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Monitoring the levels of deoxynivalenol (DON) in cereals in Lebanon and validation of an HPLC/UV detection for the determination of DON in crushed wheat (bulgur)

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This paper reports for the first time a method for determining deoxynivalenol (DON) in crushed wheat (bulgur) using an high-performance liquid chromatography (HPLC) method with ultraviolet light detection. Linearity ($r^2 > 0.999$), selectivity and recovery (70–110%) were acceptable. Results show that the limit of detection (LOD) was $50\,\mu g\,kg^{-1}$ and the limit of quantification (LOQ) was $80\,\mu g\,kg^{-1}$. The level of DON was determined in 165 samples of cereals – wheat, *forkha* (wheat flour special for cake) and bulgur (crushed wheat) – marketed in Lebanon. The results showed that the contamination with DON was 8.75%, 7.5% and 5.0% in bulgur, wheat and *forkha*, respectively. The LOD and LOQ for wheat (or *forkha*) were, respectively, 40 and $50\,\mu g\,kg^{-1}$. The level of DON in all samples was below $1250\,\mu g\,kg^{-1}$ as recommended by European Union Directives (Commission Regulation (EC) No. 1126/2007), except for one sample of wheat which contained $2307\,\mu g\,kg^{-1}$. These data suggest that the Lebanese population is exposed to DON through food ingestion at concentrations lower than the tolerable daily intake (TDI) and suggest that measures must be performed routinely to avoid high levels of DON contamination to be found on Lebanese market.

Keywords: high-performance liquid chromatography (HPLC); mycotoxins – trichothecenes; cereals and grain

Introduction

Mycotoxins are fungal secondary metabolites toxic to vertebrates and other animal groups in low concentrations. They often occur in agriculture products and threaten food safety. In terms of economic impact and scientific interest, trichothecenes are undoubtedly major mycotoxins (others being aflatoxins, fumonisins and zearolone). (Hussein and Brasel 2001; Bennett and Klich 2003). Trichothecenes are produced by many fungi such as *Fusarium*, *Myrothecium*, *Stachybotrys* and others. The main source of trichothecene contamination in food and feedstuff is cereals (maize, oats, barley and wheat) (Balzer et al. 2004). Structurally, trichothecenes have been divided into toxin groups designated as A, B, C, and D. Type B trichothecenes, which contain a keto group at carbon 8 of the epoxytrichothecene nucleus, are detectable as contaminants of cereal grains such as wheat, barley and corn. Of these, deoxynivalenol (DON, or vomitoxin) is the most prevalent worldwide in crops used for food and feed consumption (Sudakin 2003; Larsen et al. 2004).

Due to its stability in processing and cooking, the levels of human exposure to DON can be high (Jackson and Bullerman 1999). In many countries a legal limit of $1250\,\mu g\,kg^{-1}$ of DON in cereals has been established (Commission Regulation (EC) No. 1126/2007). This level of contamination in feedstuffs can cause serious health problems and diseases. Even at low levels, DON may cause animals to refuse feed; at higher levels, it induces vomiting, leading to growth depression, increased susceptibility to infections, diarrhoea, and haemorrhage (Rotter et al. 1996; Pestka et al. 2004).

Cereals contributed to 35% of daily energy intake in the adult urban population in Beirut, Lebanon (Nasreddine et al. 2006). Following the Food and Agricultural Organization (FAO) statistical yearbooks from 2001 to 2003, the production of Lebanese cereals was 146,000 tonnes; in return, 910,000 tonnes were imported, especially from the United States and Europe (mainly France and Germany). These statistics means that more than 85% of cereals consumed in Lebanon are imported from exposed countries, as described by Leblanc et al. (2005), Mankeviciene et al. (2007), and Rasmussen et al. (2007). Actually, no information exists about the occurrence of *Fusarium* toxins in cereals in Lebanon.

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Among these cereals, *burgul* is highly consumed in Lebanon. It is obtained by boiling wheat (thoroughly cooked), drying it in the sun, and then grinding it into particles and sieving into distinct sizes. This process might favour mould development and mycotoxin secretions.

