1** | 12.8 | n/d | n/a | <3.9 | | **21** | 21.8 | n/d | n/a | <2.3 |
| **1** | 17.5 | n/d | n/a | <2.8 | | **21** | 11.3 | n/d | n/a | <4.4 |
| **1** | 5.0 | n/d | n/a | <4.5 | | **21** | 8.2 | n/d | n/a | <6.1 |
| **2** | **6.7** | **0.7** | **10.0** | **n/a** | | **23** | **32.2** | **0.6** | **1.8** | **n/a** |
| **2** | **5.0** | **0.9** | **18.0** | **n/a** | | **23** | 19.1 | n/d | 5.7 | <2.6 |
| **2** | **17.7** | **2.5** | **14.1** | **n/a** | | **23** | 18.8 | n/d | 9.1 | <2.7 |
| **2** | **34.0** | **5.6** | **16.5** | **n/a** | | **23** | 8.4 | n/d | n/a | <5.9 |
| **5** | 17.8 | n/d | n/a | <2.8 | | **24** | **17.5** | **0.6** | **3.3** | **n/a** |
| **5** | 8.7 | n/d | n/a | <5.7 | | **24** | **22.0** | **0.9** | **4.2** | **n/a** |
| **5** | 5.0 | n/d | n/a | <10.0 | | **24** | 16.8 | n/d | n/a | <3.0 |
| **5** | 5.7 | n/d | n/a | <8.8 | | **24** | 10.7 | n/d | n/a | <4.6 |
| **7** | **25.2** | **1.0** | **4.0** | **n/a** | | **25** | 8.1 | n/d | n/a | <6.2 |
| **7** | **16.3** | **2.2** | **13.2** | **n/a** | | **25** | 6.6 | n/d | n/a | <7.6 |
| **7** | **47.6** | **4.6** | **9.6** | **n/a** | | **25** | 10.0 | n/d | n/a | <5.0 |
| **7** | 20.5 | n/d | n/a | <2.4 | | **25** | 6.8 | n/d | n/a | <7.4 |
| **8** | **19.2** | **2.5** | **13.2** | **n/a** | | **26** | 5.2 | n/d | n/a | <9.6 |
| **8** | **36.8** | **2.7** | **7.3** | **n/a** | | **26** | 6.3 | n/d | n/a | <8.0 |
| **8** | **40.4** | **3.6** | **8.9** | **n/a** | | **26** | 13.6 | n/d | n/a | <3.6 |
| **8** | **35.8** | **3.7** | **10.3** | **n/a** | | **26** | 7.7 | n/d | n/a | <6.6 |
| **9** | **5.3** | **0.6** | **11.7** | **n/a** | | **27** | **21.0** | **1.5** | **6.9** | **n/a** |
| **9** | **6.0** | **1.0** | **16.8** | **n/a** | | **27** | **29.2** | **1.8** | **6.0** | **n/a** |
| **9** | **15.2** | **1.6** | **10.5** | **n/a** | | **27** | 13.0 | n/d | n/a | <3.8 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **9** | 6.7 | n/d | n/a | <7.5 | | **27** | 9.9 | n/d | n/a | <5.0 |
| **12** | **6.6** | **0.5** | **7.6** | **n/a** | | **28** | **12.4** | **0.6** | **4.4** | **n/a** |
| **12** | **6.3** | **0.6** | **8.7** | **n/a** | | **28** | **13.7** | **1.1** | **7.9** | **n/a** |
| **12** | **7.2** | **0.6** | **8.3** | **n/a** | | **28** | **20.1** | **2.6** | **12.8** | **n/a** |
| **12** | 5.2 | n/d | n/a | <9.6 | | **28** | **20.4** | **3.5** | **17.0** | **n/a** |
| **13** | 38.9 | n/d | n/a | <1.3 | | **29** | 21.5 | n/d | n/a | <2.4 |
| **13** | 37.3 | n/d | n/a | <1.3 | | **29** | 15.1 | n/d | n/a | <3.4 |
| **13** | 17.7 | n/d | n/a | <2.8 | | **29** | 6.4 | n/d | n/a | <7.8 |
| **13** | 36.3 | n/d | n/a | <1.4 | | **29** | 7.3 | n/d | n/a | <6.8 |
| **15** | **12.4** | **1.0** | **8.1** | **n/a** | | **30** | **24.5** | **4.3** | **17.4** | **n/a** |
| **15** | **24.4** | **2.2** | **8.8** | **n/a** | | **30** | **61.8** | **8.0** | **12.9** | **n/a** |
| **15** | **25.3** | **4.1** | **16.1** | **n/a** | | **30** | **57.9** | **9.3** | **16.1** | **n/a** |
| **15** | 13.8 | n/d | n/a | <3.6 | | **30** | 5.8 | n/d | n/a | <8.8 |
| **16** | 5.9 | n/d | n/a | <8.6 | | **31** | **14.2** | **1.5** | **10.4** | **n/a** |
| **16** | 5.5 | n/d | n/a | <9.0 | | **31** | **34.8** | **4.8** | **13.8** | **n/a** |
| **16** | **13.8** | **1.6** | **11.6** | **n/a** | | **31** | 5.0 | n/d | n/a | <10.0 |
| **16** | 6.1 | n/d | n/a | <8.2 | | **31** | 5.2 | n/d | n/a | <9.6 |
| **20** | 9.2 | n/d | n/a | <5.4 | | **33** | **78.2** | **8.9** | **11.3** | **n/a** |
| **20** | 6.3 | n/d | n/a | <7.9 | | **33** | 6.2 | n/d | n/a | <8.0 |
| **20** | 13.5 | n/d | n/a | <3.7 | | **33** | 5.8 | n/d | n/a | <8.6 |
| **20** | 29.2 | n/d | n/a | <1.7 | | **33** | 7.1 | n/d | n/a | <7.0 |

The mean level of total DON in those selected was 17.8ng/ml (range 5.0 – 78.2ng/ml, n=100). Free DON was detected in 46/100 samples (mean free DON for positive samples was 2.4ng/ml; range 0.5 – 9.3ng/ml). The mean level of total DON (24.4ng/ml; 95%CI: 19.5, 29.2ng/ml) was higher for those samples where free DON was detected ($p<0.001$) compared to the samples with free DON below the limit of detection (total DON 12.2ng/ml; 95%CI: 9.7, 14.7ng/ml). In those samples positive for free DON the amount of free DON was significantly positively associated with the amount of total DON ($p<0.001$, $R^2 = 0.680$), see Figure 7a.

