Food and Chemical Toxicology xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Food and Chemical Toxicology

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

Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia

Bojan Šarkanj [a,b,1], Benedikt Warth [b,1], Silvio Uhlig [c], Wilfred A. Abia [b,d,e], Michael Sulyok [b,*], Tomislav Klapec [a], Rudolf Krska [b], Ines Banjari [f]

[a] Subdepartment of Biochemistry and Toxicology, Department of Applied Chemistry and Ecology, Faculty of Food Technology, Josip Juraj Strossmayer University, Osijek, Croatia
[b] Center for Analytical Chemistry, Department for Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna (BOKU), Austria
[c] Section for Chemistry and Toxicology, Norwegian Veterinary Institute, Oslo, Norway
[d] Laboratory of Pharmacology and Toxicology, Department of Biochemistry, Faculty of Science, University of Yaounde I, Cameroon
[e] Department of Food Technology, Faculty of Science, University of Johannesburg, 2028, Doornfontein Campus, P.O. Box 17011, Gauteng, South Africa
[f] Subdepartment of Nutrition, Department of Food and Nutrition Research, Faculty of Food Technology, Josip Juraj Strossmayer University, Osijek, Croatia

ARTICLE INFO

Article history:
Received 24 May 2013
Accepted 20 August 2013
Available online xxxx

Keywords:
Mycotoxins
Deoxynivalenol glucuronide
Biomarker
Pregnant women
Human urine
Croatia

ABSTRACT

In this pilot survey the levels of various mycotoxin biomarkers were determined in third trimester pregnant women from eastern Croatia. First void urine samples were collected and analysed using a "dilute and shoot" LC–ESI–MS/MS multi biomarker method. Deoxynivalenol (DON) and its metabolites: deoxynivalenol-15-glucuronide and deoxynivalenol-3-glucuronide were detected in 97.5% of the studied samples, partly at exceptionally high levels, while ochratoxin A was found in 10% of the samples. DON exposure was primarily reflected by the presence of deoxynivalenol-15-glucuronide with a mean concentration of 120 μg L^{-1}, while free DON was detected with a mean concentration of 18.3 μg L^{-1}. Several highly contaminated urine samples contained a third DON conjugate, tentatively identified as deoxynivalenol-7-glucuronide by MS/MS scans. The levels of urinary DON and its metabolites measured in this study are the highest ever reported, and 48% of subjects were estimated to exceed the provisional maximum tolerable daily intake (1 μg kg^{-1} b.w.).

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Mycotoxins are secondary metabolites produced by toxigenic fungi that commonly contaminate agricultural products worldwide. Based on frequency of occurrence and toxic effects exerted on animals, aflatoxins (AFs), ochratoxins (OTs), deoxynivalenol (DON), zearalenone (ZEN) and fumonisins (FBs) are the most relevant mycotoxins worldwide. Once humans are exposed to mycotoxins via contaminated foods, singly and or in combinations, these toxins might pose multiple threats to human health such as teratogenicity, immunosuppression and carcinogenicity (Binder et al., 2007).

The trichothecene DON is the most frequently encountered mycotoxin addressed by regulation in Europe (Binder et al., 2007). Due to its stability during processing (Jackson and Bullerman, 1999), a high level of exposure in humans is expected. Krstanović et al. (2005) determined a high contamination of barley with DON-producing *Fusarium* species in Croatia, whilst Pleadin et al. (2012a) confirmed high DON contamination of Croatian maize samples harvested in 2010., 85% of the investigated samples ($n = 40$) were DON contaminated with an average concentration of 2150 μg kg^{-1} (range 15–17,290 μg kg^{-1}). Contamination of maize from the same harvest with T-2 toxin (T-2) and FB's has also been reported (Pleadin et al., 2012b). Of the analysed samples, 67.4% were contaminated with FBs with an average concentration of 4509 μg kg^{-1} and a maximum concentration of 25200 μg kg^{-1}; 24.4% of the samples were contaminated with T-2 with average and maximum levels of 110 μg kg^{-1} and 210 μg kg^{-1}, respectively. Since high levels of mycotoxins were detected in cereals, it is expected that the potential exposure of humans is reflected by the presence of appropriate urinary biomarkers. There is limited information on DON exposure and metabolism during pregnancy in humans. Piekkola et al. (2012) reported the co-occurrence of DON and aflatoxin M_1 (AFM_1) in urine form pregnant women in Egypt in 18.3% of the analysed samples. The range of reported DON in Egypt was 0.5–59.9 ng mg^{-1} creatinine, while Hepworth et al. (2011) reported urinary DON concentrations in pregnant individuals from Bradford, UK, with a mean urinary concentration of 10 ng mg^{-1} creatinine and a maximum concentration of 117 ng mg^{-1} creatinine (Turner et al., 2012a).

* Corresponding author. Address: Center for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna, Konrad Lorenzstr. 20, A-3430 Tulln, Austria. Tel.: +43 2272 66280 409.
E-mail address: michael.sulyok@boku.ac.at (M. Sulyok).
[1] These authors contributed equally to this work.

