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► To cite this version:

Richard P Hopton, Elizabeth Turner, Victoria J Burley, Paul C Turner, Julie Fisher. Urine metabolite analysis as a function of deoxynivalenol exposure: an NMR-based metabolomics investigation. *Food Additives and Contaminants*, 2009, 27 (02), pp.255-261. 10.1080/19440040903314015 . hal-00572620

HAL Id: hal-00572620

<https://hal.science/hal-00572620>

Submitted on 2 Mar 2011

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Urine metabolite analysis as a function of deoxynivalenol exposure: an NMR-based metabolomics investigation

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2009-195.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	06-Sep-2009
Complete List of Authors:	Hopton, Richard; University of Leeds, Chemistry Turner, Elizabeth; University of Leeds, Chemistry Burley, Victoria; University of Leeds, Leeds Institute of Genetics, Health and Therapeutics Turner, Paul; University of Leeds, Leeds Institute of Genetics, Health and Therapeutics Fisher, Julie; University of Leeds, School of Chemistry
Methods/Techniques:	Analysis - NMR, Exposure
Additives/Contaminants:	Mycotoxins - fusarium, Mycotoxins - trichothecenes
Food Types:	Beer, Cereals and grain, Bread

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4 **Urine metabolite analysis as a function of deoxynivalenol exposure: an NMR-based**
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6 **metabolomics investigation**
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Abstract

Deoxynivalenol (DON) is a toxic fungal metabolite that frequently contaminates cereal crops including wheat, maize and barley. Despite our knowledge of frequent exposure through diet our understanding of the potential consequences of human exposure remains limited; in part due to the lack of validated exposure biomarkers. In this study we interrogated the urinary metabolome using nuclear magnetic resonance (NMR) spectroscopy to compare individuals with known low and high DON exposure through consumption of their normal diet. Urine samples from 22 UK adults (male 7, female 15; age range 21-59) had previously determined urinary DON levels using an established liquid chromatography – mass spectrometry (LC-MS) assay. Urine samples were subsequently analysed using an NMR-based metabolomics approach coupled with multivariate statistical analysis. Metabolic profiling suggested that hippurate levels could be used to distinguish between groups with low (3.6ng DON/mg creatinine: 95%CI 2.6, 5.0ng/mg) and high (11.1ng/mg: 95%CI 8.1, 15.5ng/mg) DON exposure, with the concentration of hippurate being significantly (1.5 times) higher for people with high DON exposure than for those with low DON exposure ($p = 0.047$). This to our knowledge is the first report of a metabolomics derived biomarker of DON exposure in humans.

Key words: deoxynivalenol, urine, NMR, metabolic profiling, biomarker

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Introduction

Mycotoxins are frequent contaminants of cereal crops such as wheat, maize and barley that are intended for human consumption (CAST 2003). Deoxynivalenol (DON) was the most frequent *Fusarium* mycotoxin contaminant of cereals reported for European Union member countries (SCOOP 2003); with 57% of the 11,022 cereals tested being positive, and 13% at a level exceeding the guidelines established for DON (SCOOP 2003). DON is stable to much of the processing and cooking methods (Jackson & Bullerman 1999), thus DON contamination persists in the food chain (Sugita-Konishi et al. 2006), and human exposure is frequent (SCOOP 2003, Canady et al. 2001). The toxicity of DON is clearly established in animal studies, and includes gastrointestinal toxicity, immune modulation, vomiting, feed refusal and growth retardation (Pestka & Smolinski, 2005). There are differences between animal species in their susceptibility to DON, and this in part reflects the capacity of each species intestinal microflora to detoxify DON to de-epoxy DON (Yoshizawa et al. 1986); a detoxification pathway that Sundstol-Eriksen & Pettersson, (2003) have suggested human intestinal microflora may lack. A review of human epidemiological data indicates that gastroenteritis, growth faltering, an increased susceptibility to infections and immune toxicity were possible consequences of DON exposure (Pestka & Smolinski, 2005). Food poisoning incidents in China between the years of 1984 and 1991 implicate a role for DON in human toxicity (Luo. 1994). A similar incident in India involving thousands of people consuming contaminated food supports this hypothesis (Bhat et al. 1989). However, the lack of a validated exposure biomarker restricts the power of such ecological observations. One approach is to develop biological measures that are directly correlated with intake.

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4 These may take the form of measures of the parent compound or their metabolites (Lindon
5 et al. 2004) in biological fluids, including urine or serum. In swine approximately 50-60%
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7 of the ingested DON is excreted in the urine with the majority (>95%) as the parent
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9 compound (DON) or a DON glucuronide (Goyarts and Danicke, 2006), with de-epoxy
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11 DON observed with higher dosing regimens. DON is mostly cleared within 24-48 hours of
12
13 ingestion. A similar pattern of excretion in humans has been reported (Meky et al., 2003,
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15 Turner et al., 2008a), though no de-epoxy DON has been detected in UK urines to date
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17 (Turner unpublished observation). A urinary assay for DON (Meky et al. 2003) involving
18
19 immunoaffinity enrichment and LC-MS quantification was recently improved by the
20
21 inclusion of a labelled internal standard $^{13}\text{C}_{15}$ -DON (Turner et al. 2008a,b,c). An alternative
22
23 approach is to interrogate the urinary metabolome using NMR-based metabonomics (Coen
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25 et al. 2008, Connor et al. 2004, Lindon et al. 2004). This latter approach may additionally
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27 reflect a more integrated measure of the temporal variation of exposure.
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35 The current study aimed to correlate urinary DON level with one or more metabolite in the
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37 urine. Established NMR data collection and statistical analysis techniques were applied
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39 (Bollard et al. 2005, Coen et al. 2008 Lindon et al. 2004) to interrogate urine samples
40
41 previously analysed for urinary DON (Turner et al. 2008a). While these are preliminary
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43 data they do provide an interesting basis for the design of future studies.
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49 **Materials and Methods**