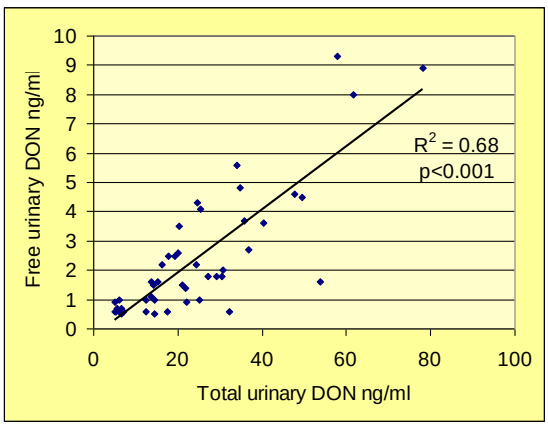



**Figure 7a: Comparison of Free DON to Total DON in urine (n=46)**

In those samples positive for free DON the percentage of free DON (mean 10.0% (range 1.8 – 18.0%) was not associated ($p=0.673$, $R^2 = 0.001$) with the level of total DON, see Figure 7b. For one individual (ID code 13) the mean total DON was 32.6ng/ml (range 17.7 – 38.9ng/ml, n=4) but despite this no free DON was observed in any of four samples collected over consecutive days, suggesting for this individual the percentage of free DON was less than 1.3%. In general the percent of free DON appeared to vary more between individuals than it did within an individual. These

data indicate a greater than 10-fold variation in percentage of free DON to total DON, and are suggestive that the variation may be dependent upon the individual rather than the total DON exposure.

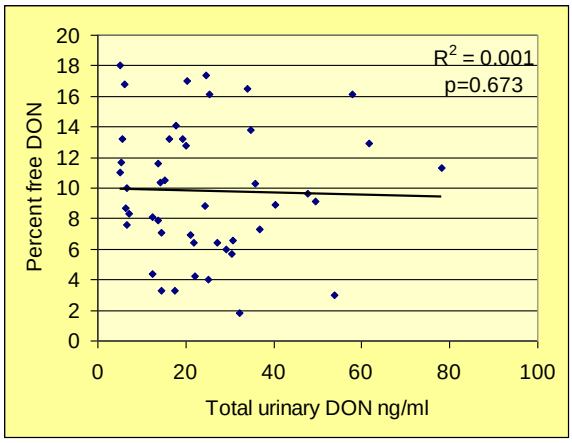



**Figure 7b: Comparison of percentage free DON to Total DON in urine (n=46)**

**PHASE 2: Objective 08 - *Fully code food diaries***
Diaries were coded using an in-house Microsoft Access based dietary analysis package which uses the UK Composition of Foods (Holland *et al*. 1992).

**Output**
The study recruited 35 individuals (17 males, 18 females; age range 20-55). The average weight was 74kg (StDev 14kg); the average height was 171cm (StDev 11cm). Thirty four were white Caucasian; one was from south East Asia. Food diaries were kept for 12 days by 30 individuals, for 11 days by three individuals and for 10 days by two individuals; thus a total of 413 days of diary information was recorded. Food diaries were fully coded according to the method of Holland et al., (1992). Wheat and maize based food items were used to generate the cereal intake on each day. These included bread, pasta, pizza, sweet snacks (biscuits, cakes, buns, pastries), breakfast cereals (excluding oat and rice cereals), flour, wheat or maize based savoury snacks. Beer was a potential source of DON exposure; consumption levels were identified but not included as part of the 'cereal' group due to its large water content. The amounts and frequency of cereal and bread consumption are presented in Table 3

Over the 12 day study all individuals consumed cereal on at least seven days (cereal as defined by the food groups listed above). The average amount of cereal consumed per person per day was 174 g/day (range 0 – 658g/day). Bread formed a significant part of this consumption with on average 110g/day consumed (range 0-535g/day). During consumption of the normal diet (over eight days) cereal was consumed on at least five days with an average consumption per person per day of 206g/day (range 0-658g/day). Bread contributed on average approximately half of the total cereal intake during this time frame. During the intervention phase 25 individuals were requested to consume bread only as the major source of wheat or maize cereals, the remaining 10 individuals were advised to avoid all major sources of wheat or maize. During the intervention cereal was consumed by 28 of 35 individuals on at least one occasion; thus compliance with the suggested diet was good but not absolute. The average cereal consumption per person per day was 117g/day (range 0-455g/day). Bread contributed the vast majority of this intake with an average per person per day of 112g/day (range 0-455g/day).

To more fully assess the level of compliance within the partial and full intervention a further break down of the data is presented. For those individual on the full intervention (requested no cereal consumption) three individuals consumed cereal on at least one occasion, one individual consumed cereal on all four occasions. However, overall the mean consumption of cereal per person per day was modest (mean 7g/day: range 0-150g/day). Average for non-compliers was 22g/day The food items consumed were not bread, and the highest quantity consumed (150g) was pasta.

**Table 3: Frequency and amount of cereal / bread consumed by 35 volunteers.**

| | **Normal Diet** | **Partial Intervention** | **Full Intervention** |
|---|---|---|---|
| | **8 days** | **4 days** | **4 days** |
| **Cereal Consumption** | | | |
| Number of consumers / total* | 35 / 35 | 25 / 25 | 3 / 10 |
| Mean days per person** | 8 (5-8) | 4 (2-4) | 1 (0-4) |
| Number of days / Total days[#] | 264 / 271 | 93 / 99 | 6 / 39 |
| Mean (range) / grams/day[##] | 206 (0-658) | 159 (0-455) | 7 (0-150) |
| **Bread consumption** | | | |
| Number of consumers / total* | 35 / 35 | 25 / 25 | 0 / 10 |
| Mean days per person** | 6 (1-8) | 4 (2-4) | 0 |
| Number of days / Total days[#] | 213 / 271 | 92 / 99 | 0 / 39 |
| Mean (range) / grams/day[##] | 110 (0-535) | 155 (0-455) | 0 |

*On at least one day

**Mean (range) of the number of days each person consumed the food item.

[#] Total number of person days when consumption occurred

[##] Mean (range) amount of food item consumed per day.

During the partial intervention all individuals consumed cereal on at least two occasions and the majority (23/25) consumed cereal on all four days. The mean cereal consumption per person per day was 159g/day (range 0-455g/day). Bread contributed the vast majority of this intake (mean 155g/day; range 0-455g/day). For the partial intervention eight individuals consumed food items on one occasion that was not bread and one individual consumed non bread items on two days. Overall the mean consumption of non bread cereal was modest (mean 5g/day; range 0-125g/day). The consumption of pasta was the largest single non-bread food item consumed on any day (108g). For the intervention phase as a whole there were 122 of 138 (88.4%) person days where compliance with the suggested diet was maintained. Overall the intervention phase had good reported compliance.

For those individuals on the partial intervention (n=25) the level of DON contamination of duplicate bread samples (Objective 05) and the amount of bread consumed by each individual on each day (Objective 08) was used to determine the daily DON intake. In addition the average DON intake over the four day period was calculated. The average daily DON intake was 10.6 μg/day (range 0 – 42.5 μg/day).

**PHASE 2: Objective 09 - *Conduct statistical analysis of biomarker data with respect to both food diaries and DON level in bread samples***.

