Occurrence of free and conjugated Fusarium mycotoxins in cereal based food

Oliver Vendl, Colin Crews, Susan Macdonald, Rudolf Krska, Franz Berthiller

► To cite this version:

Oliver Vendl, Colin Crews, Susan Macdonald, Rudolf Krska, Franz Berthiller. Occurrence of free and conjugated Fusarium mycotoxins in cereal based food. Food Additives and Contaminants, 2010, 27 (08), pp.1148-1152. 10.1080/19440041003801166 . hal-00604040

HAL Id: hal-00604040

<https://hal.science/hal-00604040>

Submitted on 28 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Occurrence of free and conjugated Fusarium mycotoxins in cereal based food

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2009-377.R1
Manuscript Type:	Original Research Paper
Methods/Techniques:	Chromatography - LC/MS
Additives/Contaminants:	Mycotoxins - fusarium
Food Types:	Beer, Bakery products, Bread, Cereals and grain

SCHOLARONE™
Manuscripts

Occurrence of free and conjugated *Fusarium* mycotoxins in cereal based food

Oliver Vendl ^a, Colin Crews ^{b*}, Susan MacDonald ^b, Rudolf Krska ^a and Franz Berthiller ^a

^a *Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Applied Life Sciences, Vienna, Konrad Lorenz Straße 20, 3430 Tulln, Austria*

^b *The Food and Environment Research Agency (FERA), Sand Hutton, York, YO41 1LZ, United Kingdom*

* Corresponding author

Email: colin.crews@fera.gsi.gov.uk

Phone: +44 (0)1904 462549

Fax: +44 (0)1904 462111

Abstract

A collection of 84 cereal based food products in 25 composites, including beer, was screened for the presence of deoxynivalenol, zearalenone and their respective metabolites deoxynivalenol-3-glucopyranoside, 3-acetyl-deoxynivalenol, zearalenol-4-glucopyranoside, α -zearalenol, β -zearalenol, α -zearalenol-4-glucopyranoside, β -zearalenol-4-glucopyranoside and zearalenone-4-sulfate. The most abundant analyte was zearalenone-4-sulfate, which was found in 13 composites, albeit in low concentrations. Furthermore, deoxynivalenol was detected in eight, zearalenone in seven and deoxynivalenol-3-glucopyranoside in two composites. None of the remaining six analytes were found in any matrices, which suggests that, if at all present, concentrations of these latter metabolites are very low and hence do not impose any danger to the consumer. The highest mycotoxin content was found in bran flakes with 254 ng g⁻¹ deoxynivalenol, 6 ng g⁻¹ zearalenone-4-sulfate and 44 ng g⁻¹ zearalenone.

Keywords: masked mycotoxins, deoxynivalenol, zearalenone, LC/MS, cereal based food,

Fusarium

Introduction

The mycotoxins deoxynivalenol (DON) and zearalenone (ZON) are common and well known contaminants of cereal products arising from infection of grain with *Fusarium* sp. These two compounds are produced by the fungus as part of its physiology of pathogenic invasion and, as the cereal ears are the location of infection, these mycotoxins have been found to contaminate cereal-based products (Leonard and Bushnell 2004).