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51 Twenty-two urine samples were available from a small intervention trial conducted with
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53 UK adults. In brief, food diaries were kept over a two day period as described previously
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55 (Turner et al. 2008a). On day three a first morning void sample was collected. Aliquots of
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4 each sample were cryopreserved at -40°C within 6 hours of collection. Urinary DON was
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6 determined using an in-house immunoaffinity – LC-MS assay (Turner et al. 2008a). The
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8 cryopreserved samples (-40°C) were thawed, centrifuged (2000g; 15 min; 4°C), and
9
10 duplicate aliquots were taken for NMR experiments and re-frozen at -32°C prior to
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12 analysis.
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16 In preparation for the NMR studies the samples were thawed at room temperature. To 400
17
18 μl of urine, sodium phosphate buffer (200 μl pH 7.3 0.2M) was added. To 540 μl of this
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20 solution, deuterium oxide (10 μl) (Fluorochem, Old Glosop, UK) was added. A solution
21
22 containing 0.1% w/v solution of the sodium salt of 3-(trimethylsilyl)propanoic -2,2,3,3-d4
23
24 acid (TSP) (Sigma-Aldrich, Poole UK) and 1% w/v of sodium azide, in D_2O was added (50
25
26 μl). The resulting solution was vortexed before transferring all 600 μl to a 5mm NMR tube
27
28 (528PP-WILMAD, Sigma-Aldrich, Poole, UK). The TSP provided a chemical shift
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30 reference ($\delta_{\text{H}} = 0.00$ ppm).
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35 ^1H NMR spectra were acquired at 499.97 MHz on a Varian Unity Inova 500 spectrometer
36
37 at 20°C . Spectra were collected using a 1-dimensional NOESY sequence [RD- 90° - t_1 -
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39 90° - t_m - 90° – collect free induction decay]. The relaxation delay, RD, was 2.0 seconds, t_m
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41 was 100 ms and t_1 was set to 3 μs and the water signal was saturated during RD and t_m . 128
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43 scans were collected in 32,768 data points, with a spectral width of 10,000 Hz and an
44
45 acquisition time of 3.28 seconds. Spectral quality was assessed through measurement of
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47 water and TSP signals at half height and at 0.55 % peak height. An exponential line
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49 broadening of 1.0 Hz was applied to each data set, prior to zero filling to 65,536 points and
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51 Fourier transformation. Subsequently phasing and baseline correction was performed as
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53 required. Data were ‘binned’ into 224 equal portions between -0.05 – 9.47 ppm. The bins
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4 within the range 4.35 – 6.01 ppm were set to zero to remove effects of variable water
5 suppressions and urea concentrations. This was conducted using Vnmrj 1.1D software
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9 (Varian Inc., Palo Alto, California, USA). The data were normalised with respect to the
10 total area of each spectrum. This removed variation in urine concentration from sample to
11 sample (Craig et al 2006). Alternative methods for both data reduction and normalization
12 are available (Forshed et al 2003, Cloarec et al 2005), however the methods adopted here
13 remain the most popular (Gu et al, 2007, Wang et al, 2006, Smith et al 2009).
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21 Partial Least Squares Discriminant Analysis (PLS-DA) was performed on the data which
22 had been mean centred and Pareto scaled using SIMCA-P + 11 (Umetrics, Umea, Sweden).
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26 The PLS-DA model generated was validated with > 10% extra samples. Class membership
27 in the prediction set was achieved using a value of >0.5 to determine correct class
28 membership.
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33 The urine samples were dichotomised into two groups based on the urinary DON levels
34 reported by Turner et al. (2008a). The resultant model was built on 16 individuals, eight
35 with low urinary DON (LUD) and eight with high urinary DON (HUD) level; and then
36 validated with a further six urine samples, of which there were three in each category of
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DON level.

Tests of normality were performed, after which comparison of mean values of absolute
integrals for the appropriate regions of the spectra were performed between high and low
DON exposure groups, using the Mann - Whitney test and SPSS 15.0 (SPSS Inc. Chicago,
Illinois, USA). The *p* values were adjusted using the false discovery rate in the software R
2.7.0 (R Foundation for Statistical Computing, Vienna, Austria), and values of <0.05 were
determined as statistically significant.

