
Article

Variation of Deoxynivalenol Levels in Corn and Its Products Available in Retail Markets of Punjab, Pakistan and Estimation of Risk Assessment

Abstract: The study focused on investigating the natural incidence of deoxynivalenol (DON) in corn and products from corn producing districts of Punjab, Pakistan. The analysis was carried out using HPLC with UV detector and immunoaffinity cleanup columns. The detection limit (LOD) and limit of quantification were 25 and 50 µg/kg, respectively. Total 1220 samples of corn and products were analyzed to detect the DON, and 539 (44.2%) samples were observed to be contaminated with DON ($n \geq \text{LOD}$). Furthermore, 92 (7.5%) samples of corn and corn products have DON levels, elevated than the proposed limits of the EU. The data is significantly different from a normal distribution for DON in corn and products samples and from different locations ($p < 0.05$) for Shapiro-Wilk and Kolmogorov-Smirnov values. However, a significant difference in DON levels was found between corn and corn derived-products types ($p \leq 0.05$). The lowest and highest exposure, and hazard quotient (HQ) of 0.92 and 9.68 µg/kg bw/d were documented in corn flour samples.

Keywords: DON; corn; corn products; exposure; risk assessment

Key contribution: A total of 1220 samples of corn and corn products from three districts of Punjab were analyzed for DON. Samples 539 (44.2%) of corn and corn products were found to be positive with DON ($n \geq \text{LOD}$). Samples 92 (7.5%) of corn and corn products having levels of DON greater than the proposed limits of EU (1000 µg/kg; 750 µg/kg). The lowest and highest exposure & HQ level of 0.92 and 9.68 µg/kg bw/d were documented in corn flour samples

1. Introduction

Corn (*Zea mays*) is the third main producing crop in Pakistan after wheat and rice. Its share in total cereal cultivations area is 8.5%, 2.2% to the value-added in the agriculture sector, and 0.4% to gross domestic product (GDP). Corn plays a vital role in the country's economy by its multiple uses for domestic, commercial, and industrial purposes. In 2017-18, corn production is predicted 5.8 million tons, 3 percent higher than the previous year reflects its demands in-country. The hybrid corn is cultivated about 40% of the corn area, and its share in total corn production is around 70%. About 65% of corn is utilized in the poultry industry, 10% in dairy feed, 15% in wet milling, and remaining for human consumption and seeds [1]. Corn is the cereal prone to be attacked by fungus and thus subsequently for mycotoxins production (e.g., DON) [2, 3].

Mycotoxins are recognized as secondary metabolites and produced by fungi, mainly *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Cladosporium* [4-7]. *Fusarium* is widely dispersed in nature and cause spoilage or deterioration of food and feeds [8]. DON, also identified as vomitoxin, is a secondary fungal metabolite to the class of trichothecenes and produced by the genus *Fusarium*, especially *F.graminearum* [9-13]. The previous studies have documented the toxic effects of trichothecenes, such as feed refusal, hemorrhage vomiting, diarrhea, anemia and immunosuppression [14-16].

In previous study, 63 out of 100 samples were found to be contaminated with DON as documented by Raza et al. [17], from rural, semi-rural and urban areas from central cities of Punjab, Pakistan. The results had indicated that 63% samples of corn were contaminated with DON, and from these samples almost 66% samples have levels higher than 1250 µg/kg. Furthermore, 49.2% samples have levels high than 1401 µg/kg. The

results have indicated that the levels were higher in samples from rural areas samples for both in corn (1512 µg/kg) and wheat (1585 µg/kg) grains. Similarly, the effect of seasonal variation on the levels of DON in wheat and corn samples were investigated from Punjab, Pakistan [18]. The results have shown that 87 (61.2%) samples of corn and corn products from summer and 57 (44.5%) from winter season were found to be contaminated with concentration ranged from 50 to 2967 µg/kg, and 50 to 2490 µg/kg, respectively.