Current methods used for DON determination in cereals include high-performance liquid chromatography (HPLC), gas chromatography (GC) with electron capture detection (ECD), or mass spectrometry (MS) and enzyme-linked immunoabsorbant assay (ELISA) methods (Kotal and Radová 2002; Schollenberger et al. 2007; Gallo et al. 2008). To the present authors' knowledge, there is no description of a method for the determination of DON in bulgur. The aim of the present study is to validate an HPLC method with ultraviolet light (UV) detection for the determination of DON in bulgur and wheat, and to measure the presence of DON in a variety of cereals (wheat, bulgur (crushed wheat) and *forkha* (wheat flour special for cake)) marketed in Lebanon.

Materials and methods

Materials and reagents

The standard solution of DON was supplied from Riedel-de-Haen® Seelze, Germany. Immunoaffinity columns (RBR DONTest columns) were supplied by Libios (Bully, France). Analytical-grade acetonitrile, methanol, and water were obtained from Merck® (Germany); polyethylene-glycol 6000 (PEG) was obtained from Sigma. The liquid chromatograph Hewlett Packard HP 1100 model (Agilent, Germany) equipped with a quaternary pump (G1311A), auto-injector (G1313A) and UV detector (G1315B) was used with a stainless steel reverse-phase 150×4.6 mm, 5 µm particle size C18 Omnispher Varian® HPLC column (Varian, the Netherlands).

Samples

A total of 165 representative samples of cereals (wheat, *burgul* and *forkha*) were purchased from five different *mouhafazat*s all over Lebanon from food stores (supermarket, wholesaler and craft industry) during spring 2006. Some wheat samples were obtained from Beirut silos and sampled taking 1 kg and mixing it thoroughly before grinding. Blank wheat samples were obtained from Laboratoires d'analyses et de contrôles (INZO), France. A total of 1 kg per sample was finely ground and sifted at 0.5 mm. The following commodities were collected: wheat ($n = 65$), *burgul* ($n = 80$), and *forkha* ($n = 20$).

Extraction procedure

Sifted cereals (25 g) were mixed with 5 g of PEG and 100 ml of water (HPLC grade) and agitated for 30 min. The mixture was then filtered on 125 mm-diameter filter papers (Whatman® No. 4). Following extraction, the sample extract was passed through the immunoaffinity column (Libios, Bully, France). The column was washed and the toxin released from the antibody using methanol. The eluate was blown-down using a nitrogen-based evaporating system.

High-performance liquid chromatography (HPLC)

The flow rate was 1 ml min^{-1} with a column at room temperature. The wavelength of the UV used for detection was set at 218 nm. The retention time for DON under these conditions was approximately 7.5 min.

Validation of HPLC/UV detection for the determination of DON in wheat and bulgur (crushed wheat)

The validations for the extraction and the HPLC/UV detection of DON in wheat and bulgur were made in accordance with the French standard for testing agricultural and alimentary products (NF V 03-110). Linearity, specificity, selectivity, reproducibility, and determinations of the limit of detection (LOD) and limit of quantification (LOQ) were determined in accordance with the French standard (Table 1).

Statistical analysis

The data were analysed using the Fischer, the Cochran, and the Student's *t*-tests.

Results

Validation of the HPLC/UV detection of DON in bulgur according to French standards for testing agricultural and alimentary products (NF V 03-110)

Linearity

Linear calibration curves were constructed for five different assays. The regression equation was calculated in the form:

$$y = ax + b,$$

where y and x are the area under the curve and the corresponding concentration of DON, respectively. The high correlation coefficients ($r^2 > 0.999$) and the regression parameters listed in Figure 1 indicated good linearity over a relatively wide concentration range. The experimental value of F_1 Fisher's test (8190.87) was higher than the theoretical value ($F_{1th(0.01;1;24)} = 7.823$),

Table 1. Guidelines for linearity, precision, selectivity and both the limits of detection and quantification according to NF V03-110.