0278-6915/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.fct.2013.08.043

Please cite this article in press as: Šarkanj, B., et al. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. Food Chem. Toxicol. (2013), http://dx.doi.org/10.1016/j.fct.2013.08.043

UNCORRECTED PROOF

DON has been shown to pass the placental barrier in sows, and it was linked with lower birth weight (Tiemann et al., 2008) and immunosuppression (Jakovac-Strajn et al., 2009). Due to its ability to cross the placental barrier during *in vivo* studies in animals (Goyarts et al., 2007; Tiemann et al., 2008), and *ex vivo* studies in humans (Nielsen et al., 2011), DON might cause toxicity in the foetus. Time periods of high cereal contamination with DON were associated with induced labour at an early stage of pregnancy (Pestka and Smolinski, 2005). Therefore, DON is assumed to constitute a hazard to both pregnant women and their foetus, especially since the foetus has a less developed detoxification capacities. However, it is not known how the observed and other anticipated changes in metabolism, distribution and excretion of toxicants during pregnancy affect the potential risk (Anderson, 2005). A major detoxification route for DON in animals and humans is via glucuronidation (Smolinski and Pestka, 2005; Maul et al., 2012; Meky et al., 2003; Turner et al., 2011, 2012a; Warth et al., 2011, 2012a, 2013a), though the specific uridine-diphosphate glucuronosyltransferases (UGTs) for DON have not as yet been identified. It has been proposed that during the last two trimesters of human pregnancy at least one specific UGT, UGT1A4, activity is increased. However, in rats glucuronidation was reduced for some substrates (Inoue et al., 2005) but alterations to efflux transporter expression resulting in increased urinary excretion of glucuronides (Cao et al., 2002).

A DON glucuronide was first suggested by Meky et al. (2003) and Turner et al. (2008), and subsequently both DON-3-GlcA and DON-15-GlcA have been identified and characterised in human urine and using human liver microsomes (Maul et al., 2012; Warth et al., 2012a). The structure of a suggested third species, DON-7-GlcA, in highly contaminated human urine samples (Warth et al., 2013a) and from microsomal assays (Maul et al., 2012), awaits further confirmation; whilst the structural elucidation of three DON glucuronides formed using rat liver microsomes included the DON-3-GlcA, DON-15-GlcA, and a novel DON-8-GlcA (Uhlig et al., 2013). Klapec et al. (2012) determined OTA and OTα in the first void urine samples of Croatian pregnant women, and according to food frequency questionnaire data, the greatest contributors to dietary OTA intake were cereal products and fruit juices. Samples from that survey were re-examined in the study at hand to assess multiple mycotoxin exposures using a newly developed multi-biomarker LC–MS/MS method (Warth et al. 2012b).

In humans, many mycotoxins and their metabolites are effectively excreted via the urine which enables the estimation of exposure through urinary concentrations (Solfrizzo et al., 2011; Warth et al., 2013b) provided that a dose–response relationship has been established. Following easy, non-invasive sampling, urine analysis requires sensitive methodology due to low levels. Considering the limited data on mycotoxin levels in pregnancy (Hepworth et al., 2011; Klapec et al., 2012; Piekkola et al., 2012), the aim of this pilot study was to investigate mycotoxin exposure in 40 healthy third trimester pregnant women from eastern Croatia. Special emphasis was given to the tentative identification of a recently discovered third DON glucuronide.

2. Materials and methods

2.1. Chemicals and reagents

Methanol (LC gradient grade) and glacial acetic acid (p.a.) were purchased from Merck (Darmstadt, Germany), acetonitrile (ACN; LC gradient grade) from VWR (Leuven, Belgium). Creatinine was from Sigma (Schnelldorf, Germany). Deoxynivalenol-3-*O*-glucuronide (DON-3-GlcA) and zearalenone-14-*O*-glucuronide (ZEN-14-GlcA) were synthesised by optimised procedures and the structures were confirmed by nuclear magnetic resonance (Fruhmann et al., 2012; Mikula et al., 2012). Deoxynivalenol-15-*O*-glucuronide (DON-15-GlcA) was separated from a naturally contaminated human urine sample to determine its MS response relative to that of DON-3-GlcA as described elsewhere (Warth et al., 2012a). Other mycotoxin standards were purchased from Romerlabs (Tulln, Austria) (DON, de-epoxy-deoxynivalenol (DOM-1), nivalenol (NIV), T-2, HT-2, OTA, AFM_1, FB_1 and FB_2) and Sigma (ZEN, α- and β-zearalenol (α- and β-ZEL)). Solid standards were dissolved in pure methanol (DON-3-GlcA, NIV) or ACN (DON, ZEN-14-GlcA, ZEN, α- and β-ZEL). Pre-dissolved standards were delivered in ACN or ACN/H_2O (FB_1 and FB_2) and stored at −20 °C. A combined multi-standard working solution containing 10.0 mg L^{-1} DON, DON-3-GlcA, DOM-1, NIV and HT-2, 5.0 mg L^{-1} FB_1 and FB_2, 2.5 mg L^{-1} ZEN-14-GlcA, α-ZEL, β-ZEL and T-2, 1.0 mg L^{-1} ZEN and 0.125 mg L^{-1} AFM_1 and OTA was prepared in ACN according to Warth et al. (2012b).

2.2. Participants and sample collection

During February 2011, 40 healthy non-smoking pregnant women who all reside in the eastern area of Croatia (from and around the city of Osijek; age: 26–33 years old), all in their final trimester of gestation, voluntarily participated in this study. Detailed description of the study design has been published before (Klapec et al., 2012) in a work focusing on urinary OTA and OTα. In order to expand the knowledge on the simultaneous exposure with other mycotoxins, especially to DON, which was assumed to be the main contaminant in Croatian cereals, additional analyses were performed at the Centre for Analytical Chemistry, (BOKU, Austria). The samples were kept at −20 °C and later transported in frozen conditions to Austria.

2.3. Multi-mycotoxin biomarker analysis and LC–ESI–MS/MS parameters

2.3.1. Sample preparation

Urine samples were allowed to reach ambient temperature. Each urine sample was thoroughly mixed, 1 mL transferred into an Eppendorf tube and centrifuged for 3 min at 5600*g*. An aliquot of the supernatant (100 μL) was mixed with 900 μL of dilution solvent (ACN/H_2O = 10/90). Five microlitres (5 μL) of the diluted sample were injected into the LC–ESI–MS/MS system.