Results

Demographic study data are provided in Tables 1 and 2. There were more females (n=11) than males (n = 5) in the model, and in the overall study, females (n =15), males (n = 7). There was no correlation between gender and the concentration of the biomarker (see below) of DON exposure (see supplementary figure). There was a significant difference in DON levels between LUD and HUD levels in each group ($p<0.001$). Regular medication was taken by seven individuals. Three women were taking oral contraceptives (two in LUD and one in HUD). Two women in the LUD group were taking hormone replacement therapy. One woman in the LUD did not describe her medication and one man in the HUD was taking non-steroidal anti-inflammatory medication.

The geometric mean level of urinary DON for the six test samples was 9.8ng DON/mg creatinine; 95%CI: 3.6, 27.1ng/mg). None of the demographic characteristics of this group were significantly different compared to those from the group used to build the model (data not shown). One woman with HUD intake was taking a thyroid medication.

“[Insert Table 1 about here]”

Typical 1D NOESY spectra from a HUD and a LUD sample are provided in Figure 1. Visually there were minimal differences between these spectra, but in any case spectra were normalised to accommodate any variation in total urine concentration (see below)..

“[Insert Figure 1 about here]”

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4 The PLS-DA scores plot (Figure 2) was built with the participants split into groups of HUD
5 and LUD. The scores plot (Figure 2) displays separation of samples on the basis of class
6 membership; LUD (open black diamond) and HUD (open black triangles). There was a
7 degree of overlap; due to the samples 18A and 21A. Food diary information revealed no
8 obvious dietary reasons for this. No samples lay outside the 95% confidence limit which
9 indicates a good model. The data were not closely clustered which may simply reflect the
10 small sample size used.
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21 “[Insert Figure 2 about here]”
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23 The separation between the LUD and HUD exposure classes was mainly in principle
24 component one (PC1) with a small amount in PC2. Through a loadings scatter plot (Figure
25 3), regions of the spectrum contributing most to the separation between the classes were
26 readily distinguished (Holmes et al. 1998). “[Insert Figure 3 about here]”. Two main areas
27 are important, the first in the region ~ 3.60 -3.90 ppm, the region where signals from
28 mannitol arise (Holmes et al. 1997). As this is a common food additive (Ji et al. 2007)
29 further correlation with this measure and urinary DON level was not investigated. In the
30 second region four signals arising from the hippurate molecule appear 7.83 - 7.55 and 3.95
31 ppm (Holmes et al. 1998, Zuppi et al. 1997). “[Insert Figure 4 about here]”. Specifically the
32 four integral regions for hippurate are contained in the bins centred around 7.84, 7.63, 7.55
33 and 3.96 ppm, in good agreement with literature values, and the integral values of these are
34 statistically significant ($p = 0.047, 0.047, 0.047$ and 0.030 respectively). These higher
35 hippurate levels in urine coincide with higher urinary DON levels. The median values for
36 the binned integrals were 0.0109 and 0.0170 for LUD and HUD respectively indicating a
37 1.5 fold increase in hippurate levels in samples found to have high levels of urinary DON .
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4 Further validation of the PLS-DA model used the remaining six samples (which had been
5 selected at random) (Table 3). The PLS-DA model (Figure 5) predicts all samples correctly
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7 in their classes. “[Insert Table 2 about here]” “[Insert Figure 5 about here]”
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11 12 13 14 **Discussion**

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16 This study compared urinary DON with its urinary metabolic profile in UK adult samples.
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18 A ¹H NMR-based chemometrics approach; metabolomics, was utilised to examine samples
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20 from individuals eating their normal diet. This methodological approach has gained
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22 popularity as a rapid and non-invasive method for biomarker detection (Lindon et al. 2000,
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24 Lindon et al. 2004, Bollard et al. 2005). The statistical analysis undertaken here has
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26 identified two possible biomarkers; hippurate and mannitol. Mannitol is used in processed
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28 foods, such as candies, cookies and chewing gum as an alternative to sucrose (sugar) as it
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30 has little effect on blood sugar levels (Ji et al. 2007). Mannitol is also used in clinical
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32 applications (Wakai et al. 2007). Thus while it was identified in this study there is no
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34 strong mechanistic basis for considering this measure further. By contrast hippurate is a
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36 more interesting candidate. Hippurate is present in urine samples due to both renal and
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38 hepatic synthesis, through conjugation of glycine and benzoic acid (Zuppi et al. 1997). It
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40 can also be formed through the degradation of shikimic acid, by intestinal microorganisms
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42 (Brewster et al. 1978). The conjugation of glycine with benzoic acid to form hippurate has
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44 been found to be an important mechanism for elimination and detoxification of aromatic
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46 carboxylic acids (Kasuya et al. 1996). Both dietary factors and microbial action can
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48 influence urinary hippurate (Lord et al. 2008). The food diary information does not suggest
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50 higher benzoate intake and therefore this is not the reason for the variation in hippurate
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4 concentration. Gut microflora activity in rats (Phipps et al., 1988) and in children (Spaapen
5 et al., 1987) were correlated with urinary hippurate levels; and DON was recently shown to
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7 modify the gut microflora in dosed swine (Wache et al. 2009). To date no studies have
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9 interrogated the metabolome of DON exposed animals and it would be interesting to
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11 further examine this putative interaction in such a model.
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16 The statistical model built was based on a small number of participants however
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18 appropriate tests of significance have been performed; the model produced no samples
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20 outside the 95% confidence limit and all the samples in the prediction set were >0.5, which
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22 is acceptable for metabolomics data. The variation in DON levels in samples used to build
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24 this model was relatively small, (mean 8.2ng/mg; range 2.4-27.1ng/mg). However, we can
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26 make rough estimates of mean intake for each of these groups. If we assume an average
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28 volume of urine per day to be 1500ml and an approximate 50% transfer of DON to urine
29
30 we can make a rough estimate of the daily intake per kg body weight. The low group would
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32 have an intake of 150ng/kg bw/day (95%CI: 110, 220), whilst high group would have an
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34 intake of 500ng/kg bw/day (95%CI: 350, 670). The lower intake is roughly in line with
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36 individuals consuming the average quantity of wheat based food items in the UK at the
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38 average level of contamination, whilst the high intake group are consuming far greater
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40 quantities of DON contaminated foods, though for both groups the intake remains within
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42 estimates predicted by SCOOP (2003).
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50 Future studies should incorporate larger numbers and involve a wider range of DON
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52 exposure. An assessment of the temporal nature of this measure during consumption of
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54 both a normal diet and during dietary intervention to restrict DON exposure would be
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56 valuable. This study highlights an NMR metabolomics approach which hopefully will
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4 enhance the epidemiological investigations of the potential effects of DON consumption by
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6 providing more rigorous exposure assessment data.
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10 11 **Acknowledgements**