**Output**

Urinary DON (ng DON/mg creatinine) was used for all statistical analysis; urinary DON was natural log transformed prior to statistical analysis, Stata version 9 was used for all statistical analysis. Univariate regression analysis of samples collected during the normal phase of the study indicated that urinary DON was modestly but significantly negatively associated with age ($p<0.001$; adjusted $R^2=0.073$), but not with weight or height ($p>0.15$). Females had higher (but not significantly higher, $p>0.25$) levels of urinary DON (geometric mean 6.1ng/mg; 95%CI: 4.6, 7.3ng/mg) compared to males (geometric mean 5.8ng/mg; 95%CI: 4.6, 7.3ng/mg). Neither cereal intake nor bread intake were significantly associated with any of these demographic measures ($p>0.25$ for all). In subsequent multivariate analysis sex and age were retained in all models.

In multivariate regression analysis (assessing the contribution of food items, in individual food item models, to urinary DON, using adjustment for age and sex, during the normal diet) bread ($p<0.001$; adjusted $R^2=0.195$), sweet snacks ($p=0.016$; adjusted $R^2=0.091$), and pizza ($p=0.019$; adjusted $R^2=0.090$) and beers* ($p=0.008$; adjusted $R^2=0.025$) were significantly associated with urinary DON; *univariate only. (NB beer consumption differed by sex). Further statistical analysis of the contribution of individual food items to levels of urinary DON was not an objective of this part of the study, and was described in detail for Phase One of the project.

During the consumption of the normal diet (n=35 individuals) 208 paired urine and the previous days cereal intake data were available for comparison. Figure 8a shows a scatter-plot of cereal intake versus urinary DON. Cereal intake was significantly ($p<0.001$) associated with urinary DON; and in models including age the adjusted $R^2=0.230$. In order to provide a more integrated measure of intake versus urinary DON, average cereal intake and average urinary DON levels were calculated. Figure 8b shows a scatter-plot of these data. Average cereal intake was significantly ($p<0.001$) associated with average urinary DON; and the adjusted $R^2=0.358$. Thus assessment of average intake with average urinary DON provides an improved model to explain the variation of urinary DON.

During the intervention phase of the study (n=35 individuals), 138 paired urine and bread intake data were available for comparison. Figure 9a shows a scatter-plot of bread intake versus urinary DON. Bread intake was significantly ($p<0.001$) associated with urinary DON; adjustment for age and sex further improved the model (adjusted $R^2=0.476$). The data modelled for average bread intake and average urinary DON further improved the explanation of the variation in urinary DON (adjusted $R^2=0.544$), see Figure 9b.