However, DON and ZON are partly metabolized by the infected host plant as well as by the producing fungus itself. Metabolism by the host plant generally aims at detoxification of these compounds which is often accomplished by attachment of hydrophilic groups, thus increasing their solubility in water (Engelhardt et al. 1999; Karlovsky 1999). In this manner, DON is converted to DON-3-glucopyranoside (D3G) (Sewald et al. 1992; Berthiller et al. 2005), and ZON can be altered to ZON-4 glucopyranoside (Z4G) (Engelhardt et al. 1988) by plants. During fungal metabolism, ZON has been found to be partially converted to ZON-4-sulfate (Z4S) (Plasencia and Mirocha 1991) by various *Fusarium* sp. as well as to α -zearalenol (α -ZOL) and β -zearalenol (β -ZOL) (Böswald et al. 1995) by different yeast strains. Further glucosylation of the latter two compounds was observed to occur as part of the host plant's metabolism, leading to α -ZOL-glucopyranoside (α -ZG) and β -ZOL-glucopyranoside (β -ZG), respectively (Berthiller et al. 2006). Despite their chemical alteration, there is evidence that the above mentioned metabolites have a similar toxic potential to those of their precursors when ingested with food, as attached functional groups like glycosylic or sulfate residues are likely to be enzymatically cleaved during digestion (Gareis et al. 1990; Plasencia and Mirocha 1991). Also D3G is at least partly cleaved to DON by various intestinal bacteria (F. Berthiller; manuscript in preparation). Furthermore, α -ZOL and β -ZOL have been shown to exhibit similar hyperestrogenic effects as their precursor ZON (Fitzpatrick et al. 1989). Analytical

approaches targeted to the parental molecules DON and ZON, respectively, might not detect their metabolites, thus resulting in an underestimation of the inherent toxicity of a contaminated sample matrix. For these reasons, chemically altered metabolites of mycotoxins are also referred to as masked mycotoxins (Gareis et al., 1990). The formation, determination and significance of masked and other conjugated mycotoxins were recently reviewed (Berthiller et al. 2009a).

Maximum levels of DON and ZON have been set by European Commission regulations (European Commission 2007), ranging from 200-750 ng g⁻¹ for DON and 20-100 ng g⁻¹ for ZON in processed cereals, but masked metabolites have not yet been taken in account for an exposure assessment of total mycotoxins from cereal based foods. In order to provide simultaneous LC-MS/MS determination of all of the above mentioned metabolites together with DON and ZON in cereal based food, we developed and validated a method of sample preparation and measurement which has recently been published (Vendl et al. 2009). In the present paper we employ this method in order to determine the occurrence of the 10 target mycotoxins in a wide range of cereal based food products, purchased in the UK and Austria, consisting of 84 samples in 25 composites of product groups.

Materials and Methods

Chemicals

DON, D3G, 3ADON, ZON, α -ZOL and β -ZOL were purchased from Biopure Referenzsubstanzen GmbH (Tulln, Austria) as calibrants in acetonitrile. Z4G, α -ZG, β -ZG (Berthiller et al. 2009b) and Z4S (Berthiller et al. 2003) had previously been isolated by our group and were available as solutions in methanol. LC-grade acetonitrile was obtained from VWR (Leuven, Belgium). Water, which was used for the preparation of extraction solvents,

sample dilution and as a LC mobile phase, was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

Food and cereal samples

84 cereal products destined for food usage (either for direct consumption or for further processing) were purchased in retail markets in the UK and in Austria. For every product group, 3–4 commercial products from different manufacturers were collected and combined into one composite as described later. A total number of 25 product groups (composites) of cereal based food was chosen for initial screening. In case the screening results were positive, the samples were analyzed individually. Before further processing, samples were stored at room temperature in the darkness. Samples with short expiration period (particularly bread) were processed within 2 days of purchase, which was before the expiry date or best before date was reached. Other samples were stored at room temperature in darkness. Expiration dates were not exceeded, and the maximum storage time between purchase and processing was 3 weeks. Milled sample materials were stored in plastic bottles at room temperature in the dark.

Composite preparation

Dry samples (corn flakes, wheat flakes and raw pasta) were milled in a laboratory mill. Samples containing higher amounts of fat or sugar were milled frozen with dry ice. This procedure was applied to the following composites: Extruded maize snacks, biscuits, muesli, crackers, popcorn, cereal snack bars, extruded oat snacks and some particular samples of the baby food composites. Bread samples were dried at 45 °C for 48 hours and subsequently milled. Milling was performed for every sample separately. For each product group, 100 g of each milled product were combined and homogenized in a laboratory blender. The milled

fractions of the non-combined individual samples were kept for optional separate analysis for the case that the respective composite proved to be contaminated. Canned maize was mashed in a blender together with an equal weight amount of 1% aqueous acetic acid (100 ml for 100 g sample), which corresponded to the water and acid content in the extraction solution. For the beer composite, equal volumes (100 ml each) of beer samples were combined and degassed in a sonic bath for 10 min.

