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ASSESSMENT OF DEOXYNIVALENOL (DON) MYCOTOXIN IN CORN AND WHEAT GRAINS CONSUMED IN CENTRAL PUNJAB, PAKISTAN

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Abstract

Deoxynivalenol (DON) belongs to trichothecene mycotoxins having acute and chronic health hazards to human beings. The purpose of the current study was to assess DON concentration in corn and wheat samples (n = 200) collected from central Punjab, Faisalabad. Occurrence of DON in samples was analyzed by HPLC using UV-Vis detector in isocratic mode. Results indicated that 63% of corn samples were effected with DON residue. Out of these contaminated samples, 66% had higher residue level (1250 µg kg^{-1}) than the maximum limit as permissible by the European Commission Law (ECL) for DON. Among contaminated samples, 49.2% were found highly contaminated with DON with value of more than 1401 µg/kg. In the case of wheat grains, contamination of the DON was noticed in 56% samples with an average residue value of 1352 µg/kg. DON contamination level ranged from 1442-1524. The level of DON was compared among urban, rural and semi-urban areas and was detected to be highest in rural areas in both corn (1512 µg/kg) and wheat (1585 µg/kg) grains. The high concentration of trichothecene (DON) levels in corn and wheat grains may pose substantial health problems for the population of central Punjab, Pakistan and need urgent attention to address the issue of mycotoxins in staple food.

Key words: Cereals, HPLC-UV-Vis, Deoxynivalenol, health hazards.

Introduction

Mycotoxins (trichothecene) are Organic compounds and are formed by fungi having the ability to induce diseases of cancer, liver cirrhosis and failure of the immune system in human beings after taking contaminated corn and wheat (Wu *et al.*, 2014). A bunch of published data on the incidence of toxins in foodstuffs, either from one or multiple species of fungus, is available (Stoev, 2015) and presence of these toxins is a major worry due to danger of their exposure, which can be anticipated to exercise carcinogenicity and greater toxicity (Grenier & Oswald, 2011). Investigation have shown that around 25% of total crops of world per annum are affected by various types of mycotoxins (Negedu *et al.*, 2011).

Food safety is the prime concern globally and extensive work plans have been chalked out to save the people from toxic effects of minute residues of pesticides, heavy metals, polyaromatic hydrocarbons, mycotoxins, etc in food materials destined to humans. Typically, mycotoxins growth accelerated by food or feed substrates, moisture contents, temperature and time. Contamination can take place through the food chain from field to fork i.e. harvesting, processing, transportation, storage, and consumption (Anukul *et al.*, 2013). For raw substances, the pre-harvest residue level of mycotoxins is the utmost complicated portion of risk management.

Trichothecene mycotoxins, which are generally produced from *Fusarium* species, are of great importance due to their acute and chronic effects on living species especially in humans (Zinedine *et al.*, 2007; De Boevre *et al.*, 2012). On the Basis of toxigenicities of numerous mycotoxins, their regulatory levels have been established by different national governments and followed in international and national food trade.

Oxygen and moisture levels are major environmental factors affecting aflatoxin growth. Typically, aflatoxins G_1 & G_2 production normally occurs at 28°C, whereas growth of B_1 & B_2 aflatoxins having optimum temperature 11-37°C (OBrian *et al.*, 2007). Therefore, due to increased global and continental changes in temperature for the last ten years, in temperate regions, higher temperatures can assist the production of fungi which causes rapid growth of mycotoxins (Do *et al.*, 2015).

