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In: World Mycotoxin Journal, 7 (3), 285-290, 2014.

Deoxynivalenol Content in Wheat Dust versus Wheat Grain: a Comparative Study

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Abstract
The present study, set up in the growing season 2011-2012, was designed to obtain quantitative data on the occurrence of deoxynivalenol in wheat grain and the corresponding wheat dust. The field experiment consisted of a complete randomized block design with five wheat varieties sown on a field on which maize was grown in the previous season. The impact of the tillage method and the influence of the wheat variety resistance on the deoxynivalenol content of wheat and wheat dust were investigated. The accumulation of deoxynivalenol in wheat dust was confirmed and a sigmoidal relationship between the deoxynivalenol content in wheat dust versus wheat grain was determined. Deoxynivalenol reduction was obtained by ploughing and by sowing moderately resistant wheat varieties. As wheat dust provides equal results and solves the problem of heterogeneity during sampling of conventional wheat matrix, the sampling of wheat dust can be considered as a promising alternative.

Keywords: deoxynivalenol, wheat dust, tillage, wheat variety

Abbreviations: EC, European Commission; ELISA, Enzyme-linked immune-sorbent assay; ESI, Electrospray ionization; ESI+, Positive electrospray ionization; FHB, Fusarium head blight; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; SD, Standard deviation; SRM, Selected reaction monitoring

1. Introduction

Mycotoxins are secondary metabolites produced by a wide range of fungi e.g., *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp., under natural conditions. The contamination of food and feed with mycotoxins poses a serious threat to human and animal health. Despite good agricultural practices followed by good manufacturing practices represent the primary line of defense against elevated toxin contents, it is not possible to completely avoid mycotoxin contamination in food and feed. *Fusarium* spp. can produce a heterogeneous blend of toxins, of which trichothecenes form predominant mycotoxin group in small grain cereals in Europe. Among the 150 related trichothecenes, deoxynivalenol (Figure 1), also known as vomitoxin, shows the highest incidence. Besides rice and corn, wheat is the most important cereal for human nutrition and, in some regions, the major source of deoxynivalenol intake (JECFA 2001) (Lancova *et al*., 2008; Krska *et al*., 2001; Beyer *et al*., 2006; Murphy *et al*., 2006). Therefore, The regulatory limits for DON in food and feed are defined in the European Commission Regulation (EC) No. 1881/2006 and the European Commission Recommendation 2006/576/EC, respectively.

Figure 1: Chemical structure of deoxynivalenol

The infection of wheat heads by *Fusarium* species during anthesis causes Fusarium head blight (FHB). Until now, 17 species of *Fusarium* have been described to be potentially associated with FHB symptoms (Leonard and Bushnell, 2003). In Europe, *F. graminearum* and *F. culmorum* are known as the most important FHB pathogens of wheat because of their high pathogenic character in comparison with other species and their high mycotoxin production level, in particular deoxynivalenol. As mentioned by Hooker et al. (2002), weather conditions during flowering are the main factors contributing to the variation in deoxynivalenol content. Rainfall and warm temperatures during wheat anthesis promote the FHB incidence (Hooker *et al*., 2002; Edwards, 2004; Beyer *et al*., 2006). In addition, weather conditions during the vegetative growth of wheat during autumn and winter influence the FHB infection pressure (Landschoot *et al*., 2012). Besides weather conditions, good agricultural practices contribute to minimize the FHB problem. FHB infection can be controlled by a combination of the following methods: crop rotation, tillage, choice of the wheat variety, and use of fungicides. Besides wheat, *Fusarium* species infect maize and after harvest crop debris provides an excellent source for the survival of pathogens. Therefore, including maize in crop rotation systems will increase the risk of FHB epidemics and deoxynivalenol contamination. Tillage, a general mechanism for removing the crop debris, reduces the inoculum potential of *Fusarium* species, and as such leads to a reduction in deoxynivalenol content. A third method which can be considered in order to reduce the FHB incidence is the wheat variety. At the moment, no complete FHB-resistant crop is known, even though the susceptibility of commercial wheat varieties to FHB pathogens and their deoxynivalenol content differs. The lack of an absolute type of resistance originates from the multigenic nature of FHB resistance encoded by quantitative trait loci which are scattered throughout the genome. Consequently, cultivated wheat varieties display a continuous variation in resistance depending on the country of origin due to differences in the genetic pool within each country's breeding program and the different environmental and agronomic conditions in which crops are cultivated (Edwards, 2004; Miller *et al*., 1998; Beyer *et al*., 2006; Lori *et al*., 2009; De Boevre *et al*., 2012). The improvement of host genetic resistance is therefore seen as essential to achieve meaningful control although advances in this research are hampered by the multigenic nature of the resistance (Gilbert and Haber, 2013). Besides crop rotation and variety choice, fungicides remain an indispensable measure to tackle the FHB problem. Research trials conducted in the nineties showed the effectiveness of the