Parameters	NF V 03-110 guidelines
Linearity	The regression test is considered satisfactory (Fisher's test is acceptable, i.e. the F_1-values are higher than $F_{\text{critical value}}$ with a 1% α risk for 1 and $p(n-1)$ degrees of freedom, where p is the number of levels tested; and n is the number replicates). Nor is there any error of the model in the selected domain (F_2 is less than Fisher's $F_{\text{critical value}}$ with a 1% risk for $p-2$ and $p(n-1)$ degrees of freedom)
Limits of detection and quantification	The detection limits and quantification limit are determined as LOD = 3 and LOQ = 10 times the background noise plus the blank/sensitivity
Precision (repeatability and reproducibility)	The internal reproducibility of the method evaluates its reliability when the measurements are made over several days by more than one operator (two operators). The Cochran test was used to check that all sample variances did not differ statistically and that the precision was stable all over the scope of the method. The value obtained is compared with the critical value of Cochran test, with an error risk α of 1%
Selectivity	A Student's t-test has to be used to verify whether the slope of the line of regression is equal to 1 and is acceptable for all the molecules (if t_{obs} is less than $t_{\text{critical value}}$ as read from the tables for a risk of $1-\alpha/2$ with $\alpha = 1\%$ with $p-2$ degrees of freedom). Likewise, the ordinate at the origin of the line of regression does not differ from zero for each of the analytes

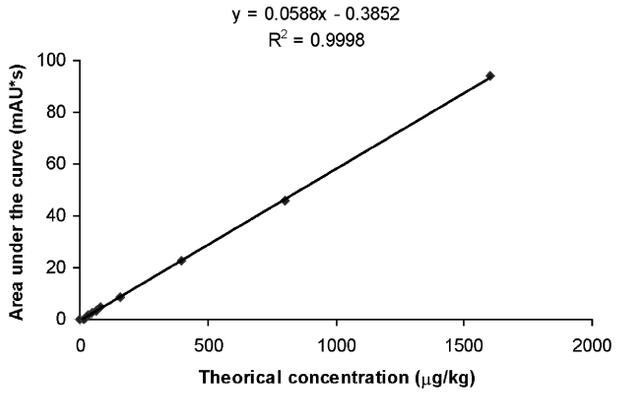

Figure 1. Linearity of the HPLC/UV detection of DON in crushed wheat. y and x express the values of peak area and different concentration of DON, respectively. AU, amount of absorbance, s, seconds.

indicating that the slope of the regression line was significant. Since the obtained experimental value of F_2 Fisher's test (0.473) was lower than the theoretical value ($F_{2\text{th}(0.01;4;24)} = 4.218$), method errors were not significant. The linear model was validated. F_1- and F_2-tests were realized at $p-2$ and $p\,(n-1)$ degrees of freedom, respectively, where p is the number of calibration points (six); and n is the number of repetitions (five).

Limits of detection/quantification (LOD/LOQ) for bulgur

Blank crushed wheat samples were spiked and the smallest detectable and measurable concentrations were calculated. Results showed that the LOD was $50\,\mu g\,kg^{-1}$ and the LOQ was $80\,\mu g\,kg^{-1}$. Most of the recoveries ranged from 70% to 110% and were significant (Table 2).

Precision (repeatability and reproducibility)

Both intra-day and inter-day reproducibilities for the determination of the DON in bulgur were established. Blank bulgur was spiked with DON. A Cochran test was performed and the results showed that the precision was maintained over the whole application range (Table 3). A value of 0.94 was obtained, which is lower than 0.968 obtained in Cochran's table ($\alpha = 1\%, n = 2, p = 4$).

Selectivity (interference of other compounds and/or matrix components)

Spiking blank crushed wheat samples was carried out. The concentrations covering the range 80–500 $\mu g\,kg^{-1}$ showed that selectivity was maintained. Selectivity was tested by comparing the theoretical concentrations prepared by spiking with the measured concentrations measured by HPLC/UV (Figure 2).

Validation of the HPLC/UV detection of DON in wheat according to French standards for testing agricultural and alimentary products (NF V 03-110)

Similarly, we validated the method for the determination of DON in wheat as recommended in the French standard (NF V 03-110). The LOD and LOQ obtained were 40 and $50\,\mu g\,kg^{-1}$, respectively. The method was precise, as seen by the Cochran test, and selective on the entire range (concentrations ranging from 50 to $1600\,\mu g\,kg^{-1}$). A representative chromatogram is shown in Figure 3.

Survey of DON levels in 165 samples

Foodstuffs were selected randomly from the five districts (North, Mount-Lebanon, Beirut, the South

Table 2. Recovery of the HPLC/UV detection of DON in crushed wheat.