The countries and organization have established maximum limits for DON in raw and processed cereal products. The Codex Alimentarius Commission has established permissible limit of 2000 µg/kg in raw barley, wheat, and corn. Similarly, EU has also established a limit of 2000 µg/kg in unprocessed wheat, and oat. However, a permissible legal limit of 1000 µg/kg for raw or those cereals which would be unprocessed, and 750 µg/kg for foodstuffs, intended for consumers and dry pasta and 200 µg/kg for snacks and breakfast cereals used for children [19]. The tolerance daily intake for DON is proposed as 1 µg/kg/bw per day.

Pakistan is located in a tropical part, and therefore, the environmental conditions are encouraging for fungal growth and production [5, 20]. Although corn is a major cash crop and previous studies have shown, this substrate is more vulnerable to fungi attack and contamination. However, there is not enough information available on the prevalence of DON in corn-derived foods in Pakistan. Consequently, the existing study was designed to examine the incidence levels of DON in corn and corn-derived products and compare the levels with EU recommended regulations. The exposure assessment of DON in corn and products will also help disseminate the information to farmers, traders, and other stakeholders in Pakistan.

2. Results

2.1. Method Validation

The recoveries analysis of DON in corn and corn products were performed as presented in Table 1. The recoveries were in the range of 81.3 to 91.0%, and the relative standard deviation was varied from 11 to 28%. The detection limit (LOD) and limit of quantification (LOQ) were 25 µg/kg and 50 µg/kg, respectively. The straight-line equation was constructed, and the coefficient of determination (0.9992) was obtained.

Table 1. Recovery percentage of DON in corn and products.

DON level µg/Kg	Corn (mix)				Corn flour				Cornbread				Sweet Corn			
	Recovery (%)	RSD (%)	Precision (%)	Reprod ^b	Recovery (%)	RSD (%)	Precision (%)	Reprod ^b	Recovery (%)	RSD (%)	Precision (%)	Reprod ^b	Recovery (%)	RSD (%)	Precision (%)	Reprod ^b
100	84.5	16	11	21	81.5	11	12	19	89.5	14	9	20	85.2	16	10	21
150	89.5	11	18	19	86.5	14	09	15	83.5	18	15	26	89.5	17	14	17
300	88.0	22	15	22	87.4	17	16	22	90.5	23	10	18	89.5	15	9	21
400	91.0	13	18	24	90.9	12	14	20	89.5	22	12	22	88.4	19	17	26
800	88.8	23	10	22	86.1	16	18	26	88.4	21	15	20	85.2	21	15	24
3000	85.0	21	18	28	84.0	20	20	27	85.5	20	18	27	81.3	28	18	28

RSD = relative standard deviation, LOD 25 µg/kg, LOQ= 50 µg/kg, a = Repeatability, b = Reproducibility.

2.2. Incidence of DON in Corn and Products

The occurrence of DON in 1220 samples of corn and corn products (449 samples from Gujranwala, 378 from Hafizabad, and 393 samples from Sheikhpura) were presented in Table 2-4, respectively. Samples 539 (44.2%) were found to be positive ($n \geq \text{LOD}$) with DON in corn and products. In 449 samples from Gujranwala, 197 (43.9 %) samples of corn and corn products were found to be positive with DON (Table 2).

Table 2. Occurrence of DON in corn and corn products from Gujranwala, Punjab, Pakistan.

Sample type	Samples N	Positive N (%)	Mean ($\mu\text{g/kg}$) \pm S.D.	Range ($\mu\text{g/kg}$)
Corn 1	40	18 (45.0)	1256.2 \pm 124.5	25- 5687.5
Corn 2	45	22 (48.9)	1126.2 \pm 90.8	25- 5568.6
Corn 3	54	15 (27.8)	1049.7 \pm 110.5	25-5678.5
Corn 4	41	22 (53.7)	909.8 \pm 120.5	25-4550.5
Corn flour	60	34 (57.6)	854.7 \pm 40.7	25-8990.5
Sweet corn	83	30 (36.1)	556.5 \pm 80.6	25-4550.5
Corn bread	35	18 (51.4)	86.6 \pm 14.5	25-450.5
Corn oil	56	22 (39.3)	88.8 \pm 15.5	25-980.5
Popcorns	35	16 (45.7)	41.4 \pm 10.5	25-95.5
Total	449	197 (43.9)		25-8990.5

Corn 1= corn variety; corn 2= corn variety; corn 3 = Corn variety; corn 4= Corn variety, N (%) = n (percentage of samples), LOD= 25 $\mu\text{g/kg}$.