Extraction

Extraction of samples was performed according to the procedure that has been published previously (Vendl et al. 2009). Briefly, for dry composites, 1.00 ± 0.01 g of ground and homogenized combined sample material was extracted with 4 ml extraction solvent (composition: acetonitrile/water/acetic acid in the ratio 79/20/1 v/v/v) for 90 min on a rotary shaker (Burgwedel, Germany) circulating at 190 rpm. Homogenized canned maize was extracted directly after mashing and homogenization by adding 400 ml acetonitrile/acetic acid (99/1) to 100 ml of the aqueous, acidic homogenate. The resulting mixture was distributed into four sealed 250 ml Erlenmeyer flasks and extracted for 90 min on a rotary shaker at 190 rpm. Beer was injected directly after degassing.

LC-MS/MS instrumentation and methodology

An 1100 Series HPLC System (Agilent, Waldbronn, Germany) linked to a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, USA) was used to analyze the extracts. Electrospray ionisation (ESI) was used in negative mode at a 550°C source temperature. HPLC and MS/MS parameters were set according to Vendl et al. (2009). Briefly, separation was performed on a Phenomenex Synergi Polar RP-18 column (150×3 mm, 4 µm) at 35 °C using an aqueous ammonium acetate (5mM) / acetonitrile gradient at a flow rate of 1 ml min⁻¹

¹. Two SRM transitions were recorded for each analyte: D3G (m/z 517.2 $\rightarrow$ 457.2; 427.0), DON (m/z 355.1 $\rightarrow$ 59.1; 295.1), 3ADON (m/z 397.1 $\rightarrow$ 59.1; 337.0), Z4S (m/z 397.1 $\rightarrow$ 317.1; 131.0), β -ZG and α -ZG (m/z 481.2 $\rightarrow$ 319.0; 275.1), Z4G (m/z 479.2 $\rightarrow$ 317.0; 131.0), β -ZOL and α -ZOL (m/z 319.1 $\rightarrow$ 130.0; 160.0), ZON (m/z 317.1 $\rightarrow$ 131.0; 175.0).

Results and discussion

Twenty-five composites of cereal based food products were screened for the occurrence of DON, ZON and their respective major masked metabolites. Among the targeted analytes, DON, Z4S and ZON were the most abundant, as DON was detected in 8, Z4S in 13 and ZON in 7 composites (see table 1). The highest quantities of DON (254 ng g⁻¹), Z4S (6.1 ng g⁻¹) and ZON (44.2 ng g⁻¹) were found in bran flakes. Relatively high levels of DON were also found in crackers (248 ng g⁻¹) and wheat flour (237 ng g⁻¹). Both latter matrices also showed Z4S contents of over 2 ng g⁻¹, although neither of them displayed significantly high levels of ZON. Due to its high ionization efficiency, Z4S is detectable at very low levels and could therefore be determined quantitatively in 10 composites, namely wheat flour, whole-meal wheat bread, maize meal, biscuits, wheat flakes, bran flakes, muesli, crackers, cereal snack bars and polenta. To our knowledge, a comparable wide range of food samples had never before been screened for the existence of Z4S, hence this study brings first evidence for a seemingly wide abundance of Z4S, although at low concentrations. ZON, which was quantifiable in wheat grain and bran flakes, was also detected in whole-meal wheat bread, extruded maize snacks, corn flakes, wheat flakes, crackers and cereal snack bars. Besides the three above analytes mentioned, D3G was detected in wheat bread and in whole-meal wheat bread but levels were below LOQ. the occurrence of D3G (along with DON) in unprocessed wheat and maize kernels after harvest was recently reported (Berthiller et al. 2009c). After bran flakes, wheat flower and crackers had the highest content of target analytes, therefore