Theoretical concentration ($\mu g\,kg^{-1}$)	Obtained concentration ($\mu g\,kg^{-1}$)	Symmetry	Number of plates per column	Recovery (%)
80	53	0.89	7791	66
80	75	0.85	8342	93
80	89	1.07	6407	111
80	60	0.79	9389	76
80	60	0.89	7680	75
80	59	0.86	9536	74
80	55	0.99	9119	69
80	57	0.87	8533	71
80	52	0.85	10634	65
80	53	0.94	9611	66

Note: Blank samples were spiked with DON (80μg/kg). Concentrations of DON were measured. Symmetry, the number of plates per column and recoveries are shown in table 2.

Table 3a. Precision of the HPLC/UV detection of DON in crushed wheat.

	Concentration ($\mu g\,kg^{-1}$)							
	Day 1		Day 2		Day 3		Day 4	
$\mu g\,kg^{-1}$	1	2	1	2	1	2	1	2
80	53	75	89	60	60	59	55	57
100	92	78	109	85	70	77	71	77
160	107	149	148	124	123	111	115	186
200	160	139	213	140	171	167	180	160
400	287	295	294	290	276	282	272	279
500	350	428	379	349	363	411	353	333
1000	650	742	693	690	740	682	680	730
2000	1411	1422	1409	1407	1377	1411	1398	1356

Note: For each concentration, the extraction was done twice daily by two different operators and for 4 days. The results are expressed in table 3a.

Table 3b. Precision of the HPLC/UV detection of DON in crushed wheat.

	Mean	Standard deviation	Variance	Coefficient of variation
80	63	12	150	19.3
100	82	13	167	15.7
160	133	27	714	20.1
200	166	24	553	14.1
400	284	8	70	3.0
500	371	33	1093	8.9
1000	701	33	1090	4.7
2000	1399	22	467	1.5

Note: The mean, the standard variation, the variance and the coefficient of variation were calculated and are expressed in Table 3b.The standard deviation and the coefficient of variation expressed the repeatability and the reproducibility of the method respectively

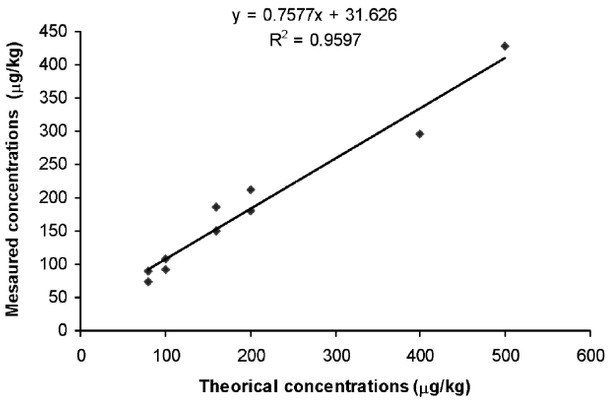

Figure 2. Selectivity of the HPLC/UV detection of DON in crushed wheat. *y* and *x* represent the values of theoretical and measured concentrations of DON, respectively.

and the Bekaa Valley) in order to assess the contamination by DON in foodstuff in the Lebanese market (Table 4). Results given in Figure 4a and b show the levels of DON in the tested samples from each district.

The level of DON in all samples was below $1250\,\mu g\,kg^{-1}$ as recommended by European Union recommendations (Commission Regulation (EC) No. 1126/2007), except for one sample of wheat (from Beirut) which contains $2307\,\mu g\,kg^{-1}$.

The levels of DON in bulgur, wheat and *forkha* were positive in Beirut, Mount-Lebanon, the North and the South; all the samples tested in the Bekaa were under the LOD. In Beirut, six samples were positive for DON (52, 56 and 289 $\mu g\,kg^{-1}$ for bulgur contamination; and 52, 56 and 2307 $\mu g\,kg^{-1}$ for wheat contamination). In Mount-Lebanon only one sample of wheat was contaminated (811 $\mu g\,kg^{-1}$). In the North, three samples of bulgur contained 56, 82 and 153 $\mu g\,kg^{-1}$. In the South, three samples of bulgur, wheat and *forkha* contained 177, 48 and 110 $\mu g\,kg^{-1}$, respectively. The level of DON measured in 165 samples of cereals showed that the contamination of DON in Lebanon was 8.75%, 7.5% and 5.0% in bulgur, wheat and *forkha*, respectively, for all the samples tested. The prevalence of DON was 10% in the most populated district (Beirut), as well as in the North and the South of Lebanon. All samples collected from Bekaa contained no detectable levels of DON.