The maximum average of 1256.2 \pm 124.5 $\mu\text{g/kg}$ of DON was found in corn type 1 samples and varied from 25 to 5687.5 $\mu\text{g/kg}$. Table 3, 166 (43.9%) out of 378 samples of corn and products from Hafizabad, were positive with the maximum average of (1489.4 \pm 190.8 $\mu\text{g/kg}$) in corn type 4 samples, ranged from 25-5540.5 $\mu\text{g/kg}$. Furthermore, 176 (44.8%) out of 393 samples of corn and products from Sheikhupura were found to be contaminated with DON, and the maximum average (1489.4 \pm 180.5 $\mu\text{g/kg}$) was found in corn type 4 samples with concentration ranged from 25-5540.5 $\mu\text{g/kg}$. About 92 (7.5%) samples of corn and corn products were found to be contaminated with DON greater than EU [19] proposed limits as represented in Figure 1.

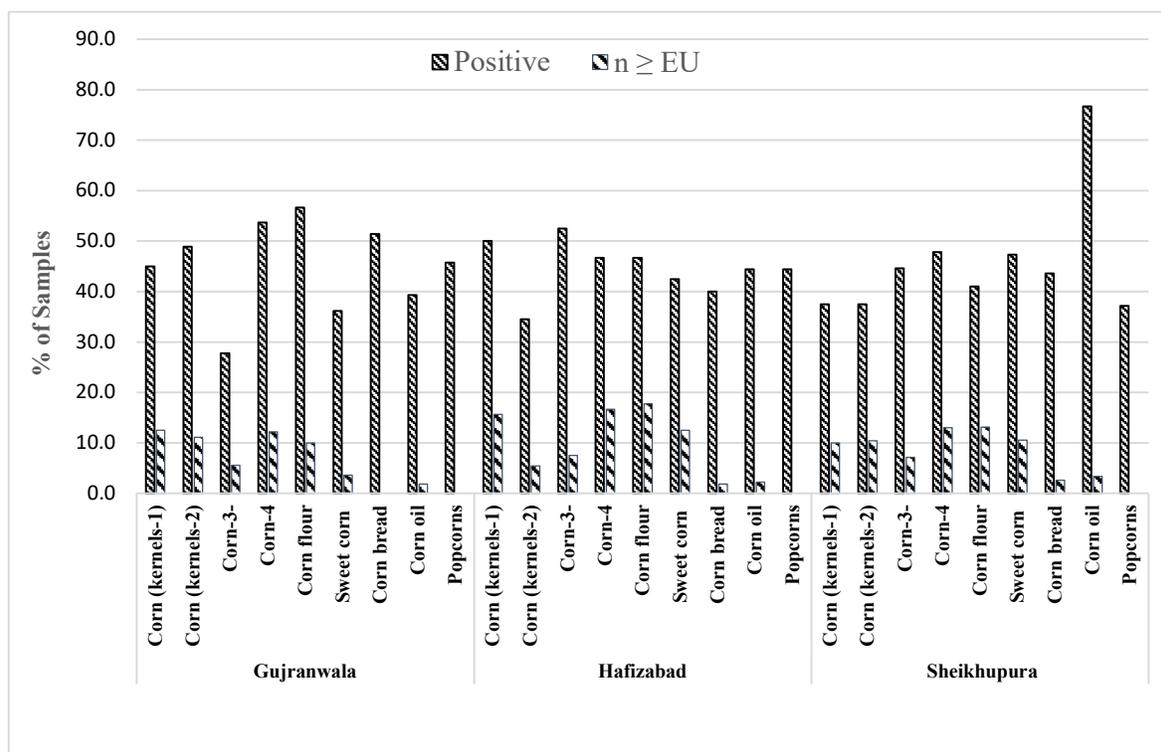


Figure 1. Percentage of sample of corn and corn products having levels of DON higher than recommended limits of EU (1000 $\mu\text{g/kg}$ for unprocessed corn and 750 $\mu\text{g/kg}$ for foodstuff) .