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14 We thank the White Rose Doctoral Training Centre, UK (RPH).
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Figure captions

Figure 1. Low field region of the ^1H -NMR spectrum of a urine sample taken from a volunteer found to have (top spectrum) a low urinary DON (LUD) level and (bottom spectrum) a high urinary DON (HUD) level.

Figure 2. PLS-DA scores plot generated from ^1H NMR data from urine samples taken from volunteers found to have high levels of urinary DON (HUD) (open triangles) and low urinary DON (LUD) (open diamond) exposure. There is overlap between the classes at sample 18A and 21A – food diaries did not reveal an explanation for this.

Figure 3. Loadings plot resulting from the PLS-DA analysis (figure 2) indicating the regions (the numbers in the rectangle and the ellipse are chemical shift values) of the NMR spectrum responsible for discrimination between the two (urinary DON exposure) classes. This 2-dimensional plot illustrates that discrimination is across both principal component 1 (P[1]) and 2 (P[2]).

Figure 4. Expansion of the 1-dimensional loadings plot (P[1]), highlighting signals in the NMR spectrum arising from hippurate which are responsible for discrimination between the two (DON level exposure) classes..

Figure 5. Scores plot indicating that the six samples used to validate the PLS-DA model are correctly classified. The numbers shown on the plot are for cross-referencing to specific volunteers. The open triangles indicate the high urinary DON (HUD) exposure class, the diamonds indicate the low urinary DON (LUD) exposure class.