2.3.1. Analysis of urine samples & LC–ESI–MS/MS conditions

Sample analysis was performed using an AB SciexQTrap® 5500 LC-MS/MS system (Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source interfaced with an Agilent 1290 series HPLC system (Waldbronn, Germany). The optimised dilute and shoot approach described by Warth et al. (2012b) was applied for measurements of urinary biomarkers. This method was optimised to monitor high and moderate exposures to major mycotoxins rather than to detect very low background levels. Key performance parameters of detected metabolites are displayed in Table 1. ESI–MS/MS was conducted using selected reaction monitoring (SRM), and two individual transitions were monitored for each analyte with the exception of DON-GlcA, where an additional fragment was chosen. The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the spiked urine samples using the Analyst software script based on a signal to noise ratio of 3:1 and 10:1, respectively. Two quality control (QC) samples (pooled blank urine and blank urine spiked with multi standard solution diluted 1:200) were included in each batch of 20 samples within an LC–MS/MS measurement sequence.

For recalculation of DON and DON-GlcA results to %TDI (percentage of stated provisional maximum tolerable daily intake of

UNCORRECTED PROOF

Table 1
Key performance parameters of the LC–MS/MS–ESI method applied for urine analysis (Warth et al., 2012b).

	LOD matrix [μg L^{-1}][a]	LOQ matrix [μg L^{-1}][b]
DON	4	13
D3GlcA	6	20
D15GlcA	3	11
OTA	0.05	0.17

[a] LOD based on a *S*/*N* ratio of 3:1 in spiked urine sample. Values correspond to concentration in urine, taking the 1:10 dilution into account.
[b] LOQ based on a *S*/*N* ratio of 10:1 in spiked urine sample. Values correspond to concentration in urine, taking the 1:10 dilution into account.

1 μg kg^{-1} body weight (WHO, 2001)) the following equations were used:

$$\%\mathrm{TDI} = \frac{\left(\frac{\text{DON-15-GlcAMW(DON)}}{\text{MW(DON-15-GlcA)}} + \frac{\text{DON-3-GlcAMW(DON)}}{\text{MW(DON-3-GlcA)}} + \text{DON}\right) V(\text{urine per day})}{\text{ERBWCF}} \cdot 100 \quad \text{(A1)}$$

$$\%\mathrm{TDI} = \frac{\left(\frac{\text{DON-15-GlcA296}}{472} + \frac{\text{DON-3-GlcA296}}{472} + \text{DON}\right)2}{0.72\text{BW2}} \cdot 100 \quad \text{(B1)}$$

$$\%\mathrm{TDI} = \frac{(\text{DON-15-GlcA} + \text{DON-3-GlcA})0.627 + \text{DON}}{\text{BW}} \cdot 13,889 \quad \text{(C1)}$$

where DON-15-GlcA is the concentration of deoxynivalenol-15-glucuronide (μg L^{-1}); DON-3-GlcA the concentration of deoxynivalenol-3-glucuronide (μg L^{-1}); MW (DON) the molecular weight (MW) of deoxynivalenol (DON) = 296 (g mol^{-1}); DON the concentration of deoxynivalenol (μg L^{-1}); V (urine per day) the urinary output during pregnancy 2 L (Mikhail and Anyaegbunam, 1995); ER the urinary DON elimination rate (ER) of 72% (Turner et al., 2010); BW the body weight; CF is the concentration factor, first void urine is typically more concentrated than 24 h urine; in a recent pilot survey this factor was calculated to be approximately two (Warth et al., 2013a)

It is important to mention that the urine volume and the concentration factor are realistic estimates rather than validated values, due to the lack of available validated data on DON metabolism and concentration factor in pregnant women. The urinary DON elimination rate determined by Turner et al. (2010) must likewise be regarded as the best available average value.

2.4. Creatinine analysis

Urinary creatinine levels were determined on the same LC–ESI–MS/MS instrument by the rapid method described by Warth et al. (2012b). Urinary mycotoxin concentrations were later normalised for creatinine and expressed as μg g^{-1} creatinine alongside the concentrations in μg L^{-1} for ease of comparison with similar studies.

2.5. Analysis of DON in wheat samples

Two wheat samples from the 2010 season, thought to be the best representative available for the mycotoxin contamination of that specific year and region, were obtained from local siloes (nearby Osijek). The samples were extracted and analysed by a LC–ESI–MS/MS method described previously by Sulyok et al. (2006) in order to confirm contamination with DON and its derivatives (deoxynivalenol-3-glucoside (DON-3-Glc), 3- and 15-acetyldeoxynivalenol (3-ADON, and 15-ADON). Briefly, samples were grind, and mycotoxins were extracted from 5 g of homogenised sample with 20 mL of extraction solvent (acetonitrile:water:acetic acid = 79:20:1) for 90 min on a rotary shaker (180 rpm). Afterwards 0.5 mL of extract was diluted with 0.5 mL of dilution solvent (acetonitrile:water:acetic acid = 20:79:1), and 5 μL of the diluted sample was injected into the LC–ESI–MS/MS system.

2.6. Statistical analysis

Differences between subgroups were tested with pairwise Mann–Whitney U-test, and a p value $\leqslant 0.05$ was considered statistically significant. The average and median urinary concentration of DON and its metabolites (Table 2) was recalculated by replacing all <LOD results with LOD × 0.5, and all <LOQ values with LOQ × 0.5 (Turner et al., 2012b; Warth et al., 2012a). Statistical analysis was performed using Statistica 8.0 (StaSoft, Tusla, OK, USA) and Microsoft Office Excel 2013 (Microsoft, Redmond, WA, USA).