Table 3. Occurrence of DON in corn and corn products from Hafizabad, Punjab, Pakistan.

Sample type	Samples N	Positive N (%)	Mean ($\mu\text{g}/\text{kg}$) \pm S.D.	Range ($\mu\text{g}/\text{kg}$)
Corn 1	32	16 (50.0)	1187.3 \pm 210.5	25-6660.5
Corn 2	55	19 (34.5)	1081.6 \pm 140.3	25-6590.6
Corn 3	40	21 (52.5)	808.2 \pm 70.5	25-5640.5
Corn 4	30	14 (46.7)	1489.4 \pm 190.8	25-5540.5
Corn flour	45	21 (46.7)	1030.8 \pm 105.4	25-7860.5
Sweet corn	40	17 (42.5)	471.1 \pm 20.5	25-2345.5
Corn bread	55	22 (40.0)	97.3 \pm 15.5	25-500.5
Corn oil	45	20 (44.4)	88.3 \pm 9.5	25-540.5
Popcorns	36	16 (44.4)	42.2 \pm 4.5	25-80.5
Total	378	166 (43.9)		25-7860.5

Corn 1= corn variety; corn 2= corn variety; corn 3 = corn variety; corn 4= corn variety, N (%) = n (percentage of samples), LOD= 25 $\mu\text{g}/\text{kg}$.

Table 4. Occurrence of DON in corn and corn products from Sheikhpura, Punjab, Pakistan.

Sample type	Samples N	Positive N (%)	Mean ($\mu\text{g}/\text{kg}$) \pm S.D.	Range ($\mu\text{g}/\text{kg}$)
Corn 1	48	15 (37.5)	1187.3 \pm 230	25-6660.5
Corn 2	48	18 (37.5)	1081.6 \pm 145.5	25-6590.6
Corn 3	56	25 (44.6)	808.2 \pm 60.5	25-5640.5
Corn 4	46	22 (47.8)	1489.4 \pm 180.5	25-5540.5
Corn flour	61	25 (41.0)	1030.8 \pm 170.5	25-7860.5
Sweet corn	38	18 (47.4)	471.1 \pm 30.5	25-2345.5
Corn bread	39	17 (43.6)	97.3 \pm 15.5	25-500.5
Corn oil	30	23 (76.7)	88.3 \pm 9.5	25-540.5
Popcorns	35	13 (37.1)	42.1 \pm 4.5	25-90.5
Total	393	176 (44.8)		25-7860

Corn 1= variety of corn; corn 2= corn variety; corn 3 = variety of corn; corn 4= variety of corn, N (%) = n (percentage of samples), LOD= 25 $\mu\text{g}/\text{kg}$.

The data is significantly different from a normal distribution of DON's levels in different types of corn and products and to different locations, considering the high values of skewness and kurtosis and $p \leq 0.00$ for Shapiro-Wilk Kolmogorov-Smirnov values. The ANOVA of DON levels in different corn and corn products was statistically significant ($p \leq 0.000$). The amounts of DON in corn and corn products from different locations were non-significant ($p \geq 0.05$), as shown in Table 5a. The least significant difference (LSD) of which type of corn and corn products were significant compared to other types are shown in Table 5b.

Table 5a. ANOVA of DON levels in corn and corn products and from various locations.

		Sum of Squares	df	Mean Square	F	Sig
DON level	Between Groups	96192972.7	8	12024121.6	8.132	0.000
	Within Groups	783676623.6	530	1478635.1		
	Total	879869596.4	538			
Location	Between Groups	4.737	8			
	Within Groups	367.4	530	0.592	0.854	0.555
	Total	372.2	538	0.693		

Table 5b: LSD analysis of different types of corn and corn products within groups samples.