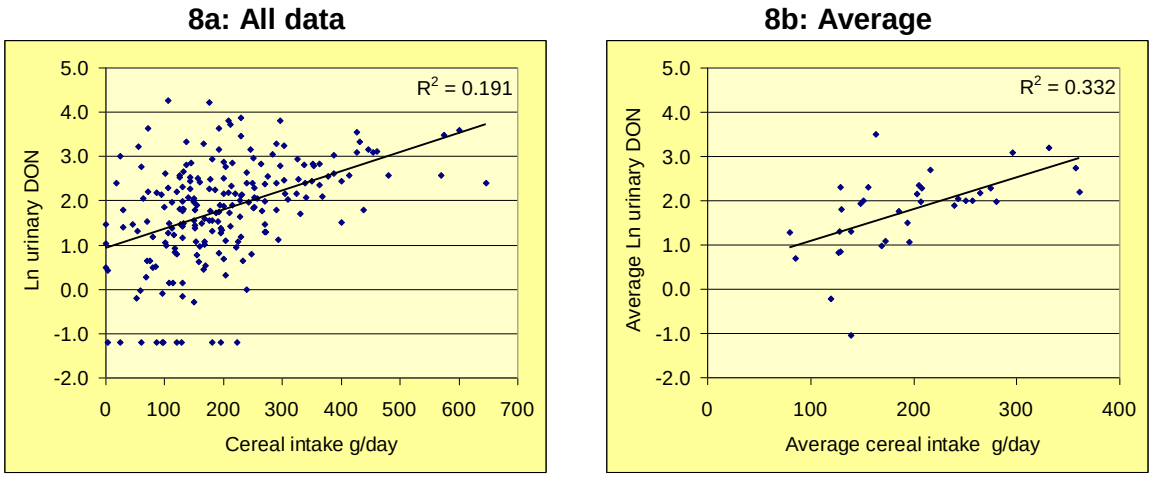



**Figure 8. Normal diet: Scatter-plot cereal intake vs. urinary DON (p<0.001)**

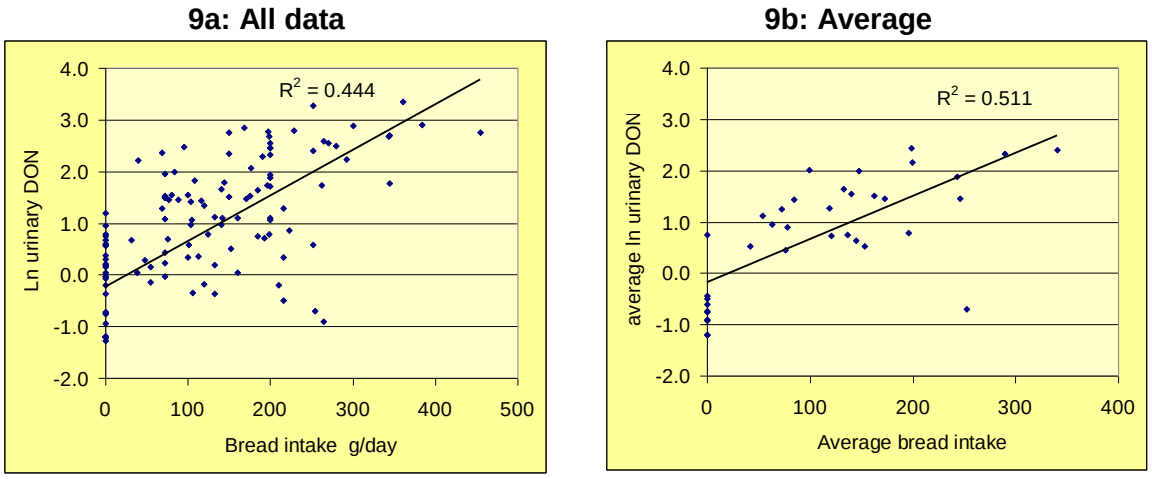



**Figure 9. Intervention (n=35): Scatter plot bread intake vs. urinary DON (p<0.001)**

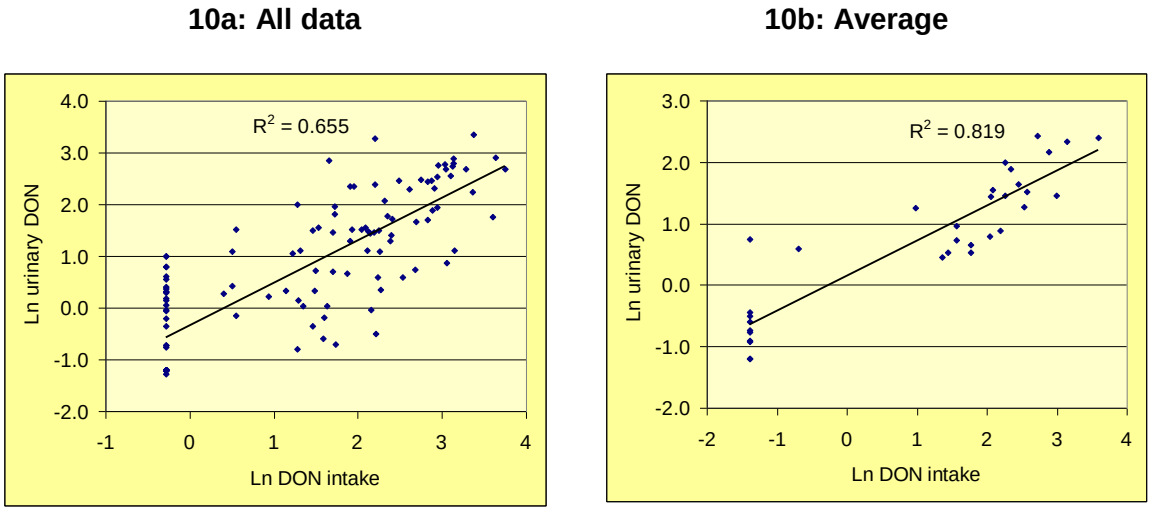


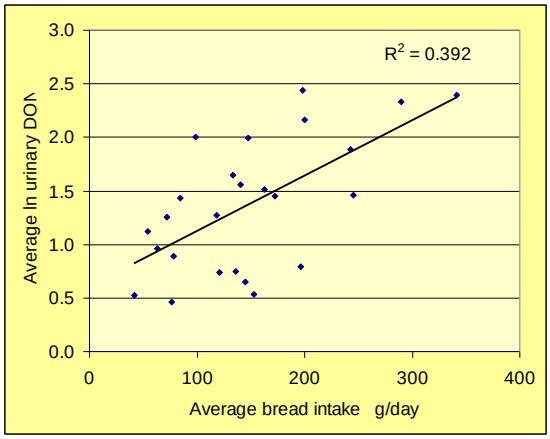

**Figure 10. Intervention (n=35): Scatter plot DON intake vs. urinary DON (p<0.001)**

During the intervention phase of the study (n=35 individuals) 125 paired urine and DON intake data were available for comparison. Figure 10a shows a scatter-plot of

DON intake versus urinary DON. DON intake was significantly ($p<0.001$) associated with urinary DON (adjusted $R^2=0.675$). In order to provide a more integrated measure of intake versus urinary DON, average DON intake and average urinary DON levels were calculated. Figure 10b shows a scatter-plot of these data. The use of average DON intakes with average DON in urine provided an improved model to explain the variation in urinary DON (adjusted $R^2=0.831$).

A further analysis of data obtained during the intervention phase restricted the comparison to bread consumers only (n=25 individuals); data for average intakes versus average urinary DON only are presented. Figure 11a shows a scatter-plot of average bread intake versus average urinary DON. Average bread intake was significantly ($p<0.001$) associated with average urinary DON (adjusted $R^2=0.392$, corr coef 0.010 (95%CI: 0.071, 0.013), t=5.64, $p<0.001$). Figure 11b shows a scatter-plot of average DON intake versus average urinary DON. Average DON intake was significantly associated with urinary DON (adjusted $R^2=0.519$, corr coef 0.118 95%CI 0.076, 0.158, t=6.23, $p<0.001$).

**11a: Bread**




**11b: DON**

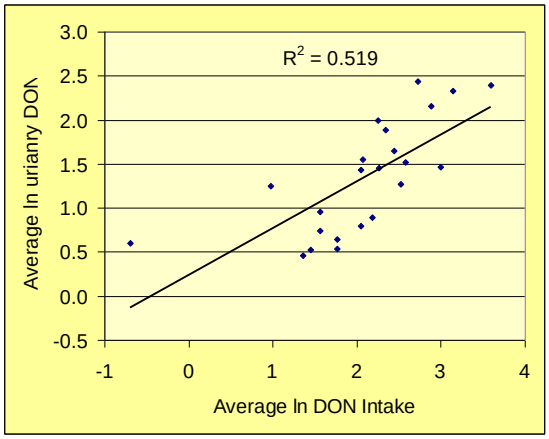



**Figure 11. Intervention (25 bread consumers): Scatter plot of average intake vs. urinary DON (p<0.001)**

**Discussion Phase Two**

Mycotoxins occur frequently in cereal crops, with an estimated 25% of agricultural commodities contaminated (CAST 2003). For most types of mycotoxin our understanding of the health consequences of exposure are hampered by a lack of validated exposure assessment tools. Due to the ubiquitous DON contamination of cereals in temperate regions of the world, and its resistance to degradation during processing DON exposure in the UK will be frequent. In Phase One of the study the utility of a highly robust urinary assay to measure DON was demonstrated. Further, this measure was applied to assess 300 urine samples stratified for cereal intake. Urinary DON was significantly associated with cereal intake ($p<0.0001$); but the variation in urinary DON was relatively poorly explained by the variation in cereal intake.

There may be a number of reasons for this. Firstly, DON contamination of wheat and maize food items is heterogeneous. In Phase One of this study no DON measures of food were available. Secondly, there are potential reporting errors when obtaining food diary information. Thirdly, urine measurements were based on samples collected on a single day, and thus may not represent more typical levels, better obtained by measures over several days.

In order to address these issues, Phase Two of the study incorporated repeat collection of urine samples alongside food diary information collected throughout the study. During the first eight days all individuals (n=35) consumed their normal diet. During the subsequent four days (intervention phase) some individuals (n=10) were requested to consume no major sources of wheat or maize in their diet (full intervention); and a further 25 individuals were requested to consume only bread as their major source of wheat or maize (partial intervention). For these latter individuals duplicate bread samples were collected daily during the intervention, so that measures of DON intake could be measured and compared to the level of urinary DON.

During the consumption of the normal diet urinary DON was frequently detected (309 of 348 positive), and the overall mean was 10.1ng/mg creatinine (range nd – 70.7ng/mg). Urinary DON was positively significantly associated with cereal intake ($p<0.001$) using regression analysis. When age and sex were included in the model with cereal intake the adjusted $R^2$ was (0.23), thus 23.0% of the variation in urinary DON level was explained using this model. These data are similar to the previous assessment of cereal intake and urinary DON in 300 individuals (Phase One) in which cereal intake explained ~18% of variation in urinary DON. When a more integrated measure of cereal intake and urinary DON was assessed (in models including age and sex), the model explained 35.8% of the variation in urinary DON. Thus by obtaining repeat measures of both intake and the biomarker, the variation in urinary DON level was better explained.

During the intervention phase of the study compliance with the suggested dietary restriction was good. Overall 95% of the cereal based diet during the intervention was bread, and no individuals on the full intervention consumed bread. The levels of urinary DON reduced significantly ($p<0.001$) after 24 hours of the full intervention being introduced to about 15% of the previous days level and overall urinary DON levels were less than 10% of that during consumption of the normal diet. This rapid clearance of DON suggested that this biomarker represents exposure predominantly from consumption during the previous 24 hours. A more detailed assessment within a 24 hour period would more fully explore the kinetics of clearance for this toxin.