analyses of individual samples of these three matrices were carried out in addition to the composite screening. Although analysis of combined samples was expected to provide statistically more reliable results on the contamination of a particular product group, screening of individual samples gave a good idea of the actual mycotoxin distribution within one composite. Certain samples might be heavily contaminated and might not contain any mycotoxins at all, or, in contrast, the target analytes might be rather equally distributed among all samples of one product group. Table 2 shows the values of these additional measurements. Within the composite “bran flakes”, all three samples contained DON, Z4S and ZON within a relatively similar concentration range. In contrast, within both composites “crackers” and “wheat flour”, two samples showed significantly higher mycotoxin concentrations than the third one of the composite. DON and Z4S were determined in all measured individual samples. ZON was found in all three samples of the bran flakes composite, but in only one out of three samples of wheat flour. In crackers, ZON was determined in two of the three samples. None of the remaining 6 analytes (3ADON, Z4G, α -ZOL, β -ZOL, α -ZG and β -ZG) were detected in any composite or sample, suggesting that, in the case of contamination, these metabolites occur at levels far below the parental compounds DON and ZON, respectively. Z4S has been found to be produced in fungal cell cultures in molar ratios from 1:12 to 1:1 compared to ZON (Plasencia and Mirocha 1991). In this work, Z4S was present at levels between 9.7% of the ZON level in the wheat flour sample A and 17.9% of the ZON level in the crackers sample B2. In case of those composites, where ZON was detected but not quantified, values of Z4S contents suggested Z4G:ZON ratios of over 1:10 considering maximum ZON levels of 10 ng g^{-1} (=LOQ). For Z4G:ZON, correlations of contents have been reported in a study on wheat, stating Z4G values of about 10% of the ZON level in those samples in which both analytes were determined (Schneeweis et al., 2002). On the other hand, Z4G could not be determined in 50% of those samples that contained ZON. In consideration

of these statements, values of Z4G would lie below limits of detection for those composites that exhibit ZON concentrations between LOD and LOQ.

With regard to food safety, the maximum levels for DON and ZON set by the European Commission were not exceeded in any product group, however, in the case of bran flakes the underestimation of toxicity imposed by masked mycotoxins becomes evident: The value of ZON concentration (44.2 ng g^{-1}) lies below the EC limit, but an additional content of 6.1 ng g^{-1} Z4S has to be taken into consideration. Assuming that Z4S is totally hydrolyzed to ZON during digestion (slightly reducing its mass after cleavage of the sulfate group), the actual toxicity exposure corresponds to a ZON concentration of about 49 ng g^{-1} , which is closer to the maximum EC regulation level. This gives good evidence that cereal based food meeting regulatory levels for DON and ZON might not display the actual mycotoxin intake of the consumer.

A method to determine ZON and DON conjugates together indirectly after cleavage of the conjugates to the precursor toxins would be helpful and allow comparison of the total toxin values. However, alkaline hydrolysis would destroy the lactone ring of ZON and its derivatives. Acidic hydrolysis would be unlikely to work for D3G, as this compound is strongly resistant to acids even at pH values <1 at heightened temperatures. This leaves enzymatic hydrolysis as the only available option. β -glucosidases are not able to cleave D3G to DON, but cellulases have shown at least partial cleavage with pure D3G (data not shown). Further studies will concentrate on indirect methods of analysis for conjugated mycotoxins.

Conclusion

In none of 25 product groups of cereal based food did DON and ZON levels, exceed European Commission regulation limits. However, in the case of the bran flakes samples, if Z4S were to be completely hydrolyzed to ZON during digestion, the result would be an exposure of 49 ng g⁻¹ ZON, which is close to the maximum regulation level. Out of eight major masked metabolites, six were not detected in any of the analyzed matrices.