3. Results and discussion

The urine samples were analysed for a total of 15 mycotoxin-derived biomarkers. Table 1 provides performance characteristics of detected mycotoxin biomarkers with apparent recoveries ranging from 88% to 104%; and LODs ranging from 0.05 to 6 μg L^{-1}. A total of 39/40 (97.5%) samples contained DON (and/or its metabolites), and a much smaller fraction of samples contained detectable OTA concentrations (4/40; 10%). Other mycotoxin biomarkers were not detected (Fig. 1). The largest median concentration was determined for DON-15-GlcA which was about five times higher than DON-3-GlcA and eight times higher than free DON levels (Table 2). The total DON exposure frequency estimated in this study (>95%) was in line with the recent findings in Austria reported by Warth et al. (2012a); and a United Kingdom study, in both pregnant (Hepworth et al., 2011) and nonpregnat individuals (Turner, 2008). The observed high frequency of DON-15-GlcA:DON ratio relative to DON-3-GlcA:DON ratio confirms that DON-15-GlcA is the dominant conjugated metabolite of DON. Since many samples contained very high quantities of DON and its conjugates, a second dilution step (1:20) was carried out on the three most contaminated samples besides the common dilution of 1:10. The obtained results confirmed the high concentrations with low average RSD values of 3.0% for DON-3-GlcA and 2.8% for DON-15-GlcA.

For the estimation of daily DON exposure it was assumed that first void urine is more concentrated (by a factor of two) than 24 h urine based on a current *in vivo* mass balance experiment (Warth et al., 2013a). However, this assumption must be critically considered as it is based on a single experiment on one male individual and is only relevant when biomarker concentrations are not normalised to creatinine. As far as OTA is concerned, the detected concentrations for all positive samples were below the LOQ. Co-exposure to DON and OTA was evident in 4/40 (10%) of the samples. Solfrizzo et al. (2011) reported a higher co-occurrence of DON and its metabolites with OTA and found both mycotoxins in 30% of the samples in a small-scale pilot study from southern Italy (3/10 samples) using a more sensitive method. Forty-eight% of the recovered levels of total DON exceeded the provisional maximum tolerable daily intake (PMTDI) levels (1 μg kg^{-1} b.w. day^{-1}) (WHO, 2001), a proportion that is exceptionally high and exceeds all studies performed on different continents so far (Hepworth et al., 2011; Piekkola et al., 2012; Turner, 2010; Turner et al., 2012a,b; Warth et al., 2012a).

Participants were divided into 2 homogenous subgroups to investigate effect of residence and education on the sum of total

UNCORRECTED PROOF

Table 2
Mycotoxin levels in urine samples of pregnant women from Croatia ($N = 40$).

	Mycotoxin [μgL^{-1}]			
	DON-15-GlcA[a]	DON-3-GlcA[a]	DON[a]	OTA
Average	120.4	28.8	18.3	<LOQ
Median	55.2	10.0	6.7	<LOQ
Max	1237.7[b]	298.1	275.0	<LOQ
<LOD	1	7	9	36
<LOQ	4	18	21	4
Number of positive (%)	39/40 (98%)	33/40 (83%)	31/40 (76%)	4/40 (10%)

[a] Recalculated, all values < LOD were replaced with 0.5 × LOD; and <LOQ with 0.5 × LOQ.
[b] Samples above highest calibration point (400 μg L^{-1}) were diluted and remeasured.

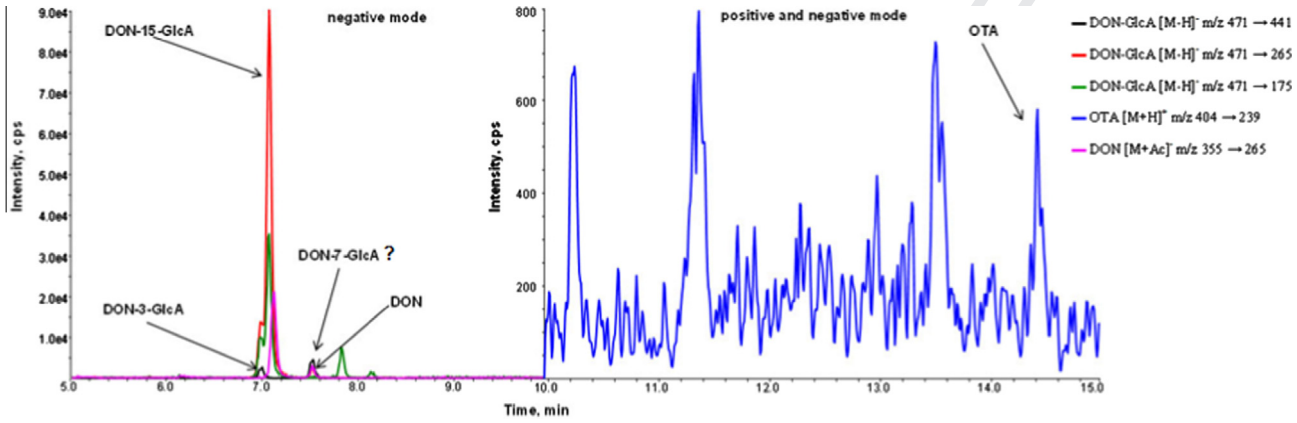

Fig. 1. Chromatogram from selected reaction monitoring of DON (275.0 μg L^{-1}), DON-3-GlcA (298.1 μg L^{-1}), DON-15-GlcA (1237.7 μg L^{-1}) and OTA (<LOQ) in a naturally contaminated urine sample obtained from a Croatian pregnant woman. Peaks without label are interferences (matrix peaks).

Table 3
Total DON equivalents and percentage of PMTDI in urine samples of pregnant women from Croatia.