During the intervention phase of the diet bread intake was significantly positively associated with urinary DON ($p<0.001$) and the model explained 47.6% of the

variation in urinary DON, when additionally adjusted for age and sex. In models that assessed the average bread intake and average urinary DON level, with adjustment for age and sex, the models explained 54.4% of the variation in urinary DON. During this more restricted period of the study bread, as the major source of wheat and maize based cereal intake, provided a better explanation of urinary DON compared to overall cereal intake during the consumption of the normal diet. This may in part reflect that some of the cereal groups included in the period of normal diet did not appear to significantly contribute to the level of urinary DON, for example pasta and breakfast cereal. The survey of mycotoxin intake in the EU (SCOOP 2003) indicates these items are potential sources of DON thus the lack of an association may reflect infrequent intake, which would provide limited statistical power. In addition, in the case of breakfast cereals, they were consumed almost 24 hours before the subsequent urine collection and thus part of the 'dose' may have been excreted prior to the subsequent morning void being collected, in comparison to a food item consumed later in a given day.

During the intervention phase of the diet duplicate bread samples were collected to provide estimates of DON intake. DON intake was significantly positively associated with urinary DON ($p<0.001$) and DON intake better explained the variation in urinary DON (65.5%) compared to bread 47.6%, in models adjusted for age and sex. Subsequently data was modelled using average DON intake and average urinary DON. These models provided a better explanation for the variation in urinary DON (83.1% and 54.4% respectively).

**These data indicate that the variation in urinary DON level was well explained by DON intake, providing considerable confidence in the use of this measure as a biomarker of DON exposure.**

***Estimates of intake***

The current recommended tolerable daily intake for DON is 1000ng/kg bw/day. During the intervention phase we have an accurate measure of this intake based on bread consumption for 25 individuals. No individuals exceeded the TDI during the bread only intake phase (maximum intake was 582ng/kg bw day). There are two methods of estimating 24 hour urinary output of a xenobiotic from a spot urine sample. Firstly, an estimate of average urine production for an adult can be used to convert the concentration of the xenobiotic (ng/ml urine) to a total daily level. Whilst the estimate is useful, it can vary significantly dependent upon fluid intake, physical activity and body weight. Secondly, an estimate of creatinine clearance can be used. Urinary creatinine excreted per day is strongly influenced by body mass, age and sex, and can be calculated from the following equations for males and females:

Daily Excretion of creatinine[1] (mg) in females = (22 – (age/9)) * body weight

Daily Excretion of creatinine[1] (mg) in males = (28 – (age/6)) * body weight

Whilst both methods must be regarded as estimates it is generally considered that the creatinine adjustment method is more robust. For each individual in the partial intervention an estimate of daily urinary creatinine was calculated based on the sex, age and body weight. This value was used to estimate the daily urinary DON

[1]Using http://www.clinicalculator.com/english/nephrology/excrea/excrea.htm

excreted using the DON concentration in ng DON/mg creatinine. The average DON intake per day (calculated from the bread intake recorded in diaries and the DON contamination level of duplicate bread samples) for each person and the average urinary DON excreted per day were then used to calculate the average percentage excretion of DON via the urine, see Table 4.

The percentage transfer was not associated with sex, age, weight or DON intake level, p>0.4 in all comparisons, though sample numbers limits this statistical comparison. The overall average excretion rate per day was 72.3% (95%CI: 59.1, 85.5); a value slightly higher than our previous estimates based on studies in pigs. Using this estimated transfer value and the levels of urinary DON (ng DON/mg creatinine) estimates of DON intake were then made for all individuals based on the levels of urinary DON and the estimated transfer rate, and the individuals body weight, during the normal phase of the diet, Table 5. The overall estimated mean intake was **298ng/kg bw/day** (95%CI: 218, 362ng/kg bw/day). The estimated intake exceeded the recommended TDI for 6 / 35 (17%) of individuals on one or more occasion, and a further 13 individuals had one or more days when estimated intake exceeded 50% of the TDI.

**Table 4. Estimated percentage transfer of DON to urine – partial intervention**

| Sample ID | Mean DON Intake μg/day | Mean percent DON transfer (%) |
|---|---|---|
| **1** | 13.15 | 65.4 |
| **2** | 18.62 | 78.8 |
| **3** | 5.85 | 34.5 |
| **4** | 12.10 | 44.6 |
| **5** | 10.35 | 122.3 |
| **6** | 4.78 | 89.2 |
| **9** | 9.53 | 109.0 |
| **10** | 5.20 | 117.8 |
| **12** | 2.66 | 49.6 |
| **13** | 11.17 | 70.4 |
| **14** | 20.04 | 59.7 |
| **15** | 9.61 | 94.4 |
| **16** | 10.35 | 70.0 |
| **17** | 8.85 | 21.2 |
| **18** | 23.16 | 79.0 |
| **21** | 36.47 | 51.1 |
| **22** | 6.37 | 89.0 |
| **25** | 8.48 | 61.9 |
| **26** | 10.78 | 111.4 |
| **27** | 7.73 | 34.4 |
| **28** | 3.35 | 103.1 |
| **30** | 17.90 | 75.3 |
| **31** | 7.97 | 75.9 |
| **34** | 5.86 | 71.2 |
| **35** | 8.92 | 30.1 |

**In Phase One of the study a crude estimate of DON intake was made for 300 individuals based on their urinary DON levels. Now with a more accurate**

**measure for percent transfer of DON into urine, the mean estimate previously reported (494 ng/kg bw/day) is refined to 222ng/kg bw/day**; with 2/300 individuals predicted to exceed the recommended TDI. However, it should again be noted that the latter individuals were a stratified sample based on cereal consumption and no samples were taken from individuals in the highest decile of cereal intake in the NDNS survey