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Figure 1

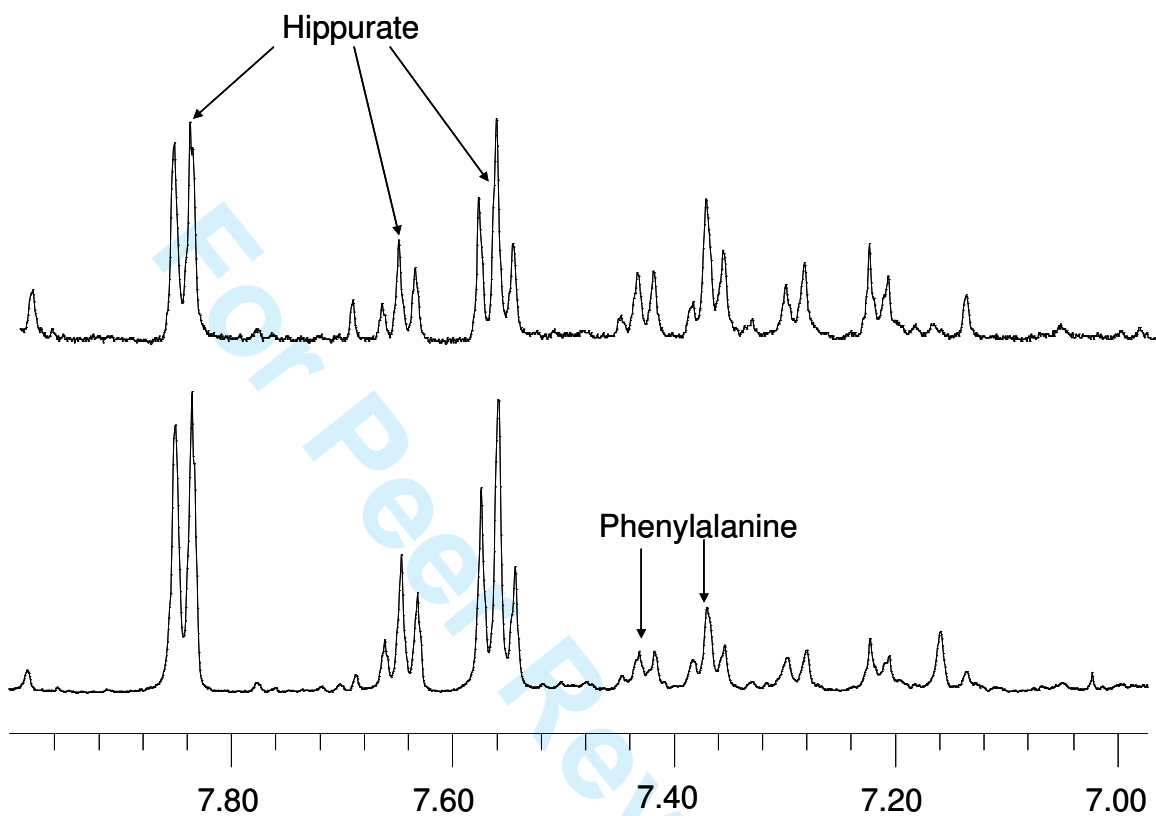
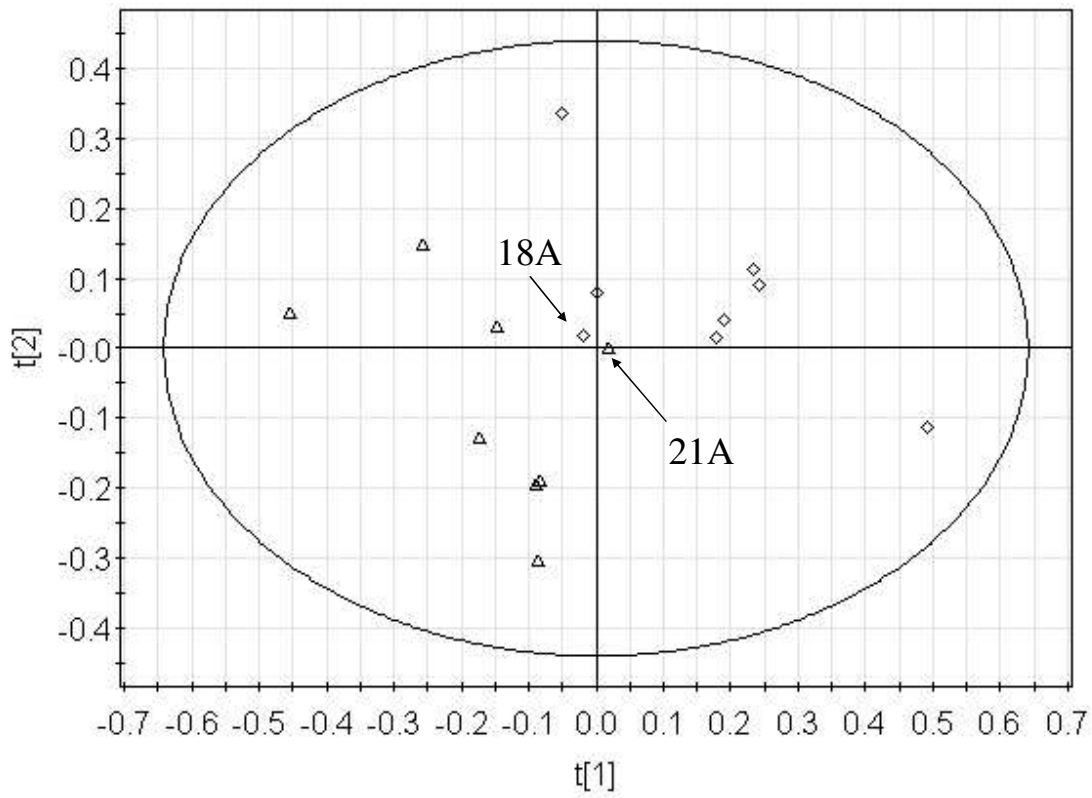
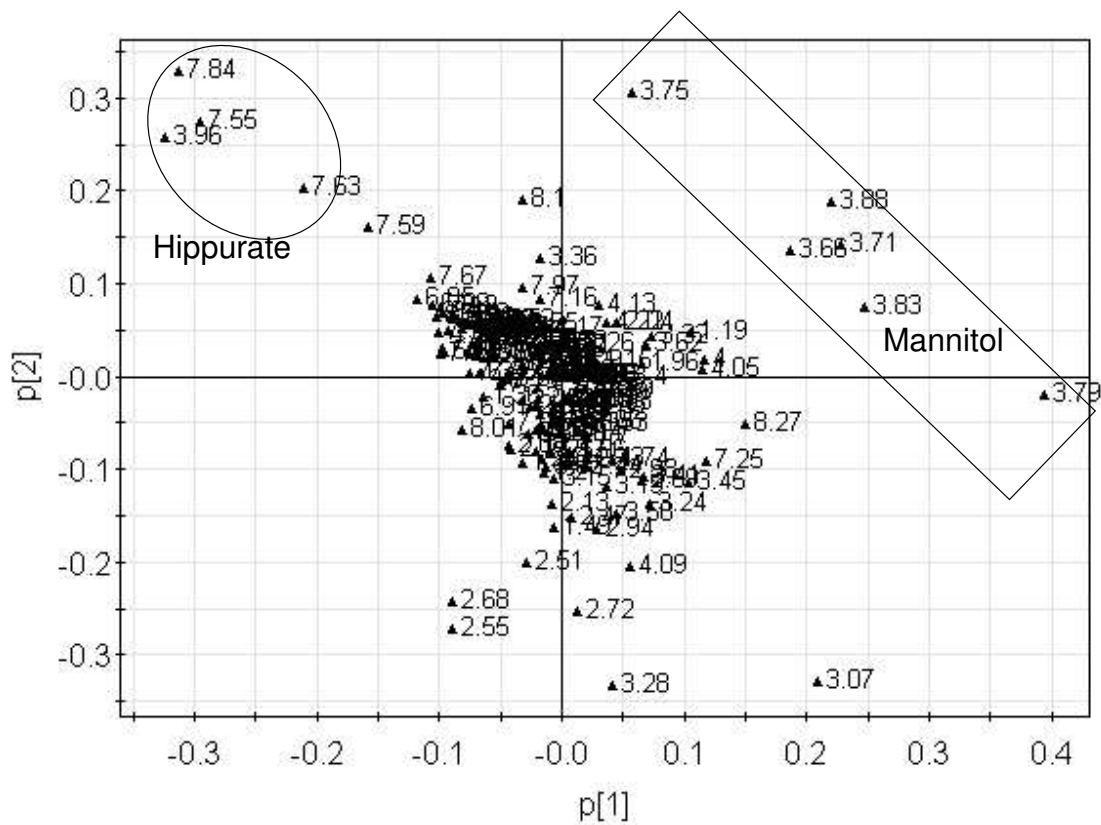