triazoles prothioconazole, metconazole and tebuconazole plus prothioconazole in suppressing FHB disease and controlling mycotoxins. The best control of the disease can be achieved with fungicide applications at wheat anthesis and the efficacy declines rapidly with time after and before the plant growth stage (Beyer *et al*., 2006; Gilbert and Haber, 2013). Although the impact of fungicides on fungal biomass is a straightforward reduction, the implication of fungicide application with regard to toxin production is a story that has not been fully elucidated and several research groups report on increased toxin production upon fungicide application or a selection towards specific chemotypes of the fungus (Audenaert *et al*., 2010; Zhang *et al*., 2013).

Wheat dust is produced by the transport of grain through closed systems, known as air dust and can occur in storage facilities, which is called settled dust. As described earlier (Tangni and Pussemier, 2006; Tangni and Pussemier, 2007; Sanders *et al*., 2013), there is an accumulation of deoxynivalenol on dust particles. Because of the homogeneous distribution of deoxynivalenol through the small dust particles, the sampling and subsequent analysis of dust is a good measure to solve the problem of heterogeneity within a bulk lot. A fact of concern is the respiratory, work-related effects of deoxynivalenol on dust particles which are not always well documented (Krysinska-Traczyk *et al*., 2001; Sanders *et al*., 2013). Until now, all research developments consider the influence of agricultural practices and weather conditions on FHB and associated mycotoxins in wheat grain. Whereas, wheat dust can be highly contaminated and no studies are available describing methods lowering the deoxynivalenol content in this kind of matrix. The present study was designed to obtain quantitative data on the occurrence of deoxynivalenol in wheat grain and dust taken into account the different methods controlling FHB infection like crop rotation, tillage and wheat cultivar.

2. Materials and methods

2.1 Reagents and chemicals

The individual mycotoxin calibration standards deoxynivalenol (10 mg) and de-epoxy deoxynivalenol (50 ng/µL) were purchased from Fermentek (Jerusalem, Israel) and Coring System Diagnostics (Gernsheim, Germany), respectively. The deoxynivalenol standard was dissolved in methanol in a concentration of 1 µg/µL and stored at 4°C. Working solutions of deoxynivalenol (10 ng/µL) and de-epoxy deoxynivalenol (2.5 ng/µL) were prepared in methanol and stored at -18°C.

Water was obtained from a Milli-Q SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol (LC-MS grade) was obtained from BioSolve BV (Valkenswaard, The Netherlands). Acetonitrile (Analar Normapur), *n*-hexane (Hipersolv Chromanorm) and ammonium acetate (analytical reagent) were purchased from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was supplied from Merck (Darmstadt, Germany).

2.2 Field trial

The experimental field trial was set up to investigate the influence of maize-wheat rotation, tillage method and wheat variety on the deoxynivalenol content of wheat grain and the corresponding wheat dust. Therefore, a randomized complete block design with two soil tillage systems, five wheat varieties and four replications was set up. During the growing season 2011, maize was grown on the experimental field. After harvesting the maize, one half of the field was ploughed before sowing, while on the other part wheat was sown after minimal tillage. In November 2011, five winter wheat varieties, namely Azzerti and Homeros (susceptible) and Sahara, Mulan and Tabasco (moderately resistant) were sown. One

fungicide treatment was applied with a fungicide containing a strobilurin and a triazole. The field trial is explained in detail in Landschoot et al., 2013 (Landschoot *et al*., 2013).