Discussion

A large-scale European study on occurrence of *Fusarium* toxins in cereals (Schothorst and van Egmond 2004), with the participation of twelve European countries, showed that 57% of the samples were positive for deoxynivalenol (DON), especially in wheat and wheat flour. To the authors' knowledge, no assessment of DON contamination in cereals consumed in Lebanon has been carried out, whereas most of the cereals are imported from several countries. It was thought to measure the contamination of cereals with DON in the Lebanese market. The present study (1) reported for the first time a method for the determination of DON in bulgur; (2) evaluated a method for the determination of DON in wheat; and (3) measured the presence of DON in 165 samples of cereals consumed in the Lebanese market.

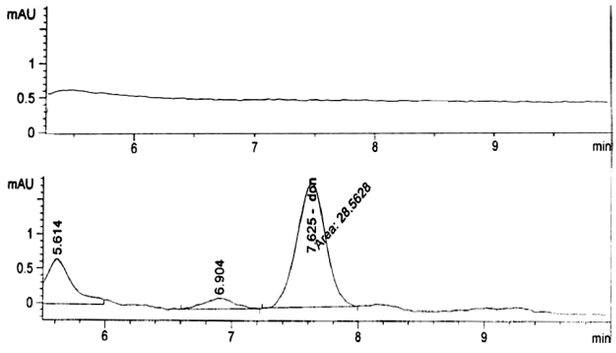

Figure 3. Representative chromatogram of a spiked sample of wheat. Chromatograms represent a blank sample and a spiked sample with 1000 $\mu g\,kg^{-1}$ of DON. The wavelength of ultraviolet light detection was set at 218 nm. The retention time for DON was approximately 7.5 min. The *y*- and *x*-axes represented the amount of absorbance and the time (min), respectively.

Many analytical (HPLC or GC) or immunochemical methods (ELISA) have been developed for the detection of trichothecenes in food and feed. The advantages and disadvantages of each method depend on their capability to separate impurities from the analytes, the time for preparation sample, and economic aspects (Schneider et al. 2004; Gallo et al. 2008). Due to its keto group in conjunction with the double-bond between C_9 and C_{10}, DON absorbs in the UV range where most of the impurities from the cereal matrix have a significant UV maximum. To avoid impurities that can interfere with UV detection, we used an immunoaffinity clean-up column with a selective separation of DON which has been successfully used with DON or other mycotoxins (Assaf et al. 2004; Trebstein et al. 2008). For the separation and detection of DON, we chose to work with an HPLC-UV method. The HPLC-UV method for the detection of DON in cereals showed the best agreement of results between laboratories (Josephs et al. 2004) and it was chosen to be the European method for the detection of DON in cereals (PREN 15891 – AFNOR).

The method used for the determination of DON in wheat and bulgur was set according to the French standard for testing agricultural and alimentary products (NF V 03-110). This method was adapted from the method for the determination of DON in wheat used in 'laboratoires d'analyses et de contrôles', INZO, France. It showed good linearity ($r^2 > 0.999$),

Table 4. Samplings from the five districts all over Lebanon.

District	Bulgur	Wheat	Forkha	Percentage of samples
Beirut	132 [52–289] (3/26)	805 [52–2307] (3/26)	0 (0/3)	33
Mount-Lebanon	0 (0/19)	811 (1/17)	0 (0/5)	25
North	97 [56–153] (3/16)	0 (0/10)	0 (0/4)	18
South	177 (1/14)	48 (1/9)	110 (1/6)	18
Bekaa	0 (0/5)	0 (0/3)	0 (0/2)	6

Note: This table represents the average level, contamination range and number of positive samples/number of samples for each matrix in each district as well as the percentage of sampling for wheat, bulgur and *forkha* from the five districts in Lebanon.