Don Level	Corn 1	Corn products	Mean difference	Std. Error	Significance
		Sweet corn	629.92	230.05	0.006
		Corn bread	1066.63	236.89	0.000
		Com oil	1073.58	230.05	0.000
		Popcorns	1130.06	251.06	0.000
	Corn 2	Sweet corn	542.88	218.65	0.013
		Corn bread	979.59	225.83	0.000
		Com oil	986.53	218.65	0.000
		Popcorns	1043.03	240.66	0.000
	Corn 3	Corn bread	842.21	224.01	0.000
		Com oil	849.16	216.76	0.000
		Popcorns	905.65	238.95	0.000
	Corn 4	Sweet corn	483.11	219.64	0.028
		Corn bread	919.82	226.79	0.000
		Com oil	926.77	219.64	0.000
		Popcorns	983.26	241.56	0.000
	Corn flour	Corn bread	772.55	210.76	0.000
		Com oil	779.50	203.05	0.000
		Corn popcorns	835.99	226.58	0.000
	Sweet corn	Com Type 1	-629.91	230.05	0.006
		Corn type 2	-542.88	218.65	0.013
		Corn type 4	-483.11	219.64	0.028
		Corn bread	436.70	220.65	0.048
		Com oil	443.65	213.29	0.038
		Popcorns	500.15	235.81	0.034
	Corn bread	Com Type 1	-1066.62	236.89	0.000
		Corn type 2	-979.59	225.83	0.000
		Corn type 3	-842.21	224.01	0.000
		Corn type 4	-919.82	226.79	0.000
		Corn flour	-772.55	210.76	0.000
		Sweet corn	-436.70	220.65	0.048
	Corn oil	Com Type 1	-1073.57	230.05	0.000
		Corn type 2	-986.53	218.65	0.000
		Corn type 3	-849.15	216.76	0.000
		Corn type 4	-926.73	219.64	0.000
		Corn flour	-779.50	203.05	0.000
		Sweet corn	-443.65	213.29	0.038
	Popcorns	Com Type-1	-1130.06	251.06	0.000
		Corn type 2	-1043.03	240.66	0.000
		Corn type 3	-905.65	238.95	0.000
		Corn type 4	-983.26	241.56	0.000
		Corn flour	-835.99	226.58	0.000
		Sweet corn	-500.15	235.81	0.034

d.f. = degree of freedom; F= F test; significance= p value

2.3. Estimation of Exposure Assessment of DON in Cornflour

The assessment of exposure of DON levels in corn flour from different locations is presented in Table 6. The DON's highest exposure was 9.68 $\mu\text{g}/\text{kg}$ bw/d, and the lowest exposure was 0.92 $\mu\text{g}/\text{kg}$ bw/d in corn flour samples from Gujranwala, District.

Table 6. Exposure assessment of DON in corn flour from corn producing cities of Punjab, Pakistan.

Consumption (kg)	Gujranwala				Hafizabad				Sheikhupura				
	DON levels		Exposure		DON levels		Exposure		DON levels		Exposure		
	Mean $\mu\text{g}/\text{kg}$	Highest level $\mu\text{g}/\text{kg}$	Mean $\mu\text{g}/\text{kg}$ bw/d	Highest $\mu\text{g}/\text{kg}$ bw/d	Mean $\mu\text{g}/\text{kg}$	Highest level $\mu\text{g}/\text{kg}$	Mean $\mu\text{g}/\text{kg}$ bw/d	Highest $\mu\text{g}/\text{kg}$ bw/d	Mean $\mu\text{g}/\text{kg}$	Highest level $\mu\text{g}/\text{kg}$	Mean $\mu\text{g}/\text{kg}$ bw/d	Highest $\mu\text{g}/\text{kg}$ bw/d	
Corn flour	0.07	854.7	8990.5	0.92	9.68	1030.8	7860.5	1.11	8.47	1030.8	7860.5	1.11	8.47
HQ ¹			0.92				1.11					1.11	
HQ ²				9.68				8.47					8.47

HQ¹= mean level of DON, HQ²= highest level of DON.