2.3 ELISA screening

To estimate the deoxynivalenol content in the fresh harvested wheat derived from the different wheat varieties under the different tillage conditions, wheat grains were firstly analysed for deoxynivalenol presence using the Veratox DON 5/5 enzyme-linked immunosorbent assay (ELISA) test kit following the manufacturer's protocol (Biognost, Neogen, Leest, Belgium).

2.4 LC-MS/MS sample preparation and extraction

The mixing of the collected field samples was based on the fact that there was no significant influence of the pre-crop; only tillage or no tillage seemed to be important. So wheat derived from the same variety and with the same soil treatment were mixed to derive ten different wheat samples. The ten samples were mixed in a vertical screw mixer and were divided in four subsamples. One part of each subsample was ground using the M20 grinder (Ika Werke, Staufen, Germany). The equipment was decontaminated after each milling step by the use of water and Dissolol®. Another part of the subsample was transferred to a laboratory bin equipped with a vacuum pump sucking the dust from the wheat kernels.

Sample extraction of the ground wheat and the corresponding wheat dust samples was performed according to Sanders et al., 2013. Concisely 2.5 g of wheat or 1 g of wheat dust was extracted with 10 ml acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by hexane defatting (10 ml) using the Agitator decanter overhead shaker (Agitelec, J. Toulemonde and Cie, Paris, France) for 60 min. After centrifugation (3,000 g, 15 min) the hexane layer was removed and the aqueous layer was filtered and evaporated to dryness (N_2, 40 °C). Finally, the residue was redissolved in 100 µl injection solvent, consisting of methanol/water/acetic acid (41.8/57.2/1, v/v/v) with 5 mM ammonium acetate (Sanders *et al*., 2013).

2.5 LC-MS/MS methodology

The liquid chromatography-tandem mass spectrometric (LC-MS/MS) analysis of the wheat and wheat dust samples was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) with Masslynx version 4.1 and Quanlynx version 4.1. software (Micromass, Manchester, UK) used for data acquisition and processing. Chromatographic separation was performed using an Acquity UPLC BEH C_{18} column (1.7 µm, 100 mm x 2.1 mm i.d.) supplied by Waters (Milford, MA, USA). A mobile phase consisting of water/methanol/acetic acid (94/5/1, v/v/v) (A) and methanol/water/acetic acid (97/2/1, v/v/v) (B), both containing 5 mM ammonium acetate was used at a flow rate of 0.3 mL/min. The gradient elution programme started at 99% mobile phase A with a linear decrease to 50% mobile phase A in 3 min. A linear increase to 99% mobile phase B was established in 0.5 min. An isocratic gradient of 99% mobile phase B was initiated at 3.5 min for 2 min. The duration of each UPLC run was 8.5 min. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. Data acquisition was performed using selected reaction monitoring (SRM) and the transitions were described in detail by Sanders et al., 2013. The developed LC-MS/MS method was successfully validated according to Commission Decision 2002/657/EC of 12 August 2002 (Sanders *et al*., 2013). The identity of deoxynivalenol within the wheat and wheat dust samples of all wheat varieties was controlled according to the identification criteria set in Commission Decision 2002/657/EC. Every analytical run consisted of a standard control mix,

seven samples of the calibration curve and a maximum of 20 samples. All calculations were performed and processed using Microsoft Office Excel 2010 and IBM SPSS 21.

2.6 Dust correlation study

For the statistical evaluation the SPSS software version 21 was used. Normality assumptions were checked using the Shapiro-Wilk test and differences between groups of data were tested with an independent samples t-test. The deoxynivalenol content of the wheat and corresponding wheat dust samples of the different varieties and soil treatments was set out in a scatterplot together with the data presented in Sanders et al., 2013. Verifying a possible correlation, the Pearson correlation coefficient was determined for the total batch of samples (Sanders *et al*., 2013).