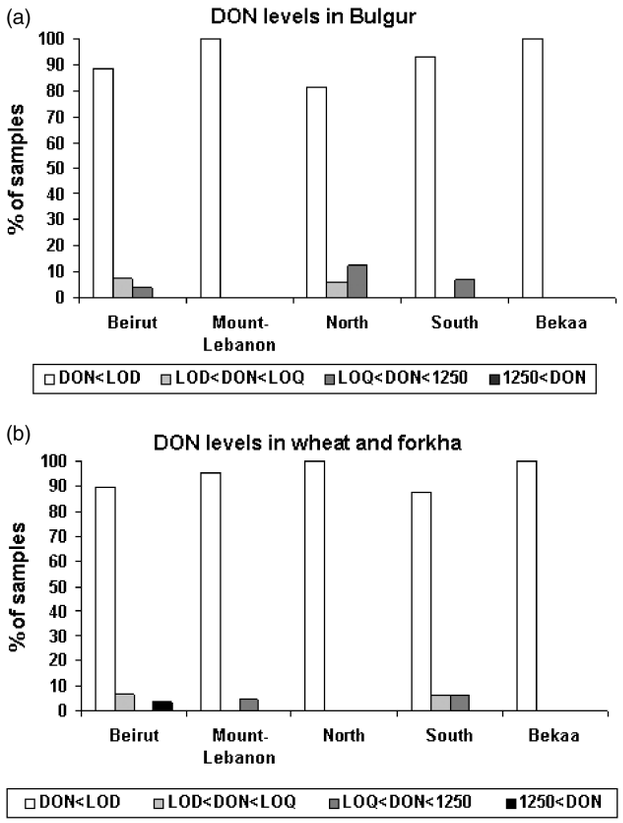

Figure 4. DON levels in bulgur, wheat and *forkha* in the five districts of Lebanon. These histograms represent the percentage of samples contaminated or not by DON in the five districts; results are expressed as the percentage of samples tested in every district. (a) Percentage of bulgur samples contaminated or not; LOD = 50 μg kg^{-1}, LOQ = 80 μg kg^{-1}; and (b) percentage of wheat and *forkha* samples contaminated or not; LOD = 40 μg kg^{-1}, LOQ = 50 μg kg^{-1}.

precision and selectivity. Most of the recoveries ranged from 70% to 110%. The LOD and LOQ obtained were 40 and 50 μg kg^{-1} for wheat and 50 and 80 μg kg^{-1} for bulgur, respectively. The LOQ obtained in this method is comparable with the LOQ obtained for DON determinations in wheat and previously described, e.g. LOQ = 100 μg kg^{-1} achieved using an HPLC method described by Poapolathep et al. (2008) and LOQ = 50 μg kg^{-1} obtained with an LC-MS/MS method (Spanjer et al. 2008). The present work describes for the first time a simple method for the determination of DON in bulgur with acceptable LOD and LOQ. We suggest that this validated method for the determination of DON in wheat and bulgur could be used for a routine surveillance of DON in cereals.

We also measured the levels of DON in 165 samples obtained from the five districts of Lebanon. The results showed that the contamination of DON in Lebanon was 8.75%, 7.5% and 5.0% in bulgur, wheat and *forkha*, respectively. In all samples tested for DON, only one contained a high level, and that sample was imported. These results suggest that DON contamination in cereals consummated in Lebanon is relatively low.

The Bekaa valley is the most important farming region in Lebanon, with cereals being the main crops. The temperature in the valley is very high in summer and very low in winter, with low levels of humidity. It is well known that the risk of *Fusarium* accumulation increases with rainfall and relative humidity, while an extremely high or low temperature decreases that incidence. Interestingly, none of the samples obtained from the Bekaa was positive for DON. This may suggest that all the local growing of cereals in Lebanon contain no detectable level of DON. The low level of DON in cereals found in the Lebanese market might be originated from imported samples. As more than 65% of cereal consumption was imported especially from the United States and Europe, the measurement of DON in cereals must be performed routinely to avoid high levels of DON contamination being found in Lebanese market.

Finally, the authors are now measuring the level of all type B trichothecene (3-acetyl DON, 15-Ac DON and nivalenol) in cereals with GC-MS as described by others (Schollenberger et al. 2007) and they are validating a method of the determination of type B trichothecene in bulgur.

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