3. Discussion

3.1. Method Validation

The results have shown that the analytical parameters like accuracy and precision of DON in corn and corn products were within the European Commission's recommended guidelines [22]. According to the guidelines, the recoveries of DON should be within 60 % to 110 %, and the repeatability and reproducibility should be ≤ 20 % and ≤ 40 %, respectively. Furthermore, for DON value > 500 $\mu\text{g}/\text{kg}$ level, the recommendations are 70 to 120 % recoveries, and repeatability and reproducibility must be less than 20 % and 40 %, respectively [22-23]. The determination coefficient was ≥ 0.999 , and LOD and LOQ were 25 and 50 $\mu\text{g}/\text{kg}$, respectively. The coefficient of determination was quite similar with the value from our previous study Iqbal et al. [18]. However, the LOD and LOQ are much lower in the current study. Ok et al. [23] have documented the value of the coefficient of determination ≥ 0.999 , and LOD and LOQ were 6.4 to 10.0 $\mu\text{g}/\text{kg}$ and 21.3 to 33.5 $\mu\text{g}/\text{kg}$. Yang et al. [24] have demonstrated the LOD of 12.2 $\mu\text{g}/\text{kg}$ for DON in corn samples using SPE cleanup. In another study, relatively high LOD and LOQ for DON, i.e., 30 and 40 $\mu\text{g}/\text{kg}$, were documented in milling fractions of wheat using multifunctional column cleanup [25]. Golge and Kabak, [26] have determined the LOQ of 46.90 to 72.30 $\mu\text{g}/\text{kg}$ of DON in various cereal products, higher than the LOQ of the present study.

3.2. Incidence of DON in Corn and Corn Products

The results have demonstrated comparatively high DON in corn and corn products from three districts, i.e., Gujranwala, Hafizabad, and Sheikhupura of Punjab, Pakistan. In our previous study, [18] we have investigated the seasonal variation of DON levels in corn and corn products and documented that samples from summer have a higher incidence, i.e., 87 (61.2%) as compared to the samples from the winter season (44.5%). The maximum average observed was 1434.8 ± 25.5 $\mu\text{g}/\text{kg}$ in corn flour samples from the summer season, and the lowest mean level was found in cornbread 620.8 ± 17.8 $\mu\text{g}/\text{kg}$, from the winter season. A high incidence level of DON compared to the present finding results was observed from Spain, and almost 68% of commercial corn-based food samples were found to be positive, with levels ranged from 29 to 195 $\mu\text{g}/\text{kg}$ [27]. From Turkey, Golge, and Kabak [26] have observed 13 wheat samples (58 to 1092 $\mu\text{g}/\text{kg}$), three barley (138 to 973 $\mu\text{g}/\text{kg}$), seven paddy rice (136 to 256 $\mu\text{g}/\text{kg}$), three wheat flour (92 to 151 $\mu\text{g}/\text{kg}$), two biscuits (31.2 to 71.3 $\mu\text{g}/\text{kg}$), and one pasta sample (59.3 $\mu\text{g}/\text{kg}$). However, two corn samples were found contaminated with DON (313 to 331 $\mu\text{g}/\text{kg}$). All the samples have levels below the recommended EU limits.

The high amount of DON (42 to 4130 $\mu\text{g}/\text{kg}$ with a average amount of 977 ± 1000 $\mu\text{g}/\text{kg}$) in wheat and cereals samples from Austria, Germany, Slovakia, and Australia [28], was documented. From Cameroon, Njobeh et al. [29] documented 65% samples out of 82 samples of dried food commodities with DON levels ranged from 13 to 273 $\mu\text{g}/\text{kg}$. High occurrence and maximum amount of DON in corn compared to the current study results were reported by Paladin et al. [30] from Croatia. They reported 85% of samples were found positive with the highest concentration of 17920 $\mu\text{g}/\text{kg}$. In Portugal, Marques et al. [31] have analyzed 307 samples of plant crops, the mean of DON concentrations 70 $\mu\text{g}/\text{kg}$, the highest amount was 17,900 $\mu\text{g}/\text{kg}$, respectively. Vidal et al. [32] from Spain have observed DON in 42% bran samples with the highest amount of 6178 $\mu\text{g}/\text{kg}$, and thirteen samples (19%) exceeded the EU allowable limit. Serbia, Jajić et al. [33] have documented that 32% of corn samples were discovered to be contaminated, and 86% of samples were discovered contaminated with DON from Hungary [34].