			∑DON equivalents [μg L^{-1} urine]	∑DON equivalents [μg g^{-1} creatinine]	∑DON equivalents [μg kg^{-1} b.w. day^{-1}]	% TDI
Residence	Urban ($N = 20$)	Average	92.4	84.7	1.8	183
		Median	55.3	52.0	1.2	123
		IQR	67.7	107.0	1.8	177
		Min	4.8	6.7	0.1	9
		Max	401.6	237.2	7.7	775
	Rural ($N = 20$)	Average	131.3	102.7	3.1	309
		Median	42.5	39.6	0.8	77
		IQR	48.1	48.7	1.3	129
		Min	7.0	9.9	0.1	14
		Max	1238.1	903.7	33.1	3307
Education	University ($N = 18$)	Average	85.3	84.0	1.8	176
		Median	55.3	63.5	1.2	120
		IQR	86.6	114.3	1.6	155
		Min	4.8	6.7	0.1	9
		Max	401.6	236.3	7.7	775
	High school ($N = 22$)	Average	133.6	101.6	3.0	304
		Median	47.6	39.1	0.8	84
		IQR	66.9	34.5	1.8	182
		Min	7.0	10.4	0.2	17
		Max	1238.1	903.7	33.1	3307
Total		Average	111.8	93.7	2.5	246
		Median	48.7	41.2	0.9	94
		IQR	72.5	83.6	1.8	181
		Min	4.8	6.7	0.1	9
		Max	1238.1	903.7	33.1	3307

UNCORRECTED

DON exposure and glucuronidation rate (Table 3). The urban subgroup consumed relatively more food products from retail markets, while the rural subgroup consumed more homegrown foods, which, presumably were not as strictly controlled as foods from commercial sources. A similar difference was determined between the urban and rural subgroups, i.e. the analyses revealed an insignificantly higher exposure to DON of the rural subgroup compared to the urban subgroup ($p = 0.63$). One woman had an exceptionally high (the highest ever reported to the best of our knowledge) concentration of total DON (DON, DON-15-GlcA and

Please cite this article in press as: Šarkanj, B., et al. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. Food Chem. Toxicol. (2013), http://dx.doi.org/10.1016/j.fct.2013.08.043

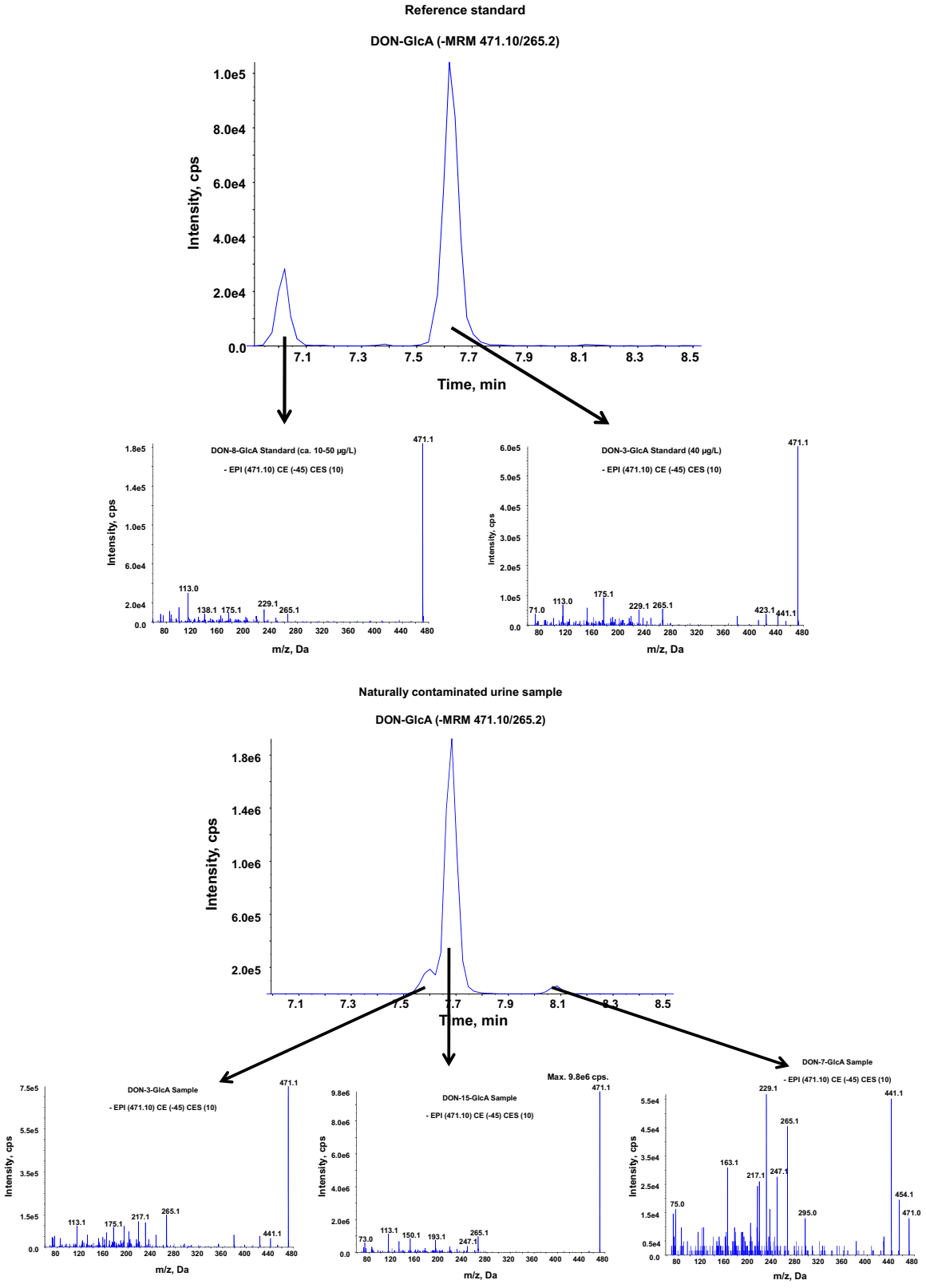

Fig. 2. Chromatogram from selected reaction monitoring and product ion scans of DON-8-GlcA and DON-3-GlcA reference standards and a naturally contaminated human urine sample. DON-8-GlcA was not detected in human urine, while DON-15-GlcA was the major conjugate followed by DON-3-GlcA; the third glucuronide is assumed to be DON-7-GlcA (RT = 8.1).