Acknowledgements

The authors thank the UK Food Standard Agency, the Christian Doppler Society, the Lower Austrian government and the Austrian Science Fund (FWF) for financial support.

References

Berthiller F, Schuhmacher R, Adam G, Krska R. 2003. LC-MS/MS von konjugierten Toxinen. ALVA Mitteilungen 1:21-24.

Berthiller F, Dall'Asta C, Schuhmacher R, Lemmens M, Adam G, Krska R. 2005. Masked mycotoxins: determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry. J Agric Food Chem. 53:3421-3425.

Berthiller F, Werner U, Sulyok M, Krska R, Hauser MT, Schuhmacher R. 2006. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) determination of phase II metabolites of the mycotoxin zearalenone in the model plant Arabidopsis thaliana. Food Addit Contam. 23:1194-1200.

Berthiller F, Schuhmacher R, Adam G, Krska R. 2009a. Formation, determination and

significance of masked and other conjugated mycotoxins. Anal Bioanal Chem. 395:1243-1252.

Berthiller F, Hametner C, Krenn P, Schweiger W, Ludwig R, Adam G, Krska R, Schuhmacher R. 2009b. Preparation and characterization of the masked *Fusarium* mycotoxins zearalenone-4O- β -D-glucopyranoside, α -zearalenol-4O- β -D-glucopyranoside and β -zearalenol-4O- β -D-glucopyranoside by MS/MS and 2D-NMR. Food Addit Contam. 26:207-213.

Berthiller F, Corradini R, Dall'Asta C, Marchelli R, Sulyok M, Krska R, Adam G, Schuhmacher R. 2009c. Occurrence of deoxynivalenol and its 3- β -D-glucoside in wheat and maize. Food Addit. Contam. 26:507-511.

Böswald C, Engelhardt G, Vogel H, Wallnöfer PR. 1995. Metabolism of the *Fusarium* mycotoxins zearalenone and deoxynivalenol by yeast strains of technological relevance. Nat Toxins 3:138-144.

Engelhardt G, Zill G, Wohner B, Wallnöfer PR. 1988. Transformation of the *Fusarium* mycotoxin zearalenone in maize cell suspension cultures. Naturwissenschaften 75:309-310.

Engelhardt G, Ruhland M, Wallnöfer PR. 1999. Metabolism of mycotoxins in plants. Adv Food Sci. 21:71-78.

European Commission Regulation No 1126/2007. Setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. Off J Europ Union L255:14-17.

Fitzpatrick DW, Picken CA, Murphy LC, Buhr MM. 1989. Measurement of the relative binding affinity of zearalenone, alpha-zearalenol and beta-zearalenol for uterine and oviduct estrogen receptors in swine, rats and chickens: an indicator of estrogenic potencies. Comp Biochem Physiol C. 94(2):691-694.

Gareis M, Bauer J, Thiem J, Plank G, Grabley S, Gedek B. 1990. Cleavage of zearalenone-glycoside, a "masked" mycotoxin, during digestion in swine. J Vet Med. 37:236-240.

Karlovsky P. 1999. Biological detoxification of fungal toxins and its use in plant breeding, feed and food production. Nat Toxins 7:1-23.

Leonard K, Bushnell W. 2004. (eds.) *Fusarium Head Blight of Wheat and Barley*. APS Press St. Paul, MN, USA.

Plasencia J, Mirocha CJ. 1991. Isolation and characterization of zearalenone sulfate produced by *Fusarium spp.* Appl Environ Microbiol. 57:146-150.