**Table 5: Summary of estimated DON intake during the normal diet**

| Sample ID | Sex | DON intake ng/kg bw/day Mean (range) | Frequency that estimated DON intake exceeded 500ng/kg bw/day | Frequency that estimated DON intake exceeded 1000ng/kg bw/day |
|---|---|---|---|---|
| **1** | M | 235 (79 – 325) | 0 / 6 | 0 / 6 |
| **2** | F | 104 (8 – 242) | 0 / 6 | 0 / 6 |
| **3** | F | 336 (54 – 1159) | 1 / 6 | 1 / 6 |
| **4** | F | 240 (76 – 570) | 1 / 6 | 0 / 6 |
| **5** | M | 304 (89 – 572) | 1 / 6 | 0 / 6 |
| **6** | F | 80 (23 – 164) | 0 / 6 | 0 / 6 |
| **7** | M | 423 (130 – 967) | 1 / 6 | 0 / 6 |
| **8** | F | 670 (297 – 1208) | 4 / 6 | 1 / 6 |
| **9** | F | 316 (107 – 637) | 1 / 6 | 0 / 6 |
| **10** | M | 91 (37 – 148) | 0 / 6 | 0 / 6 |
| **11** | F | 75 (7 – 199) | 0 / 6 | 0 / 6 |
| **12** | F | 179 (70 – 255) | 0 / 6 | 0 / 6 |
| **13** | F | 1046 (151 – 1747) | 5 / 6 | 3 / 6 |
| **14** | M | 31 (9 – 68 ) | 0 / 6 | 0 / 6 |
| **15** | M | 315 (198 – 505) | 1 / 6 | 0 / 6 |
| **16** | M | 277 (179 – 355) | 0 / 6 | 0 / 6 |
| **17** | M | 255 (9 – 1168) | 1 / 6 | 1 / 6 |
| **18** | M | 142 (76 – 252) | 0 / 6 | 0 / 6 |
| **19** | F | 213 (48 – 474) | 0 / 6 | 0 / 6 |
| **20** | M | 362 (55 – 794) | 2 / 6 | 0 / 6 |
| **21** | M | 318 (132 – 498) | 0 / 6 | 0 / 6 |
| **22** | F | 168 (43 – 664) | 1 / 6 | 0 / 6 |
| **23** | F | 490 (169 – 904) | 3 / 6 | 0 / 6 |
| **24** | M | 261 (67 – 443) | 0 / 6 | 0 / 6 |
| **25** | F | 313 (198 – 568) | 1 / 6 | 0 / 6 |
| **26** | M | 144 (8 – 319) | 0 / 6 | 0 / 6 |
| **27** | M | 334 (149 – 702) | 1 / 6 | 0 / 6 |
| **28** | F | 421 (276 – 636) | 2 / 6 | 0 / 6 |
| **29** | F | 276 (98 – 552) | 1 / 6 | 0 / 6 |
| **30** | M | 878 (538 – 1244) | 4 / 6 | 2 / 6 |
| **31** | F | 250 (101 – 636) | 1 / 6 | 0 / 6 |
| **32** | F | 9 (7 – 18) | 0 / 6 | 0 / 6 |
| **33** | M | 321 (84 – 1189) | 1 / 6 | 1 / 6 |
| **34** | M | 164 (30 – 326) | 0 / 6 | 0 / 6 |
| **35** | F | 127 (7 – 346) | 0 / 6 | 0 / 6 |

The study also explored the potential variation in DON:DON-glucuronide ratio. DON-glucuronide is a detoxification product and the ratio of free DON to DON-glucuronide may be a phenotypic marker of susceptibility. A high ratio indicating poor detoxification, whilst a low ratio indicating good detoxification. The usual form of the

urinary DON assay includes β-glucuronidase pre-treatment to convert any DON-glucuronide to DON prior to extraction using DON immunoaffinity columns. These columns do not bind the DON-glucuronide. In order to assess the amount of free DON only, a mock enzyme digestion step was performed in which no β-glucuronidase was added to the urine during the incubation procedure. One hundred samples with a total DON level exceeding 5ng/ml were selected. Of these, free DON was detected in 46 samples. In those samples with detectable free DON the amount of free DON increased significantly ($P<0.001$) with the amount of total DON. However, the percentage of free DON was not associated with total DON. The mean percentage free DON in samples where this ratio could be assessed was 10%. Free DON was not detected for 54 samples, and given the limit of detection of the assay most of these samples would be predicted to have <10% of their total DON as free DON. Therefore free DON likely represents <10% of the total DON in this study. These data also suggested that the amount of DON-glucuronide varied by individual, though numbers of positive measures limits these observations.

**Summary Phase Two**

In Phase Two of the study there were a number of objectives

i. An assessment of the variation of urinary DON in 35 volunteers during consumption of a normal diet (n=35 x 6 days) with respect to cereal intake.

ii. An assessment of the variation of urinary DON in 25 volunteers during a partial dietary intervention in which bread was permitted (n=25 x 4 days).

iii. An assessment of the variation of urinary DON in 10 volunteers during a full intervention based on elimination of wheat based foods (n=10 x 4 days).

iv. A direct comparison of urinary DON to DON intake over several days.

v. A better estimate of DON intake during the consumption of the normal diet.

vi. An understanding of the variance in the DON:DON-glucuronide ratio as a potentially phenotypic measure of susceptibility.

The project successfully addressed all of these objectives

i. During consumption of the normal diet, consumed over six days for 35 individuals, urinary DON level was significantly associated with cereal intake ($p<0.001$).

ii. During consumption of the full intervention diet, consumed over four days for 10 individuals, the mean urinary DON level 0.5ng/ml (range nd – 3.3ng/ml). Urinary DON was reduced approximately 10 fold within 24 hours of the intervention.

iii. During consumption of the partial intervention diet, consumed over four days for 25 individuals, the mean urinary DON level was 5.9ng/ml (range nd – 28.4ng/ml).

**iv. Urinary DON was significantly associated ($p<0.001$) with DON intake.**

v. The variation in urinary DON was better explained during both the normal phase and the intervention phase using mean consumption of cereal and mean urinary DON over several days.

vi. During the intervention phase of the study average DON intake better explained the variation in average urinary DON (83.1%) than average bread intake (~54.4%).

vii. The transfer or DON to the urine was estimated to be 72.3%.

viii. The mean DON intake during the normal diet was **estimated** to be **298ng/kg bw/day.**

ix. Six of the 35 individuals were predicted to exceed the TDI on one or more occasion during this study.

x. DON-glucuronide levels appear independent of total DON concentration, but may vary by individual. The mean level of DON glucuronide was 90% of total DON.

**Conclusions**

**i. Overall the data indicate that urinary DON was sensitive enough to measure DON exposure at the individual level in the UK population.**

**ii. DON exposure was frequent, some adults in the UK were estimated to exceed the recommended TDI, with 2/35 individuals predicted to exceed the TDI on multiple days within a six day period.**

**iii. Cereal consumption and in particular bread significantly contributes to the levels of urinary DON observed in UK adults.**

**iv. Urinary DON was significantly associated with DON intake and the variation in urinary DON was well explained by DON intake.**

**v. Urinary DON is a useful biomarker with which to assess DON intake.**

**vi. DON is rapidly cleared from urine within a 24 to 48 hour period.**

## Dissemination

### Meetings

i. Turner PC. Assessment of urinary deoxynivalenol (DON) as an exposure biomarker in UK adults. European Union Commission: 6th Fusarium toxin Forum. Conference Centre Albert Borschette, Brussels, Belgium 9th – 10th February 2009.

ii. Turner PC, Rothwell JA, White KLM, Cade JE, Wild CP. Cereal intake is correlated with urinary deoxynivalenol in individuals from the UK. 10th European Nutritional Conference in Paris 10th -13th July 2007.

iii. Turner PC, White KLM, Burley V, Hopton R, Rajendram A, Fisher J, Cade JE, Wild CP. A comparison of deoxynivalenol intake and urinary deoxynivalenol in UK adults. VI Latin American Congress on Mycotoxins. City of Merida, Yucatan, Mexico June 27th - July 1st 2010.