DON-3-GlcA were quantified to be 275 µg L^{-1} 1238 µg L^{-1} and 298 µg L^{-1}, respectively) which we estimate relates to a DON exposure of 33.1 µg kg^{-1} b.w. day^{-1}. This particular woman belonged to the rural, high school educated group, and consumed great quantities of homemade foods (including cereals and meat products). In comparison, the highest concentration of DON equivalents in the Austrian population was 2.2 µg kg^{-1} b.w. day^{-1} (Warth et al., 2012a) although in that study no correction for the higher

Please cite this article in press as: Šarkanj, B., et al. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. Food Chem. Toxicol. (2013), http://dx.doi.org/10.1016/j.fct.2013.08.043

concentrated first morning urine was done what would decrease the estimated exposure by a factor of two. Due to heavy rainfall during the 2010 season, and thus high DON concentrations in cereals (Pleadin et al., 2012a), it is likely that she had consumed highly contaminated home grown food prior to urine donation. However, no foodstuffs could be procured from the woman after these results were obtained in order to analyse these for mycotoxin contamination and prove this assumption.

The highest reported urinary DON concentration, normalised to creatinine, in a UK pregnant subpopulation was 116 μg g^{-1} (Hepworth et al., 2011). The maximum concentration in this cohort of Croatian pregnant women revealed a nine times higher exposure to DON (Table 3).

The sum of DON equivalents and FFQ data were distributed per quartiles and compared in order to identify trends (data not shown) but due to low sample number there was no statistically significant correlations between food intake and urinary DON equivalents, BMI, or age.

The detection of free DON and conjugated forms in the studied urine samples is indicative of DON as the principal food mycotoxin in this population. To confirm potential sources of high DON results, two representative wheat samples (yearly representative storage samples from local siloes, harvest year 2010) were analysed and high DON contamination was proven (942.4 μg kg^{-1} and 4510.5 μg kg^{-1} of DON; 47.8 μg kg^{-1} and 176.6 μg kg^{-1} DON-3-Glc; 0.8 μg kg^{-1} and 14 μg kg^{-1} 3-ADON). This finding was however not surprising considering the frequently reported natural occurrence of DON and its plant/fungal metabolites in food commodities from Croatia. Similarly, one milled wheat sample was rejected for export due to high levels of DON (mean: 2376 μg kg^{-1}) (RASFF, 2010). Furthermore, Pleadin et al. (2012a) recovered mean DON levels of 2150 μg kg^{-1} (range: 15–17920 μg kg^{-1}) in 85% of maize samples from Croatia. Such high levels were likely due to the extremely high rainfall during the harvest season (Meterological and hydrological service, 2011).

The co-existence of DON (and/or its glucuronide derivatives) and OTA was detected for the first time in Croatia. In this preliminary study we confirmed the presence of DON-3-GlcA and DON-15-GlcA in urine samples of pregnant women, which are less toxic DON metabolites (Wu et al., 2007).

Besides the two known DON conjugates DON-3-GlcA and DON-15-GlcA, which have been earlier confirmed as urinary metabolites (Warth et al., 2011; Warth et al., 2012a), a third glucuronide was recently detected in *in vitro* experiments utilising the same method applied within this study (Maul et al., 2012). This metabolite was tentatively identified as DON-7-GlcA and traces were also detected in urine samples obtained from a volunteer who ingested a known, high quantity of DON over a period of four days (Warth et al., 2013a). Due to the high concentrations of DON glucuronides in the urine samples analysed within the current study, it was possible to investigate the structure of this conjugation product for the first time although its relative abundance compared to the other glucuronides was insignificant. In addition, we compared the retention time and the MS/MS spectra with an NMR confirmed standard of DON-8-GlcA, which was synthesised recently (Uhlig et al., 2013).

Selected reaction monitoring chromatograms and daughter ion scans of the NMR confirmed DON-3-GlcA (Fruhmann et al., 2012) and DON-8-GlcA (Uhlig et al., 2013) reference standards as well as of a highly naturally contaminated urine sample are illustrated in Fig. 2. It is obvious that the third glucuronide elutes about 0.5 min after the DON-3-GlcA standard while DON-8-GlcA elutes earlier. Therefore, it can be concluded that the third peak is not DON-8-GlcA but rather a 7-glucuronide as suggested by Maul et al. (2012). However, the explanation of the MS spectrum from fragmentation of the supposed deprotonated molecular ion (*m/z* 471) was not straightforward (Fig. 2). While a major *m/z* 441 fragment (−30 Da) is characteristic for loss of the CH_2OH moiety attached at C-6 (Warth et al., 2012a), a *m/z* 454 fragment (−17 Da) is hard to explain. As the molecule does not contain nitrogen the only possible combination that explains a −17 Da fragment corresponds to a hydroxyl group. This would, however, involve loss of a radical, which is rarely seen in ESI–MS/MS. High-resolution MS should be applied in order to pursue this problem further. To confirm the identity of the major DON metabolite, an aliquot of a highly contaminated sample was re-analysed using SPE and HILIC–MS, and the MS and MS^2-characteristics of the major DON conjugate in the urine sample compared to those of an NMR confirmed reference standard (Uhlig et al., 2013). This experiment verified that the structure of the so far only tentatively identified conjugate (Warth et al., 2012a) is indeed equivalent with DON-15-GlcA (data not shown).

4. Conclusion

This pilot survey has provided data on urinary mycotoxin biomarkers in samples obtained from 40 pregnant Croatian women for the first time. DON and its conjugated forms, predominantly DON-15-GlcA, were the principal metabolites detected in urine with 97.5% of the samples above the detection limit. Urinary concentrations were used to estimate DON exposure and indicated exceptionally high intakes up to 3300% of the established TDI. Traces of OTA were detected in 10% of the investigated samples. Furthermore, the structure of a currently identified third DON glucuronide was investigated and might correspond to a DON-7-glucuronide. Considering that the present investigation was carried out on a small population, we recommend the results be reconfirmed using a larger scale multi-location study. Further bio-monitoring surveys on mycotoxin contamination patterns in Croatia are necessary to properly understand the extent of exposure and to propose intervention strategies to reduce potentially associated health risks.