Cereals are a vital source of energy to sustain health globally because nearly 30% of daily calories are being received from staple foods (corn and wheat) in the developed countries and 60 to 80% in the poor countries and developing, respectively (Awika, 2011). Corn and wheat are good substrates for the attack of multiple fungi so these foods are the chief sources of mycotoxins exposure to their consumers. Deoxynivalenol (DON) belongs to type B of trichothecene mycotoxin having another name "vomitoxin" and occurs predominantly in corn, wheat, oat and some time in sorghum and rice. The main species of fungi which are the foremost producer of DON are *Fusarium culmorum* and *F. graminearum*, and both induce *Fusarium* Head Blight disease in barley, corn, oats, and wheat crops (Tanaka *et al.*, 1988). International Union for Pure and Applied Chemistry (IUPAC) suggested the name for DON as (3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one) with chemical formula $C_{15}H_{20}O_6$ having molar mass 296.319 g/mol^{-1}, m.p 151-153°C and white needles like structure. DON is very stable at pH 4 and 170°C (Wolf & Bullerman, 1998). International Agency for Cancer Research (IARC) declared DON as non-carcinogenic compound after executing *in-vivo* and *In vitro* experiments on different animals to prove its teratogenicity, cytotoxicity, genotoxicity, and immunotoxicity applying chronic and acute exposures (Sobrova *et al.*, 2010).

During pre-harvest and post-harvest steps (storage with high humidity) cereal materials are much affected by fungi (Piotrowska, 2013). This information might lead to mycotoxins contamination of cereals, including breakfast cereals. The detailed data about the presence of various mycotoxins formed by *Aspergillus* and *Fusarium* genus have been documented in food-stuff recently around the globe: trichothecenes, zearalenone, ochratoxin A and aflatoxins in Spain and Pakistan (Montes *et al.*, 2012; Iqbal *et al.*, 2014); ochratoxin A and aflatoxins in Greece (Villa & Markaki, 2009), different types of fumonisins in Morocco (Mahnine *et al.*, 2012); in Italy trichothecenes and zearalenone (Romagnoli *et al.*, 2010); in Canada zearalenone, trichothecenes and fumonisins (Roscoe *et al.*, 2008). DON also found in breakfast cereals in Portugal, by Cunha & Fernandes (2010) in the range of 46 and 525 mg kg^{-1}. Gareis (2003) assessed a residue level of zearalenone and deoxynivalenol in breakfast cereal consumed in Portugal, with an average residue level of 162 mg kg^{-1} and 5.1 mg kg^{-1}, respectively.

In this view, present study was planned with objectives 1) to access the level of DON contamination in cereal grains i.e. corn and wheat, 2) to establish that the DON concentration in tested samples is either within permissible limit or not 3) and to compare the DON level in tested samples collected from rural, semi-urban and urban areas. To our knowledge, this is the first attempt to detect this mycotoxin in tested cereal grains along with the focus on analyzing the effect of urban, semi-urban and rural environments on DON residue level.

Materials and Methods

Collection of samples: A total of 200 cereal grain (corn, and wheat, 100 samples for each) samples were collected randomly from rural, semi-urban, and urban regions of Faisalabad, Punjab, Pakistan (Fig. 1). The samples were collected directly from low-income residents (laborers), low holding farmers (1-2 acres), and shops located in the areas from November 2018 to April 2019. The size of every sample used in the study was approximately one (1) kilogram (kg). Corn and wheat samples were taken in plastic zipper bags, marked with lab identification codes, kept in an icebox, brought to Food Toxicology Lab, Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Punjab, Pakistan and stored in a freezer (Haier,) at– 4ºC, until further analytical evaluations.

Regents and chemicals: The certified reference standard of DON (Sigma-Aldrich, Steinheim, Germany, CAS Number 51481-10-8) was used. Other solvents like acetonitrile (ACN) and methanol (CH_3OH) were procured from Merc, Germany. Minimum analytical grade reagents and chemicals were used in this study. Solvents were used after distillation with the frictional distillation apparatus (Gallenkamp, Switzerland).

Extraction of DON: Samples of ground corn and wheat were extracted using methodology of Irakli *et al*., (2017) with minor changes. Samples (25 g) were precisely weighed using top loading balance (Sartorius, Germany) and put into 250 mL glass Erlenmeyer flasks with stopper, added 100 mL acetonitrile (ACN) and water (H_2O) (84:16, v/v) mixed with 12.5 mL n-hexane (C_6H_{14}). Samples were then homogenized using glass homogenizer (Braun Multipurpose blender, Germany) for 3 min at medium speed (500 rpm), and then undergo the centrifuged process for 15 minutes at 3500 revolutions per minute (rpm). The n-hexane (C_6H_{14}) layer appeared as the supernatant was discarded, filtered the leftover aqueous layer by using filter paper (Whatman # 4) and filtrate taken for cleanup. For DON, 9 mL extract was taken in test tube and Mycosep # 225 multifunctional cleanup column (MycoSep® 225 Trich. Art.No.: COCMY2225) was slowly pushed into glass tube and collected 4 mL purified extracts, and treated with nitrogen gas at 50º C for evaporation of extract to dryness. After drying residues was mixed with 400 µL volume of mobile phase and passed through nylon filter (0.22 µm) and for analysis of DON by HPLC (LC-10A) 20 µL of the solution was injected.