Schneeweis I, Meyer K, Engelhardt G, Bauer J. 2002. Occurrence of zearalenone-4- β -D-glucopyranoside in wheat. J Agric Food Chem. 50:1736-1738.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Sewald N, Lepschy von Gleissenthall J, Schuster M, Müller G, Aplin RT. 1992. Structure elucidation of a plant metabolite of 4-desoxynivalenol. *Tetrahedron* 3:953-960.

Vendl O, Berthiller F, Crews C, Krska R. 2009. Simultaneous determination of deoxynivalenol, zearalenone and their major masked metabolites in cereal based food by LC-MS/MS. *Anal Bioanal Chem.* 395:1347-1354.

For Peer Review Only

Table 1. Concentrations of DON, ZON and eight masked metabolites in cereal based food (ug/kg⁻¹).

Composite	country of purchase	No.	DON	D3G	3ADON	ZON	Z4S	β-ZG	α-ZG	Z4G	β-ZOL	α-ZOL
Wheat flour	UK	3	237	-	-	< 10	2.0	-	-	-	-	-
Wheat bread	UK, Austria	4	< 100	< 100	-	-	< 1	-	-	-	-	-
Rye bread	UK, Austria	4	-	-	-	-	< 1	-	-	-	-	-
Wheat bread wholemeal	UK	3	< 100	<100	-	< 10	2.2	-	-	-	-	-
Wheat semolina	UK, Austria	3	< 100	-	-	-	-	-	-	-	-	-
Maize meal and flour	UK	3	-	-	-	-	1.3	-	-	-	-	-
Extruded maize snacks	UK	3	-	-	-	< 10	< 1	-	-	-	-	-
Biscuits	UK, Austria	4	< 100	-	-	-	1.5	-	-	-	-	-
Pasta	UK	4	-	-	-	-	-	-	-	-	-	-
Beer (including wheat beer)	UK, Austria	4	-	-	-	-	-	-	-	-	-	-
Canned maize	UK	4	-	-	-	-	-	-	-	-	-	-
Breakfast cereals - cornflakes	UK, Austria	3	-	-	-	< 10	-	-	-	-	-	-
Breakfast cereals - wheat	UK	4	< 100	-	-	< 10	1.8	-	-	-	-	-
Breakfast cereals - bran flakes	UK	3	254	-	-	44.2	6.1	-	-	-	-	-
Breakfast cereals - muesli	UK, Austria	4	-	-	-	-	1.6	-	-	-	-	-
Breakfast cereals - oats	UK	3	-	-	-	-	-	-	-	-	-	-
Crackers	UK	3	248	-	-	< 10	2.3	-	-	-	-	-
Baby food - wheat based	UK, Austria	3	-	-	-	-	-	-	-	-	-	-
Baby food - oat based	UK	3	-	-	-	-	-	-	-	-	-	-
Popcorn	UK	3	-	-	-	-	-	-	-	-	-	-
Cereal snack bars	UK	3	-	-	-	< 10	1.8	-	-	-	-	-
Buckwheat grain or flour	UK	3	-	-	-	-	-	-	-	-	-	-
Quinoa grain or flour	UK, Austria	4	-	-	-	-	-	-	-	-	-	-
Extruded oat snacks	UK	3	-	-	-	-	-	-	-	-	-	-
Polenta	UK, Austria	3	-	-	-	-	2.1	-	-	-	-	-

- = not detected

Table 2. Contents of DON, Z4S and ZON in individual samples of the composites “wheat flour”, “bran flakes” and “crackers” ($\mu\text{g}/\text{kg}^{-1}$).

Composites	DON	Z4S	ZON
Wheat flour sample A	307	1.8	18.2
Wheat flour sample B	249	3.5	nd
Wheat flour sample C	186	1.0	nd
Bran flakes sample A	243	6.2	48.9
Bran flakes sample B	241	5.5	40.1
Bran flakes sample C	262	5.8	36.5
Crackers sample A	324	2.2	14.5
Crackers sample B	330	2.9	16.0
Crackers sample C	132	0.7	< 10

nd = not detected