Conflict of Interest

The authors declare that there are no conflicts of interest.

5. Uncited references

Öhman et al. (2008) and Reimers et al. (2005). Q4

Acknowledgements

The authors thank the European Commission for the financial support within the Project MycoRed (KBBE-2007-22269-2), the Lower Austrian Government, and Croatian Ministry of Science, Education and Sports (Grant No. 113-1130473-0334). Benedikt Warth is grateful for the support of the PhD graduate school program – Applied Bioscience Technology of the Vienna University of Technology in cooperation with the University of Natural Resources and Life Sciences, Vienna (BOKU). Furthermore, the hospitality and welcoming atmosphere of the staff of the Department for Agrobiotechnology (IFA-Tulln, BOKU) during the stay of the first author is greatly acknowledged.

References

Anderson, G.D., 2005. Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach. Clin. Pharmacokinet. 44, 989–1008.

Binder, E.M., Tan, L.M., Chin, L.J., Handl, J., Richard, J., 2007. Worldwide occurrence of mycotoxins in commodities, feeds and feed ingredients. Anim. Feed Sci. Technol. 137, 265–282.

UNCORRECTED PROOF

Cao, J., Stieger, B., Meier, P.J., Vore, M., 2002. Expression of rat hepatic multidrug resistance-associated proteins and organic anion transporters in pregnancy. Am. J. Physiol. Gastrointest. Liver Physiol. 283, G757–G766.

Fruhmann, P., Warth, B., Hametner, C., Berthiller, F., Horkel, E., Adam, G., Sulyok, M., Krska, R., Fröhlich, J., 2012. Synthesis of deoxynivalenol-3-ß-D-O-glucuronide for its use as biomarker for dietary deoxynivalenol exposure. World Mycotoxin J. 5, 127–132.

Goyarts, T., Danicke, S., Brussow, K.P., Valenta, H., Ueberschar, K.H., Tiemann, U., 2007. On the transfer of the Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) from sows to their fetuses during days 35–70 of gestation. Toxicol. Lett. 171, 38–49.

Hepworth, S.J., Hardie, L.J., Fraser, L.K., Burley, V.J., Mijal, R.S., Wild, C.P., Azad, R., McKinney, P.A., Turner, P.C., 2011. Deoxynivalenol exposure assessment in a cohort of pregnant women from Bradford, UK. Food Addit. Contam. Part A 29, 269–276.

Inoue, H., Tsuruta, A., Kudo, S., Ishii, T., Fukushima, Y., Iwano, H., Yokota, H., Kato, S., 2005. Bisphenol A glucuronidation and excretion in liver of pregnant and nonpregnant female rats. Drug Metab. Dispos. 33, 55–59.

Jackson, L.S., Bullerman, L.B., 1999. Effect of processing on *Fusarium mycotoxins*. Adv. Exp. Med. Biol. 459, 243–261.

Jakovac-Strajn, B., Vengust, A., Pestevsek, U., 2009. Effects of a deoxynivalenol-contaminated diet on the reproductive performance and immunoglobulin concentrations in pigs. Vet. Rec. 165, 713–718.

Klapec, T., Šarkanj, B., Banjari, I., Strelec, I., 2012. Urinary ochratoxin A and ochratoxin alpha in pregnant women. Food Chem. Toxicol. 50, 4487–4492.

Krstanović, V., Klapec, T., Velić, N., Milaković, Z., 2005. Contamination of malt barley and wheat by Fusarium graminearum and Fusarium culmorum from the crop years 2001–2003 in eastern Croatia. Microbiol. Res. 160, 353–359.

Maul, R., Warth, B., Kant, J.-S., Schebb, N.H., Krska, R., Koch, M., Sulyok, M., 2012. Investigation of the hepatic glucuronidation pattern of the fusarium mycotoxin deoxynivalenol in various species. Chem. Res. Toxicol. 25, 2715–2717.

Meky, F.A., Turner, P.C., Ashcroft, A.E., Miller, J.D., Qiao, Y.-L., Roth, M.J., Wild, C.P., 2003. Development of a urinary biomarker of human exposure to deoxynivalenol. Food Chem. Toxicol. 41, 265–273.

Meterological and Hydrological Service, 2011. Climate Monitoring and Assessment for 2010.

Mikhail, M.S., Anyaegbunam, A., 1995. Lower urinary tract dysfunction in pregnancy: a review. Obstet. Gynecol. Surv. 50, 675–683.

Mikula, H., Hametner, C., Berthiller, F., Warth, B., Krska, R., Adam, G., Fröhlich, J., 2012. Fast and reproducible chemical synthesis of zearalenone-14-β,D-glucuronide. World Mycotoxin J. 5, 289–296.

Nielsen, J.K.S., Vikström, A.C., Turner, P., Knudsen, L.E., 2011. Deoxynivalenol transport across the human placental barrier. Food Chem. Toxicol. 49, 2046–2052.

Öhman, I., Luef, G., Tomson, T., 2008. Effects of pregnancy and contraception on lamotrigine disposition: new insights through analysis of lamotrigine metabolites. Seizure 17, 199–202.

Pestka, J.J., Smolinski, A.T., 2005. Deoxynivalenol: toxicology and potential effects on humans. J. Toxicol. Environ. Health B Crit. Rev. 8, 39–69.

Piekkola, S., Turner, P.C., Abdel-Hamid, M., Ezzat, S., El-Daly, M., El-Kafrawy, S., Savchenko, E., Poussa, T., Woo, J.C.S., Mykkänen, H., El-Nezami, H., 2012. Characterisation of aflatoxin and deoxynivalenol exposure among pregnant Egyptian women. Food Addit. Contam. Part A 29, 962–971.