Figure 2



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Figure 3



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Figure 4

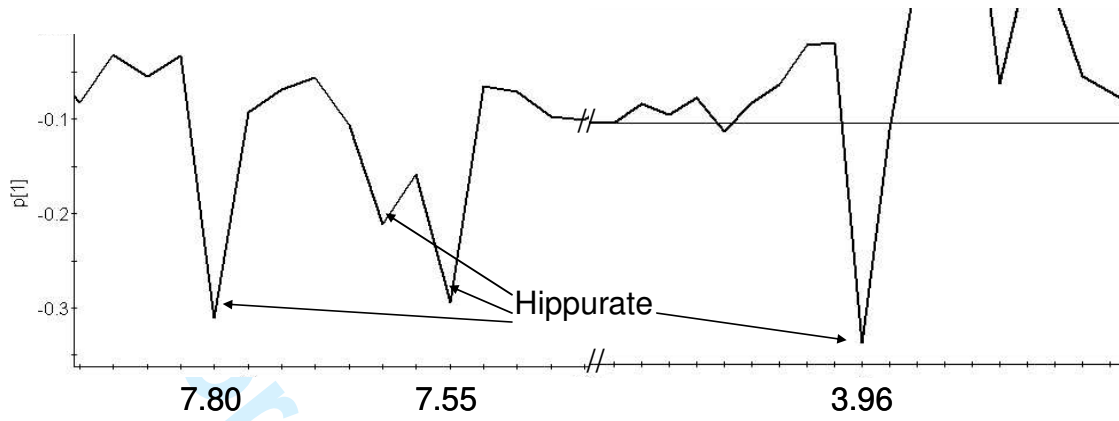
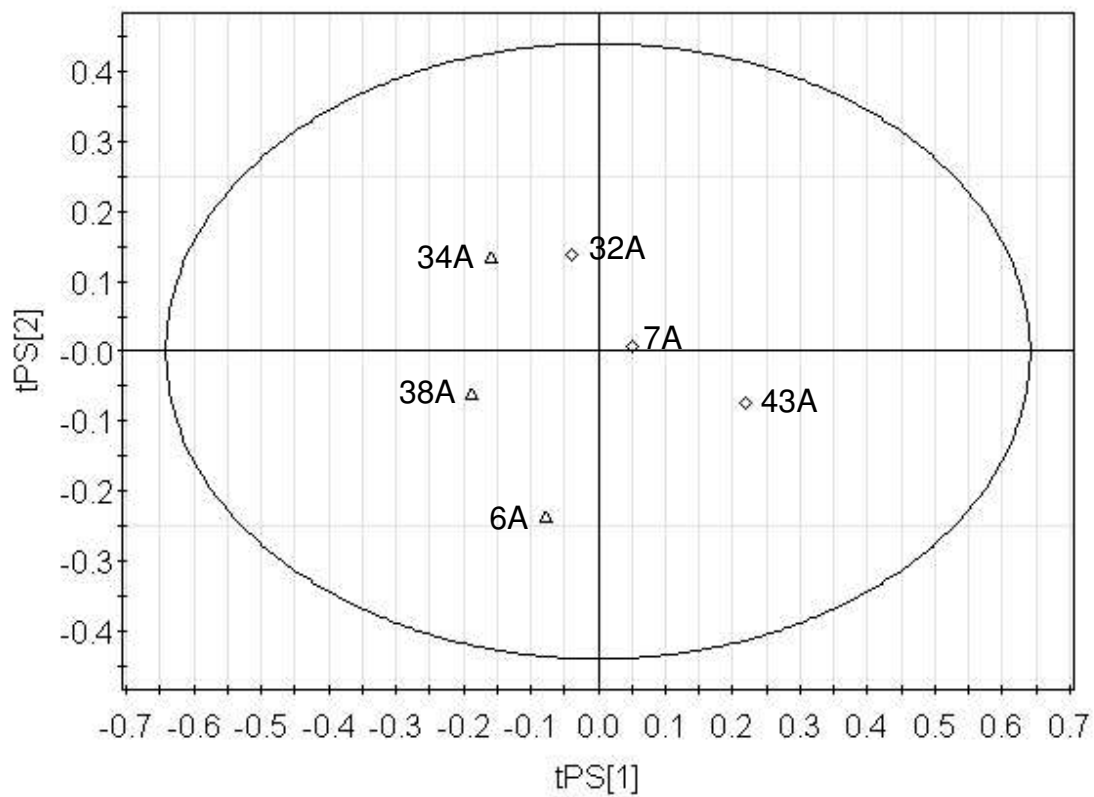