However, earlier studies have documented low levels of DON compare to the findings of the present survey. Escobar et al. [3], from Spain, have analyzed 25 corn oils samples with mean levels of DON were 31 $\mu\text{g}/\text{kg}$. Similarly, Giménez et al. [35], from Spain, have found 10 out of 25 wheat germ oil samples with mean levels of DON 41 $\mu\text{g}/\text{kg}$.

It is worth mentioning that regional variation was found in DON levels. Hot weather conditions promote the formation of fusarium-producing fungi and the production of mycotoxins [36]. The elevated prevalence of DON in the summer season compared to the

winter season in corn and corn products has been confirmed in our previous study [17]. Fernandez et al. [37], from the US, have documented lower DON levels in cereal samples than the levels reported by Ngoko et al. [38] in corn from Cameroon. The temperate environmental conditions favor the production of DON [39]. Furthermore, high levels of humidity as a combination of high temperature could enhance fungi growth rate belongs to genus *Fusarium* [40]. The variation in sampling size is another factor that should be considered a significant source of variation in mycotoxins [41]. The elevated amount of DON in corn and corn products could be explained because farmers in Pakistan still use old traditional methods in rural areas with cheap varieties of corn and not using crop rotation or no-tilled. Furthermore, the storage of corn in mud bins could also enhance the contamination of cereal crops [42].

3.3. Exposure Assessment of DON in Cornflour

In our previous study Iqbal et al. [18], from the summer season, the maximum exposure of DON was observed in wheat flour, i.e., 8.8 $\mu\text{g}/\text{kg}$ b.w/day. According to WHO, [43], if $\text{ADD} > \text{PMTDI}$, then the potential for health risk exists. The individual or population may suffer a health risk if $\text{HQ} > 1$. The health risks were limited if individual or population exposed the exposure of DON less than 1 i.e., If $\text{HQ} < 1$. The exposure of 1.052 $\mu\text{g}/\text{kg}$ b.w/day of DON was estimated in bread and toasts [44], comparable to the exposure of DON in current research. The exposure of 0.027 to 0.038 $\mu\text{g}/\text{kg}$ b.w/day was observed by Cano-Sancho et al. [45]. The high level of 1.11 of HQ from current research from Hafizabad and Sheikhpura districts could cause serious risk factors in the local population.

4. Conclusions

Evaluating the research findings and evaluating them to the legitimate limit of the EU for mycotoxins in current work reflects that the prevalence and amounts of DON were comparatively high. About 7.5% of samples of corn and corn products were contaminated with DON at levels greater than the EU suggested limits. The exposure and HQ amount of DON in corn and products were also high. Adopting good storage practices would reduce the level of DON in cereals because generally, in Pakistan, the cereals crops are stored and transported in jute bags. The jute bags could absorb moisture from the environment or surface where the crop was stored. The recommendation could be to store these crops in polyethylene bags during transport and storage area. Furthermore, the moisture, humidity, and temperature should be controlled during the storage period, and farmers should use resistant crop varieties. In-vivo study of DON levels in blood and urine in local consumers should be more desirable for the assessment of exposure and implementation of regulations in population.

5. Materials and Methods

5.1. Sampling

The 1220 samples of corn and products (corn flour, sweet corn, cornbread, corn oil, and popcorn) from three Punjab districts ($n = 449$ Gujranwala, $n = 378$ Hafizabad, $n = 393$ Sheikhpura) were collected during June 2018 to January 2019. A simple random methodology (each portion or lot has equal chance to be included) was used for collecting corn and corn products samples from farmers, market, and superstores. The gross samples were taken by hands and then homogenized and proper labelling was done. These three districts are most famous for producing corn in Punjab, Pakistan. Due to the high variability of fungi and mycotoxins in kernel samples, each sample size was not less than 2 kg. Then the kernel samples were mixed and ground in adequate particle size (Retsch, ZM 200, Haan, Düsseldorf, Germany). The lab sample portion was stored in plastic bags and kept in the freezer at $-20\text{ }^{\circ}\text{C}$.