DON analysis by HPLC: All the cleaned extracts of corn and wheat grains were analyzed using the HPLC system coupled with a UV-Vis detector (SPD-10A). C_{18} column (Discovery) was used in HPLC for analysis of DON and its temperature was kept at 40ºC by column incubator (CTO 10A, Shimadzu). For the DON analysis mobile phase of methanol (CH_3OH), acetonitrile (ACN) and water (H_2O) having a ratio (45:45:10) with 1 mL flow rate per minute were used. The wavelength (**λ**) of the UV-Vis detector was set at 220 nm. All data of analysis were achieved by CLASS 10A software through Communication Bus Module (CBM-101, Shimadzu). The concentration of DON in samples were examined by the comparison of peak area with reference standard.

Quality control and recovery analysis: Performance parameters of HPLC, like Limit of quantifications (LOQ), Limit of detections (LOD), linearity, reproducibility, and repeatability of DON were performed (Table 1). The precision of method was assessed on the same day by two parameters i.e. repeatability and reproducibility with triplicate analysis (n= 3) at two different concentrations of spiked samples. Our study results have shown good linearity with a regression coefficient $R^2 \geq 0.99$ for all working solutions. The recoveries were assessed by spiking 50, 100 and 1000µg/kg and 5, 10, and 50 µg/kg of DON respectively in non-contaminated corn, wheat, and wheat flour samples. Good recoveries had been acquired in the range of 92–96% (50, 100, and 1000 µg/kg) and 90-95% (5, 10, and 50 µg/kg). The chromatogram of the DON standard is depicted in Fig. 2.

Statistical analysis

The acquired data of DON were subjected to analyze using Excel software for calculating concentrations in corn and wheat grains. Data were elaborated as mean with standard deviation and the regression coefficient (R^2) was examined by regression analysis by means of SPSS software (IBM, PASW Statistics19, USA).

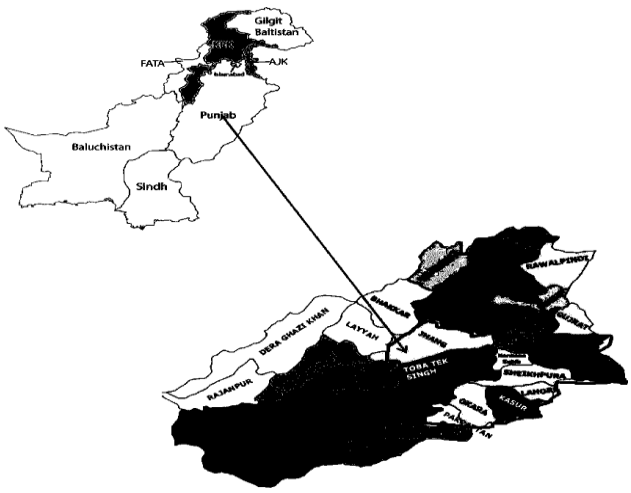

Fig. 1. Map of sampling areas from Central Punjab, Pakistan.