3. Results and discussion

3.1 ELISA screening

Four samples of each wheat variety and soil treatment were subjected to ELISA screening for deoxynivalenol determination and the results are presented in Table 1. The legal deoxynivalenol limit was exceeded in 87.5% of the samples and the highest deoxynivalenol concentration was found in the wheat variety Azzerti, sown after minimal tillage, with a mean deoxynivalenol concentration of 5,380 µg/kg. An independent-samples t-test revealed a lower deoxynivalenol content of wheat samples with soil treatment tillage for Homeros, Mulan, Sahara and Tabasco compared to the wheat samples without tillage (p-values respectively 0.007, 0.012, 0.008 and 0.019). While for Azzerti no significant difference (p-value 0.228) was seen between tillage and no tillage.

Table 1: Deoxynivalenol concentration (µg/kg) of the harvested wheat samples (n=4) determined by ELISA

		Wheat	
		Mean (µg/kg)	SD
Azz	No tillage	5 3	2 094

erti	Tillage	8 0 4 6 0 2	1 417
Homeros	Notillage Tillage	4 7 7 9 3 1 8 6	1 771 1 237
Mulan	Notillage Tillage	2 2 8 7 1 4 5 6	1 321 1 352
Sahara	Notillage Tilla	2 9 3 6 2	820 784

	ge		
		1	
		2	
		6	
T	No	3	1
a	tilla		268
b	ge	6	
a		8	
s		9	
c	Tilla	2	1
o	ge		477
		4	
		7	
		9	

3.2 LC-MS/MS analysis

The wheat samples showed deoxynivalenol concentrations between 1,450 µg/kg and 10,670 µg/kg, which means that each sample exceeded the limit for human consumption set as 1,250 µg/kg. As all LC-MS/MS results (Table 2) showed to be higher than the ELISA measurements (Table 1), an underestimation of the deoxynivalenol in wheat measured by ELISA can be concluded. However, the conclusions are analogous. The wheat variety Azzerti showed again the highest deoxynivalenol contamination level with a mean concentration of 7,596 µg/kg with tillage. A lower deoxynivalenol content of wheat samples with soil treatment tillage was discovered for Mulan, Sahara and Tabasco compared to the wheat samples without tillage by the use of an independent-samples t-test (p-values respectively 0.002, 0.004 and 0.005). For Azzerti and Homeros no significant difference in deoxynivalenol level (p-values respectively 0.972 and 0.122) was seen between tillage and no tillage. The resulted deoxynivalenol concentration of the corresponding wheat dust samples showed values above 8,000 µg/kg. This confirms the accumulation of deoxynivalenol in wheat dust as mentioned in Sanders et al., 2013 (Sanders *et al*., 2013). By the use of an independent-samples t-test, the wheat dust analysis results with regard to the tillage method (tillage and no tillage) were compared for each winter wheat variety. P-values of 0.388 and 0.471 for respectively Azzerti and Homeros, revealed no significant difference in deoxynivalenol content between tillage and no tillage for wheat dust. For Mulan, Sahara and Tabasco, p-values were determined of 0.043, 0.003 and 0.006 respectively. This means that for these wheat varieties a significantly lower deoxynivalenol content in wheat dust was observed in wheat sown after tillage.

Table 2 : Deoxynivalenol concentration (µg/kg) of the harvested wheat and wheat dust samples (n=4) determined by LC-MS/MS

	Wheat		**Wheat dust**	
	M	S	M	SD

		Mean (µg/kg)	DF	Mean (µg/kg)	
Azzerti	No till age	75.73	62.23	22.723	3524
	Tillage	75.969	10.669	21.973	5253
Homeros	No till age	50.2	61.15	25.79	3737
	Tillage	41.5	12.9	25.8	4967