5.2. Chemicals and Reagents

A pure standard of DON (100 mg/mL in ACN) from Sigma Aldrich (Saint-Louis, MO, USA) was available in the lab (Food Safety lab). High-grade purity ($\geq 99\%$) solvents of acetonitrile, methanol, and polyethylene glycol 8000 (PEG), were acquired from Sigma-Aldrich (Sigma-Aldrich, Lyon, France). The method's linearity was assessed with seven-point concentrations (100, 200, 800, 1600, 3200, 6400, and 9000 $\mu\text{g}/\text{kg}$) of DON and stored in capped vials at temperature $-20\text{ }^{\circ}\text{C}$. All other chemicals used were of analytical grade and available in the lab.

5.3. Extraction and HPLC Parameters

The extraction of DON in corn and products were carried out using our previously validated method [17]. Briefly, the sample (5 g) and PEG (1 g) were mixed in 20 mL of ultra-pure water and homogenized in 50 mL of Teflon tubes, and centrifuged at room temperature for one minute at 6500 rpm. The extraction of cornbread was carried out by adding 200 mL water on dry bread and then homogenized for 5 minutes at room temperature at 8000 rpm as discussed above. After centrifugation, the mixture was filtered and the filtrate (5 mL) was passed through to the immunoaffinity column (IAC-NIV WB, columns) (VICAM, Watertown, MA, USA). The column was washed twice with 10 mL purified water, and the DON was eluted using 1 mL of pure methanol from the IAC column. Then, 0.5 mL of pure water was added to 0.5 mL of eluting and subjected to HPLC analysis, after passing through 0.22 μm nylon syringe filters. The Shimadzu (Kyoto, Japan) HPLC system with C18 Supelco column (250 x 4.6 x 5 mm) (Discovery HS, Bellefonte, PA, USA). The UV detector (RF-530) was used setting at a detection wavelength of 218 nm. The mobile phase was 30% methanol and 70% of water with a flow rate of 1.2 mL/min.

5.4. Exposure Assessment

In the current research, the level of DON in food from literature is considered reasonable. Further, it was believed that the exposure to DON was only through corn flour due to the main ingredient in the Pakistani diet. The mean levels of DON in corn flour and highest levels were used to assess exposure analysis, and an adult body weight is regarded as 65 kg b.w. The per capita consumption of corn flour was 0.07 kg [1]). The exposure was assessed using the formula of eq 1.

$$\text{ADD}_{\text{DON}} = \frac{C_{\text{DON}} \times \text{IR}}{\text{bw}} \quad \text{eq-1}$$

ADD_{DON} = average daily dose of DON

C_{DON} = Concentration of DON in corn flour

IR = intake rate of corn flour

bw = body weight

The hazard quotient (HQ) of the mean and highest level of DON in corn flour were also estimated and equaled to exposure assessment studies. The provisional maximum tolerable daily intake (PMTDI) was 1 $\mu\text{g}/\text{kg}$ BW for DON [20]. The HQ was determined following the formula shown in eq 2.

$$\text{HQ} = \frac{\text{ADD}_{\text{DON}}}{\text{PMTDI}} \quad \text{eq 2}$$

5.5. Method Validation

All the method validation parameters were performed for the assessment of DON in corn and corn products. The recoveries analysis was performed. The fortified levels (100, 150, 300, 400, 800, 3000 $\mu\text{g}/\text{kg}$) of DON were added in uncontaminated samples of corn and product samples. The LOD and LOQ were assessed as 3:1 and 10:1 of signal-to-noise ratio, respectively.

5.6. Statistical Analysis

The results of DON levels in corn and corn products were subjected to statistical analysis using SPSS (version 26 for Windows, SPSS Inc., Chicago, USA). The DON levels in different corn samples and from different locations were checked for normal distribution (Shapiro-Wilks test). ANOVA was applied to investigate the difference of means (DON levels) between different types of corn and products and from different locations.

The least significant difference (LSD) was used to investigate differences within each type or each location. A probability value of 0.05 was used to determine the statistical significance.

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