Pleadin, J., Sokolović, M., Perši, N., Zadravec, M., Jaki, V., Vulić, A., 2012a. Contamination of maize with deoxynivalenol and zearalenone in Croatia. Food Control 28, 94–98.

Pleadin, J., Perši, N., Mitak, M., Zadravec, M., Sokolović, M., Vulić, A., Jaki, V., Brstilo, M., 2012b. The natural occurrence of T-2 toxin and fumonisins in maize samples in croatia. Bull. Environ. Contam. Toxicol. 88, 863–866.

RASFF, 2010. <https://webgate.ec.europa.eu/rasff-window/portal/index.cfm?event=notificationDetail&NOTIF_REFERENCE=2010.CGJ> (accessed 10.02.13).

Reimers, A., Helde, G., Brodtkorb, E., 2005. Ethinyl estradiol, not progestogens, reduces lamotrigine serum concentrations. Epilepsia 46, 1414–1417.

Solfrizzo, M., Gambacorta, L., Lattanzio, V.T., Powers, S., Visconti, A., 2011. Simultaneous LC–MS/MS determination of aflatoxin M1, ochratoxin A, deoxynivalenol, de-epoxydeoxynivalenol, α and β-zearalenols and fumonisin B1 in urine as a multi-biomarker method to assess exposure to mycotoxins. Anal. Bioanal. Chem. 401, 2831–2841.

Sulyok, M., Berthiller, F., Krska, R., Schuhmacher, R., 2006. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Commun. Mass Spectrom. 20, 2649–2659.

Tiemann, U., Brüssow, K.P., Dänicke, S., Vanselow, J., 2008. Feeding of pregnant sows with mycotoxin-contaminated diets and their non-effect on foetal and maternal hepatic transcription of genes of the insulin-like growth factor system. Food Addit. Contam. Part A 25, 1365–1373.

Turner, P.C., Rothwell, J.A., White, K.L.M., Gong, Y.Y., Cade, J.E., Wild, C.P., 2008. Urinary deoxynivalenol is correlated with cereal intake in individuals from the United Kingdom. Environ. Health Persp. 116, 21–45.

Turner, P.C., 2010. Deoxynivalenol and nivalenol occurrence and exposure assessment. World Mycotoxin J. 3, 315–321.

Turner, P.C., White, K.L.M., Burley, V.J., Hopton, R.P., Rajendram, A., Fisher, J., Cade, J.E., Wild, C.P., 2010. A comparison of deoxynivalenol intake and urinary deoxynivalenol in UK adults. Biomarkers 15, 553–562.

Turner, P.C., Hopton, R.P., White, K.L.M., Fisher, J., Cade, J.E., Wild, C.P., 2011. Assessment of deoxynivalenol metabolite profiles in UK adults. Food Chem. Toxicol. 49, 132–135.

Turner, P.C., Flannery, B., Isitt, C., Ali, M., Pestka, J., 2012a. The role of biomarkers in evaluationg human health concerns from fungal contaminants in food. Nutr. Res. Rev. 25, 162–179.

Turner, P.C., Gong, Y.Y., Pourshams, A., Jafari, E., Routledge, M.N., Malekzadeh, R., Wild, C.P., Boffetta, P., Islami, F., 2012b. A pilot survey for Fusarium mycotoxin biomarkers in women from Golestan, northern Iran. World Mycotoxin J. 5, 195–199.

Uhlig, S., Ivanova, L., Fæste, C.K., 2013. Enzyme-assisted synthesis and structural characterization of the 3-, 8-, and 15-glucuronides of deoxynivalenol. J. Agric. Food Chem. 61, 2006–2012.

Warth, B., Sulyok, M., Berthiller, F., Schuhmacher, R., Fruhmann, P., Hametner, C., Adam, G., Fröhlich, J., Krska, R., 2011. Direct quantification of deoxynivalenol glucuronide in human urine as biomarker of exposure to the *Fusarium mycotoxin* deoxynivalenol. Anal. Bioanal. Chem. 401, 195–200.

Warth, B., Sulyok, M., Fruhmann, P., Berthiller, F., Schuhmacher, R., Hametner, C., Adam, G., Frohlich, J., Krska, R., 2012a. Assessment of human deoxynivalenol exposure using an LC–MS/MS based biomarker method. Toxicol. Lett. 211, 85–90.

Warth, B., Sulyok, M., Fruhmann, P., Mikula, H., Berthiller, F., Schuhmacher, R., Hametner, C., Abia, W.A., Adam, G., Fröhlich, J., Krska, R., 2012b. Development and validation of a rapid multi-biomarker liquid chromatography/tandem mass spectrometry method to assess human exposure to mycotoxins. Rapid Commun. Mass Spectrom. 26, 1533–1540.

Warth, B., Sulyok, M., Berthiller, F., Schuhmacher, R., Krska, R., 2013a. New insights into the human metabolism of the fusarium mycotoxins deoxynivalenol and zearalenone. Toxicol. Lett. 220 (1), 88–94.

Warth, B., Sulyok, M., Krska, R., 2013b. LC–MS/MS based multi-biomarker approaches for the assessment of human exposure to mycotoxins. Anal. Bioanal. Chem. 405 (17), 5687–5695.

WHO, 2001. WHO food additives series 47. Presented at the 56th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA). FAO Food Nutr. Paper, 74.

Wu, X., Murphy, P., Cunnick, J., Hendrich, S., 2007. Synthesis and characterization of deoxynivalenol glucuronide: its comparative immunotoxicity with deoxynivalenol. Food Chem. Toxicol. 45, 1846–1855.

UNCORRECTED PROOF

Please cite this article in press as: Šarkanj, B., et al. Urinary analysis reveals high deoxynivalenol exposure in pregnant women from Croatia. Food Chem. Toxicol. (2013), http://dx.doi.org/10.1016/j.fct.2013.08.043