Figure 5



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Table 1. Demographics of participants for PLS-DA model building

Characteristic	Total (n=16) Mean (range)	LUD* (n=8) Mean (95% CI)	HUD* (n=8) Mean (95% CI)	p-value**
Urinary DON [#]	8.2 (2.4 – 27.1) ^a	3.6 (2.6, 5.0) ^b	11.1 (8.1, 15.5) ^b	<0.001
Sex	5m/11f	4m/4f	1m/7f	0.120
Ethnicity – delete as all white Caucasian – refer to this in text	16wC	8wC	8wC	n/a
Age	39 (21 – 59)	34 (26, 42)	43 (35, 50)	0.10
Height / m	1.69 (1.52 – 1.96)	1.67 (1.59, 1.75)	1.71 (1.63, 1.79)	0.47
Weight / kg	69 (51 – 93)	69 (60, 77)	69 (61, 78)	0.98
BMI / kg/m ²	24.2 (19.2 – 32.0)	25.0 (22.6, 27.4)	23.4 (21.0, 25.8)	0.34

* Low urinary DON (LUD), high urinary DON (HUD)

** T-test comparison of LUD and HUD groups

Urinary DON ng/mg creatinine (data extracted from Turner et al., 2008a)

a - Arithmetic mean and range

b - Geometric mean and 95%CI

95% CI – 95% confidence interval

m – male, f – female

wC – white Caucasian

n/a – not applicable

BMI – body mass index

Table 2. Demographics of participants in test group

Characteristic	Total (n=6) Mean (range)
Urinary DON [#]	9.8 (3.6 – 27.1) ^a
Sex	1m/5f
Ethnicity	6wC
Age	36 (24 – 57)
Height / m	1.66 (1.57 – 1.78)
Weight / kg	64 (55 – 88)
BMI / kg/m ²	23.1 (20.2 – 31.2)

Urinary DON ng/mg creatinine (data extracted from Turner et al., 2008a)

a - Geometric mean and 95%CI

m – male, f – female

wC – white Caucasian

BMI – body mass index

Table 3. Additional samples used to test PLS-DA model. Values > 0.50 within class

Sample code	Class 1 ^a	Class 2 ^b
38A HUD*	0.09	0.91
34A HUD	0.46	0.54
6A HUD	0.00	1.00
7A LUD**	0.59	0.41
32A LUD	0.66	0.34
43A LUD	0.74	0.26