### Manuscripts published

i. Turner PC, Rothwell JA, White KLM, Cade JE, Wild CP (2008) Urinary deoxynivalenol is correlated with cereal intake in individuals from the United Kingdom Environ. Health Persp. 116: 21-25.

ii. Turner PC, Taylor F, White KLM, Cade JE, Wild CP (2009) A comparison of 24 hour urinary deoxynivalenol with recent versus usual cereal consumption for UK adults Br. J. Nutr. 102: 1276–1279

### Manuscripts in preparation

i. Urinary deoxynivalenol is correlated with dietary intake of the mycotoxin in UK adults – suggested journal '*American Journal of Clinical Nutrition'*

ii. Assessment of DON and DON-glucuronide ratios in urine from UK adults – suggested journal '*Food and Chemical Toxicology'*

**Appendix A**

**Creating groups of high/medium/low potential fumonisin consumers from the NDNS data.**

1. Data in 'fooditem.por' were used to generate potential fumonisin intakes; data were originally from the NDNS adults 19 to 64 years (fieldwork 2000-2001) and were obtained from the FSA website. (NB. Weights of foods consumed on this database for foods were a multiplication of 10 due to an anomaly with using decimal points in the data programme. However, this was adjusted back when calculating intakes.

2. The file was initially restructured to include one line per case

3. NDNS food codes were selected as major sources of corn based on EU SCOOP report data and FSA analysis carried out in 2003 which indicated foods which may be high in fumonisins.

4. These food items were as follows:

| FDCD | FOODNAME |
|---|---|
| OTHER | |
| 8 | CORNFLOUR |
| 9 | CORNMEAL SIFTED DRY |
| 10 | CORN MEAL UNSIFTED DRIED |
| **3241** | **POLENTA HYDRATED GRILLED** |
| 3349 | POLENTA HYDRATED RAW |
| **3073** | **CORNMEAL PORRIDGE MADE WITH WATER** |
| 5611 | DUMPLINGS MADE WITH CORN MEAL FLOUR |
| 6073 | DUMPLINGS MADE WITH WHOLML FLR & CORNML |
| 8716 | GLUTEN FREE PASTA MADE WITH CORNFLOUR BO |
| 8811 | CORNFLOUR SAUCE MADE WITH DRIED MILK |
| 9055 | CORNMEAL PORRIDGE HALF MILK HALF WATER |
| CORNFLAKES & BREAKFAST CEREAL | |
| **205** | **CORN FLAKES KELLOGGS ONLY** |
| **206** | **CORNFLAKES OWN BRAND NOT KELLOGGS** |
| **227** | **FROSTIES-KELLOGGS ONLY** |
| **232** | **CRUNCHY NUT CORNFLAKES KELLOGGS & OWN BR** |
| **310** | **CHOC CRISPIE CAKES (CORNFLAKES & RICE KR** |
| 312 | COCONUT CORNFLAKE TRAYBAKE |
| **7637** | **CHEERIOS MULTI** |
| **9275** | **HONEY NUT CHEERIOS** |
| **2738** | **MULTIGRAIN (CORN RICE WHOLEWHEAT ) CRIS** |
| **3226** | **GET UP & GO OATWHEAT & CORNFLAKE CEREAL** |

**3370 CHOCOLATE FLAVOURED CORNFLAKE CEREAL SA**
**3778 WHOLEWHEAT CORN & RICE CEREAL WITH RAISI**
4289 CORNFLAKES-HIGH FIBRE EG RYVITA
**5406 CORNFLAKE CHOCOLATE AND SYRUP CAKE**
**7626 FROSTED CORNFLAKES OWN BRAND NOT KELLO**
8181 CHOCKLES CHOC CORNFLAKES & PUFFED RICE W
8615 CORNFLAKE AND SUGAR PUFF KRISPIES
9062 CHOCOLATE CORNFLAKE CRISPIE WITH LOW FAT
**9188 KELLOGS CORN POPS**

CORN SNACKS
**2268 POPCORN SALTED**
**2269 POPCORN SWEET**
**2627 CORN SNACKS EG MONSTER MUNCH WOTSITS**
**5492 REFRIED BEANS AND ONION WITH NACHOS MELT**
**6996 Nachos cheese (corn chips with melted ch**
**6038 CORNMEAL TACO SHELL BAKED & FILLED WITH**
**7876 TORTILLA CHIPS**
**3737 Maize rice flour corn snacks reduced f**
**5117 CORN SNACKS FLAVOURS WITH ARTIFICIAL SWE**
**5125 MAIZE & POTATO SNACKS WITH ARTIFICIAL SW**
**7873 CORN SNACKS WITH ADDED VITAMINS EG HAZEL**
9140 BOOTS TODDLERS MAIZE SNACKS FORTIFIED WI
**7875 CHIPSTICKS OTHER POTATO & CORN STICKS**
8601 BARDS TOFFEE POPCORN WITH ADDED VITAMINS
7879 FRAZZLES BACON RASHERS OTHER MAIZE RICE

5. Other food codes exist which will contain corn based foods but these were excluded from the calculation of intake. Excluded foods included: all sweetcorn and corn on the cob related items since fumonisin levels are relatively low in these foods. Also, sauces made with cornflour since the levels of cornflour will be low relative to other ingredients.

6. In addition, it is not completely clear which foods actually contain corn. For example, tortilla chips are made with maize however in general tortilla wraps are made with wheat flour. A number of foods were excluded on the assumption that they were not made with maize:

**3363 KFC CHICKEN AND SALAD IN TORTILLA WRAP**
**3700 CHICKEN TORTILLAS WITH SALSA CHEESE AND**
5438 BEEF MEXICAN WITH TORTILLAS PEAS AND BEA

7. On further exploration of the subject based data only those food items on the list above **in bold** were actually consumed by subjects and recorded in terms of weight of food consumed.

8. Of the list in bold above a number of food items were eaten in very low amounts by subjects and would therefore be unlikely to contribute substantially to fumonisin levels. Foods for which the *maximum amount* consumed by any subject was less than 100g (1000 on the database) total for the 7 days of intake were also excluded. These items were:

**2738 MULTIGRAIN (CORN RICE WHOLEWHEAT ) CRIS**
**3226 GET UP & GO OATWHEAT & CORNFLAKE CEREAL**
**3370 CHOCOLATE FLAVOURED CORNFLAKE CEREAL SA**
**3778 WHOLEWHEAT CORN & RICE CEREAL WITH RAISI**
**5406 CORNFLAKE CHOCOLATE AND SYRUP CAKE**
**3737 Maize rice flour corn snacks reduced f**
**5125 MAIZE & POTATO SNACKS WITH ARTIFICIAL SW**
**7873 CORN SNACKS WITH ADDED VITAMINS EG HAZEL**
**7875 CHIPSTICKS OTHER POTATO & CORN STICKS**

9. Also, only one person ate Kellogg's corn pops and no urine sample was available for this subject who was therefore not included (see criteria 11 below).

10. Foods which were combination items were assigned a % weight of corn based on information obtained from websites, packaging and McCance and Widdowson Composition of Foods tables and recipes.