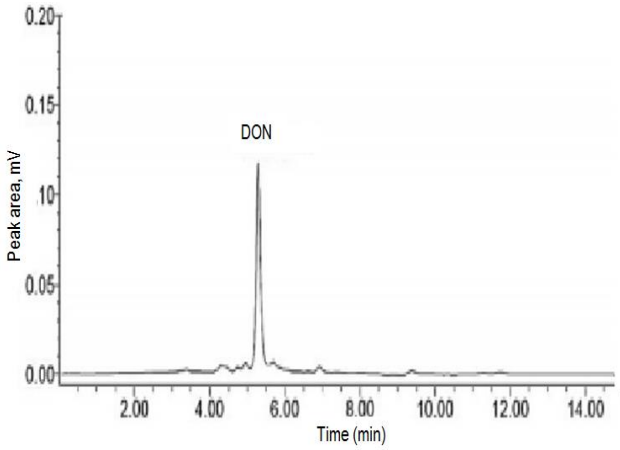

Fig. 2. Chromatogram of DON standard prepared in acetonitrile (5 µg/mL).

Table 1. HPLC quality control parameters.

Spiking level of DON (µg kg^{-1})	Recovery (%)	RSD %	Linearity (µg/kg)	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Precision	
							Repeatability	Reproducibility
50	94.40±0.55	6	1-50	0.99	0.80	2.40	6	9
100	94.80±0.84	9	5-100	0.98	0.85	2.55	8	10
1000	93.20±1.30	13	100-1000	0.99	0.90	2.70	8	14
5	92.00±2.0	11	0.5-5	0.95	0.92	2.76	12	13
10	90.67±2.08	12	1-10	0.98	0.81	2.43	10	12
50	93.00±2.64	9	1-50	0.99	0.80	2.40	13	14

LOD = Limit of detection; LOQ = Limit of quantification; LOQ = LOD x 3; RSD = Relative standard deviation; Repeatability and reproducibility are given as mean percent RSD (%)

Results and Discussion

Level of DON in corn grains: Cereal (corn & wheat) contamination by mycotoxins is one of the key problems for food safety due to its influence on animal and human health. Mycotoxins are ubiquitous in the environment produced by many fungi and *Fusarium* species are one of the main producers of mycotoxins. *Fusarium* species can grow on several agricultural products in the field and during storage under a conducive environment. Deoxynivalenol (DON) belongs to trichothecene mycotoxins having a polycyclic structure containing epoxide at carbon 12 and 13 that is responsible for inducing toxicity. Trichothecenes (DON very important mycotoxin due to its widespread diffusion on cereals,(in particular barley, oat, corn, wheat, rye and rice), animal feedstuffs (Klötzel *et al.*, 2005) and the health hazards exhibited on humans and animals (Hussein & Brasel, 2001).

Corn samples were taken from rural (ru), semi-urban (sur) and urban (ur) areas of Faisalabad, Punjab, Pakistan.

The results of corn samples from various areas of Faisalabad Division were collected and examined for the assessment of DON residue by HPLC equipped with UV-Vis detector (220 nm) are given in Table 2. The results showed that 63% of corn grain samples were effected with DON mycotoxin and high intensity of residue was noted in samples collected from rural (70%) than urban (65%) and semi-urban (57.5%) environments. The highest contamination of DON was detected in rural corn samples with a concentration of 1512 ± 224 µg kg^{-1}. Other samples belong to urban and semi-urban areas also contained mycotoxin (DON) residue with mean values of 1445 ± 223 µg/kg and 1354 ± 229 µg/kg respectively. These assessed concentrations of DON in samples of corn had high levels, much above than established limits of European Community Law i.e. 1250 µg kg^{-1} (Regulation, 2007).

Distribution data of DON mycotoxin in collected samples is displayed in Table 3. From the data, it was evident that only 21% corn samples were effected with DON below the permissible limits (1250 µg/kg) and other samples 42% had the concentration above the allowable level of European Community Law (ECL) and much samples fell > 1401 µg/kg (31%). The occurrence of samples of corn more than the ECL maximum level for the DON is elaborated in Fig. 3.