		7	3	3	
				3	
M	No	4	7	1	902
u	till		6	4	
l	age	2	7		
a		7		7	
n		4		8	
				9	
	Til	1	9	8	2
	lag		9		205
	e	4	7	1	
		8		8	
		3		2	
S	No	4	6	2	2
a	till		1	6	795
h	age	7	8		
a		0		6	
r		1		2	
a				2	
	Til	2	7	1	933
	lag		2	7	
	e	5	8		
		3		1	
		4		2	
				3	
T	No	4	5	2	1
a	till		3	7	144
b	age	7	2		
a		1		5	
s		1		5	
c				9	
o	Til	3	3	1	5
	lag		2	4	005

	e	1	4	
		4		8
		7		2
				1

3.3 Dust correlation study

The LC-MS/MS results for each wheat sample and the corresponding dust sample are presented in a scatterplot (Figure 2). As mentioned in previous research, a correlation between the deoxynivalenol concentration in dust versus the deoxynivalenol concentration in wheat was proved according to the correlation coefficient, r (0.970). In that study wheat dust showed a 13 fold accumulation of deoxynivalenol compared to wheat. In the present study the correlation between the deoxynivalenol content in wheat and dust was 0.829 (r) and a five fold accumulation of deoxynivalenol in wheat dust was observed. This difference can be explained by the fact that the wheat samples from the different varieties and soil treatments showed higher deoxynivalenol concentrations starting from 1,450 µg/kg. These extra results completed the calibration curve in the range above the limit for human consumption. When analysing the scatterplot, a logarithmic relationship between the deoxynivalenol content in wheat dust versus wheat grain was observed. Therefore, it can be concluded that the 13-fold accumulation of deoxynivalenol in wheat dust is present until 1,250 µg/kg for wheat (limit for human consumption) and 17,761 µg/kg for wheat dust. When a higher contamination level is observed, the sigmoidal curve with the following equation y=5935ln(x)-28423, needs to be considered and a five-fold accumulation of deoxynivalenol in wheat dust is observed.

Figure 2: A scatterplot of the deoxynivalenol concentration in dust (y-axis) versus wheat (x-axis)

As mentioned in Landschoot et al., (2013), maize-wheat rotation can be considered as influencing factor on the deoxynivalenol content of the wheat crop during the next growing season. During the growing season 2012 wheat sown after maize took care of a six times higher deoxynivalenol content compared to wheat sown after a crop different from maize. The susceptibility of the wheat variety had most influence on the deoxynivalenol content of wheat and wheat dust (Kruskal-Wallis test, p-value < 0.05) (Landschoot *et al*., 2013). An independent samples t-test revealed that the moderately resistant varieties Mulan, Sahara and Tabasco had a significant lower deoxynivalenol content than the susceptible varieties Azzerti and Homeros. For wheat and wheat dust no significant difference in deoxynivalenol content was observed between tillage and no tillage for the susceptible wheat varieties Azzerti and Homeros.

It can be concluded that the analysis of wheat dust is a promising alternative to deoxynivalenol analysis of grain. Indeed, this study proved that there is a significant correlation between DON in wheat dust and DON in wheat. However, this relationship is concentration-dependent, for concentrations up to 1,250 µg/kg a linear relationship can be used to predict the DON content in wheat dust. When a higher contamination level is observed, the sigmoidal curve is able to predict the DON content in a reliable way.

During sampling of cereals, uncertainty errors occur mostly due to the problem of the heterogeneous distribution of mycotoxins in food and feed. One measure mentioned by

European Commission Regulation (EC) No. 401/2006, is the reduction of particle size followed by efficient mixing (Maestroni and Cannavan, 2011; Whitaker and Slate, 2012; Whitaker and Slate, 2005). Therefore, sampling can be made more easy to perform, less labor-intensive, less costly with a lower error of uncertainty by sampling dust instead of the cereal bulk lot. Because of the small size of the dust particles and the accumulation of deoxynivalenol in the dust fraction, the sampling and LC-MS/MS analysis can be considered as a good replacement of conventional methods.

Acknowledgements
This work was executed thanks to the contribution of MFKK (Hungary), ULUND (Sweden), HGFA (Hungary), SEEDYZ (Greece), CESFAC (Spain), ASEMAC (Spain), Synagra (Belgium), EASRET (Greece), Impuls Ltd (Poland), OSV Srl, (Italy), Dunagabona Ltd. (Hungary), Dimitriaki S.A. (Greece), ETIA (France), Bioforum S. A. (Greece), EST Ltd (United Kingdom). The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement n°243633

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