* High urinary DON (HUD),

** Low Urinary DON (LUD)

a Low urinary DON classification

b High urinary DON classification

Supplementary

Figure 1

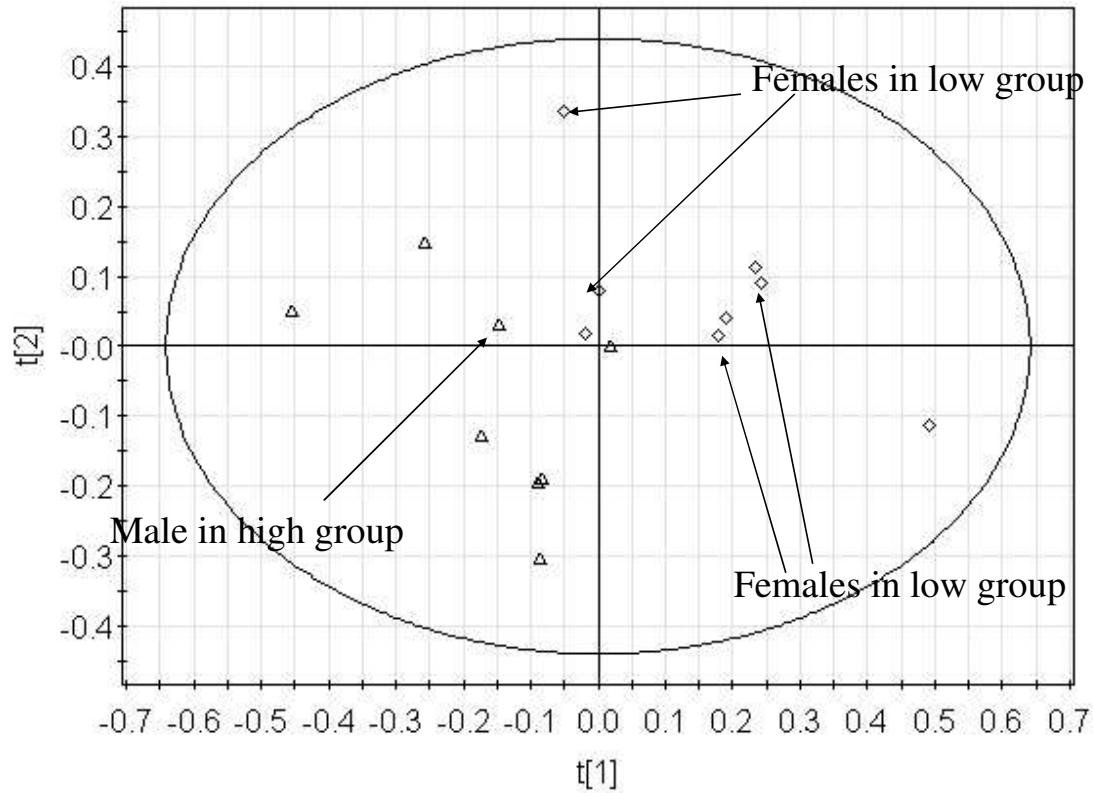


Figure Caption

Figure 1 Original model indicating gender is not the cause of discrimination between the samples. Axis font times new roman size 14. Text times new roman size 24.

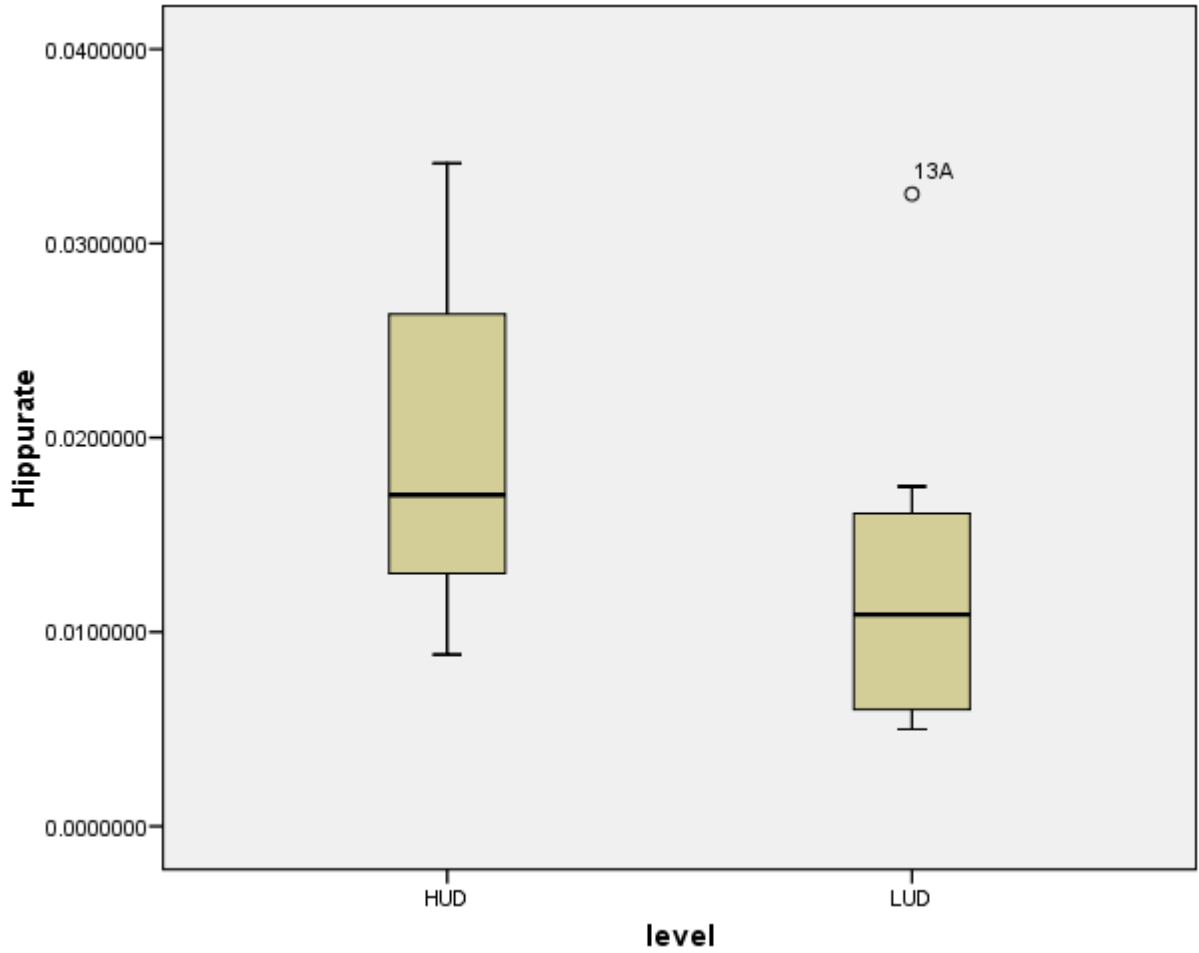


Figure 2 Variation in integrals for hippurate in HUD and LUD samples.

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