The mean contamination level of mycotoxin DON in the current study was low as compared to Pleadin *et al.*, (2013). They have determined the DON in maize collected from Croatia and noted that 45 out of 63 samples contain residues of DON within 215-1942 µg/kg having mean 1565 µg/kg, higher than the contamination found in the present study (LOD-1512 µg/kg). Tima *et al.*, (2016) investigated 29 samples, out of which 25 were contaminated. The authors reported DON in 86.2% of samples with mean level 1872 µg/kg and the maximum concentration was 2963 µg/kg. In another study conducted in Croatia during 2017, found DON contamination in 93.93% of maize samples with mean residues 564 µg/kg and the highest contamination was 2260 µg/kg (Pleadin *et al.*, 2017). The highest levels may contribute due to low temperatures and high rainfall of respective countries. The environment of Faisalabad, Pakistan, during sampling was cold with high humidity level and average rainfall was greater than 220 mm. Therefore, these environmental factors may favour for fungus attack and ultimately result in the high residue level of DON in corn.

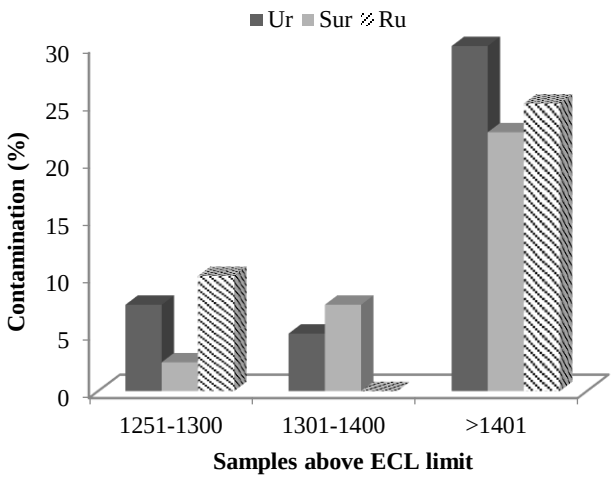

Fig. 3. The comparison of samples which exceed the ECL maximum limit in corn for DON (%).

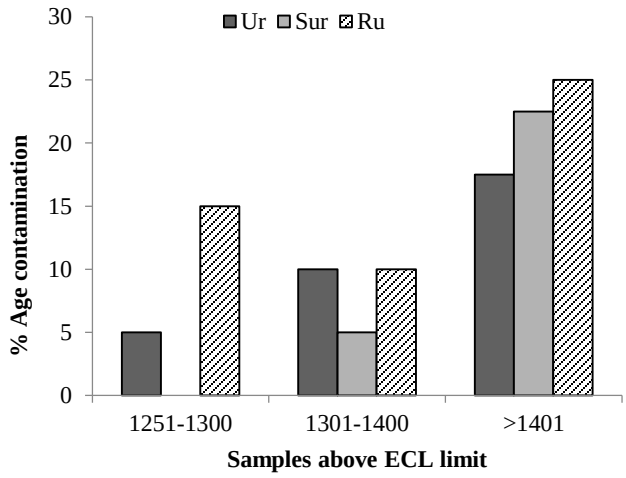

Fig. 4. The comparison of samples which exceed the ECL maximum limit in wheat for DON (%).

Table 2. DON concentration (µg kg^{-1}) in corn grain samples collected from Faisalabad Division.

Region	Total no. of samples	Contaminated samples (% age)	*Mean ± SD µg kg^{-1}
Ur	40	26 (65 %)	1445 ± 223
Sur	40	23 (57.5 %)	1354 ± 229
Ru	20	14 (70 %)	1512 ± 224

Ur = Urban; Sur = Semi-urban; Ru = Rural; *Data taken as mean of triplicate analyses ± standard deviation; values in parenthesis are % age contamination

Table 3. Distribution of DON levels (µg kg^{-1}) in corn samples.

Sampling area	Total sample	DON in corn ≤ 1250	DON in corn> 1250		
			1251-1300	1301-1400	> 1401
Ur	40	9 (22.5)	3 (7.5)	2 (5)	12 (30)
Sur	40	10 (25)	1 (2.5)	3 (7.5)	9 (22.5)
Ru	20	2 (10)	2 (10)	Nil	10 (25)
Total	**100**	**21 (21)**	**6 (6)**	**5 (5)**	**31 (31)**

*Ur= Urban; Sur = Semi-urban; Ru = Rural; Maximum permissible Limit (MPL for DON in corn is 1250 µg kg^{-1} European Community law (Regulation, 2007); Values in parenthesis show % age

Table 4. DON concentration (µg kg^{-1}) in wheat grain samples collected from Faisalabad Division.