11. Final list of foods included:

| Code | Description | % of corn | Source of data |
|---|---|---|---|
| 3241 | POLENTA HYDRATED GRILLED | 100 | USDA recipe |
| 3073 | CORNMEAL PORRIDGE MADE WITH WATER | 12 | MW recipe |
| 205 | CORN FLAKES KELLOGGS ONLY | 100 | (NB probably more like 95% from labels) |
| 206 | CORNFLAKES OWN BRAND NOT KELLOGGS | 100 | |
| 227 | FROSTIES-KELLOGGS ONLY | 72 | |
| 232 | CRUNCHY NUT CORNFLAKES KELLOGGS & OWN BR | 72 | Label |
| 310 | CHOC CRISPIE CAKES (CORNFLAKES & RICE KR | 18 | MW recipe |
| 7637 | CHEERIOS MULTI | 12 | Label 61% cereal, 5 types of cereal |
| 9275 | HONEY NUT CHEERIOS | 12 | |
| 3226 | GET UP & GO OATWHEAT & CORNFLAKE CEREAL | 30 | Estimate |

| | | | |
|---|---|---|---|
| 7626 | FROSTED CORNFLAKES OWN BRAND NOT KELLO | 72 | Label |
| 2268 | POPCORN SALTED | 100 | MW recipe |
| 2269 | POPCORN SWEET | 50 | Based on recipe – very variable |
| 2627 | CORN SNACKS EG MONSTER MUNCH WOTSITS | 70 | Based on website and corn chips |
| 5492 | REFRIED BEANS AND ONION WITH NACHOS MELT | 20 | Label from tortillas – estimate |
| 6996 | Nachos cheese (corn chips with melted ch | 20 | |
| 6038 | CORNMEAL TACO SHELL BAKED & FILLED WITH | 20 | |
| 7876 | TORTILLA CHIPS | 70 | Label |
| 5117 | CORN SNACKS FLAVOURS WITH ARTIFICIAL SWE | 70 | |

12. Total contribution from each of the included foods was added for each individual over the seven day period ("weight eaten") and then divided by seven to create a total 'corn' weight per day in grams. This was used to rank subjects to allow selection for urinary analysis. (In order to achieve reasonable intake levels the weight was divided by 10 – see note about database above).

13. Additional criteria for selection for urinary analysis were that a urine sample was listed as available in the database and that the urine sample was listed as collected during the period that the 7-day diary data were provided by subjects (URINEBWHE=1). It was felt that this latter criterion was important in ensuring the validity of the diary data in selecting by cereal intake.

14. Eligible subjects are divided into deciles and the highest and lowest deciles excluded as described in the original grant application to avoid outliers.

## Appendix B

**Two week study summary –** In **week one** all individuals had a first void urine collected on five consecutive days whilst on their normal diet. During this phase diary information were self recorded. Dr Burley (Nutritional Epidemiology Unit, University of Leeds) had provided guidance for diary completion. Data generated allowed us to assess the temporal variation of urinary DON with respect to normal dietary activity. In **week two** the primary question was to understand the correlation between DON intake (based on bread consumption) and the measure of urinary DON. Twenty-five individuals were assigned to GROUP 1 – Partial Intervention, in which this questioned was addressed, See Gantt Chart. The assignment of 25 individuals was based on an expectation of some incomplete sampling within the study. The remainder were assigned to GROUP 2 – Full Intervention, in which the kinetics of clearance was the main objective. Food diary information was additional self recorded in week two. Collection was completed by 7th December 2007 (a few individuals started on the 26th November 2007, rather than the 19th November). Seven individuals on the bread only component additionally volunteered to collect all urine subsequent to the first morning void, over two consecutive days during the bread collection. Thus there was the opportunity to compare the first morning measure for these individuals with the total urinary measure, within the same time frame. Finally, as this study allowed the tracking of individuals over time with respect to measuring urinary DON, it was decided to collect and bank blood samples for as many volunteers who were willing, in week one (Thursday) and week two (Thursday). These samples have been stored for later analysis[2].

## Gantt Chart

| | | WEEK ONE | | | | | | | WEEK TWO | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Sun | Mon | Tue | Wed | Thur | Fri | Sat | Sun | Mon | Tue | Wed | Thur | Fri | Sat |
| **GROUP 1** | Diet | NORMAL DIET | | | | | | | PARTIAL INTERVENTION | | | | | | |
| | Diary | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | | |
| | Urine | X | √ | √ | √ | √ | √ | X | X | √ | √ | √ | √ | √ | |
| | Food | X | X | X | X | X | X | X | X | √ | √ | √ | √ | | |
| **GROUP 2** | Diet | NORMAL DIET | | | | | | | FULL INTERVENTION | | | | | | |
| | Diary | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | √ | | |
| | Urine | X | √ | √ | √ | √ | √ | X | X | √ | √ | √ | √ | √ | |
| | Food | X | X | X | X | X | X | X | X | X | X | X | X | | |
| | | Sun | Mon | Tue | Wed | Thur | Fri | Sat | Sun | Mon | Tue | Wed | Thur | Fri | Sat |
| | | WEEK ONE | | | | | | | WEEK TWO | | | | | | |

[2] As part of non-Agency funded work

## Appendix C

## RHM Technology[3], Premier Foods: Analytical Methodology

### Performance Criteria

### Analysis

All mycotoxin analyses are carried out using validated methods which meet strict performance criteria.

### Recovery

All analyses are conducted with a spiked sample, i.e. to each sample matrix, on each day, a known amount of toxin is added prior to extraction, clean up and detection, these samples are used to assess recovery, and recoveries of 70-110% are classed as valid. If recovery is outside the limits of 70-110% analysis is repeated. Spiked samples are also used for quantification, thus making all results recovery corrected.

### Collaborative Trials

To maintain our expertise we participate in mycotoxin intercomparison studies, certification studies and collaborative trials organised by UK government and the EC as well as proficiency testing schemes (FAPAS). These schemes demonstrate the validity and accuracy of the methods we use to test mycotoxins. The fumonisins, zearalenone, aflatoxins, ochratoxin A and trichothecene methods have been collaboratively tested in the UK, EC and world wide during inter-comparison studies.

### Test Method BA-TM-03 - Trichothecenes.

The trichothecene mycotoxins (deoxynivalenol (DON), nivalenol (NIV), 3-acetyldeoxynivalenol (3AcDON), 15-acetyldeoxynivalenol (15AcDON), fusarenone X (FUSX), T2 toxin (T2), HT2 toxin (HT2), diacetoxyscirpenol (DAS), neosolaniol (NEO)) are determined by gas chromatography with mass spectrometry (GC/MS) using a UKAS accredited method.

**Method Parameters for Trichothecenes**

| | |
|---|---|
| Analytical sub sample size | 20g |
| Extraction<br>Solvent<br>Time<br>Mechanism | <br>Acetonitrile/water (84/16)<br>2hr<br>Shaking |
| Spike level | 200µg/kg |
| Clean Up | Charcoal/alumina columns |
| Derivatisation | TMS |

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| | |
|---|---|
| Determination | GC/MS |
| Recovery | 70-110% |
| LoD | 5µg/kg (each) |
| LoQ | 10µg/kg (each) |
| Proficiency testing | FAPAS |

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