Region	Total no. of samples	Contaminated samples (%age)	*Mean ± SD µg kg^{-1}
Ur	40	21 (52.5 %)	1352 ± 272
Sur	40	22 (55 %)	1464 ± 329
Ru	20	13 (65 %)	1585 ± 297

Ur = Urban; Sur = Semi-urban; Ru = Rural; *Data taken as mean of triplicate analyses ± standard deviation; values in parenthesis are % age contamination

Table 5. Distribution of DON levels (µg kg^{-1}) in wheat samples.

Sampling Area	Total sample (n)	DON in Wheat ≤ 1250	DON in wheat> 1250		
			1251-1300	1301-1400	> 1401
Ur	40	8 (20)	2 (5)	4 (10)	7 (17.5)
Sur	40	11 (27.5)	Nil	2 (5)	9 (22.5)
Ru	20	3 (15)	3 (15)	2 (10)	5 (25)
Total	**100**	**22 (22)**	**5 (5)**	**8 (8)**	**21 (21)**

Ur= Urban; Sur = Semi-urban; Ru = Rural; Maximum permissible Limit (MPL) for DON in wheat is 1250 µg kg^{-1} European Community law (Regulation, 2007); Values in parenthesis show % age

Level of DON in wheat: Mostly, wheat is used as a staple food throughout Pakistan after processing in the form of flour but some portion (10%) of wheat production is being used to feed livestock in raw form. Samples of wheat were taken from territories of Faisalabad Division, Punjab, Pakistan and studied for the presence of DON mycotoxin. The data of contaminated samples of wheat is presented in Table 4.

From the data, it is clear that 56% out of 100 samples were contaminated with DON. The maximum concentration was assessed in rural areas (65%) with average level of 1585 ± 297 µg/kg as compared to semi-urban (Sur) and urban (Ur). The lowest contamination percentage was found in urban area samples with a mean concentration of 1352 ± 272 µg/kg. A distribution study of DON mycotoxin was carried out and findings are given in Table 5. The results indicated that 22% of samples of wheat were contaminated with DON below the maximum concentration of ECL and the remaining 34% had the concentration above the established limits of ECL (1250 µg kg^{-1}). High intensity of samples (21%) contained residues of DON greater than 1401 µg kg^{-1}. The prevalence of DON % age in wheat samples above the ECL limit is shown in Fig. 4.

The mean concentration of DON mycotoxin in the current study was low as compared to Bryła *et al*., (2016). The authors estimated the DON in wheat collected from Poland and observed that 100% of samples were affected by DON with a concentration range of 82-2975 µg/kg having mean 770.70 µg/kg bout in the undertaken study, the level was LOD-1585 µg kg^{-1}. Studies investigated high reside levels of DON in wheat as compared to the results of the current study was reported in Finland. Hietaniemi *et al*., (2016) have been analyzed a total of 61 samples, out of which 28 (45.90%) were contaminated. The authors reported DON with mean level 420 µg/kg and the highest concentration was 2224 µg/kg. In another study carried out in Norway during 2016on the DON contamination in wheat samples (Hofgaard *et al.*, 2016). A total of 178 samples were analyzed and 91.57% of samples of wheat contained DON with a high level of 16000 µg kg^{-1}. The incidence of a

high ratio of DON in wheat may be due to low temperatures and high rainfall of countries belonging to the temperate zone. The climate of Faisalabad, Pakistan, during sampling (November 2018 to April 2019) was cold with high moisture contents and average rainfall was greater than 220 mm. Therefore, fungal attack may be accelerated by these environmental factors, causing high contamination levels of DON in wheat.

Conclusions

In conclusion, high DON mycotoxin residue levels were detected in corn and wheat samples belong to Central Punjab, Pakistan destined to residents. It is suggested that strategies should be developed for the reduction of DON contamination levels in the cereal grains. The results are very useful for consumers of the area and authorities responsible for law enforcement to apply strict regulations and to perform regular monitoring.

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