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Distribution of Fusarium mycotoxins in wheat milling process

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ABSTRACT

Fusarium head blight (FHB) is a fungal disease that affects cereals and is capable of producing mycotoxins, creating health concerns. In southern Brazil, FHB of wheat is caused by the *Fusarium graminearum* species complex that produces mainly deoxynivalenol (DON) and zearalenone (ZON) mycotoxins. There is a need for research-based information on how different contamination levels affect these mycotoxins' distribution in the milling process. The objective of this study was to analyze the Fusarium mycotoxin distribution within each milled fraction, extracted from wheat lots artificially contaminated with a crescent gradient of mycotoxins. Wheat samples produced in 2013 season in Southern Brazil region were obtained from plots of breeding program. The wheat samples were artificially contaminated with residues of cleaning and pre-cleaning process, including light and shriveled grains, obtained from a Fusarium nursery screening plot. Pilot-scale milled wheat fractions were collected, comprising finished flour and bran. The Fusarium mycotoxin content was determined by chromatography (UHPLC-MS/MS). The results obtained show that DON presented exponential growth relative to the initial levels of mycotoxin in wheat milled fractions (finished flour and bran). The DON concentration was significativelly higher in bran, when compared with milled wheat and finished flour, in the DON levels lower than 1000 μg kg^{-1}. The finished flour presented lower DON levels when compared with milled wheat, but this reduction was inadequate, to meet the current regulation limits for food.

1. Introduction

Wheat is mainly used in human consumption, thus most of it is subject to milling. The main Brazilian wheat growing area, where more than 90 percent of it is grown, is located in the South. This region lies from Rio Grande do Sul to Paraná (latitude 28° S to 23° S) and the main limiting factor for wheat production is the excess of humidity, which favors fungal diseases outbreaks. In particular, Fusarium Head Blight (FHB) is influenced by warm temperature and rainy days during the flowering time. Apart from losses in grain yield and reductions in baking and seed quality, the major concern due to FHB is contamination with toxic fungal secondary metabolites, known as mycotoxins (Buerstmayr, Ban, & Anderson, 2009). Mycotoxins are secondary metabolites produced naturally by filamentous fungi, which are considered toxic substances when present in food for humans and feed for animals (Rocha, Freire, Maia, Guedes, & Rondina, 2014). *Fusarium graminearum sensu stricto* (Fgss) is the predominant species in Brazil, detected in 90% of isolates, and is potentially a deoxynivalenol (DON) producer, according to molecular methods that characterizes genes involved in the synthesis of trichothecenes (Del Ponte, Tessmann, Spolti, Kuhnem, & Silva, 2013). FHB mycotoxins, mainly deoxynivalenol, can bind to the 60 S ribosomal subunit of eukaryotes, resulting in protein synthesis inhibition and apoptosis (Terzi, Tumino, Stanca, & Morcia, 2013).

In order to protect consumers from mycotoxicosis, many countries established maximum allowed levels for the most prevalent Fusarium mycotoxins in cereals and cereal by-products. The European Union has established the most comprehensive regulations for food and cereal safety, to facilitate world trade and protect consumer health (Cheli, Battaglia, Gallo, & Dell'Orto, 2014). In Brazil, the mycotoxin regulation is progressively more restrictive; in 2012 the upper limits established for whole wheat and white flour were 2000 μg kg^{-1} and 1750 μg kg^{-1}, respectively (ANVISA, 2011). These levels will be progressively reduced, and in 2017, they will be 1000 μg kg^{-1} and 750 μg kg^{-1} (ANVISA, 2013). The unprocessed wheat grain upper limit will be 3000 μg kg^{-1} in 2017 (ANVISA, 2013). The Brazilian regulations also limited zearalenone

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Nomenclature

DON	Deoxynivalenol
3-ADON	DON acetylated derivatives
15-ADON	DON acetylated derivatives
ZON	Zearalenone
NIV	Nivalenol
FHB	Fusarium Head Blight

(ZON) levels in cereals and by-products; in 2012, the upper levels for whole wheat and wheat flour were 400 μg kg^{-1} and 200 μg kg^{-1}, respectively (ANVISA, 2011). Both levels will be halved by 2017 (ANVISA, 2013). Currently, no regulation limits have been established for animal feed in Brazil.

In the post-harvest stage, the cleaning, aeration, debranning and milling procedures certainly have an influence in the distribution of mycotoxins in wheat fractions. In the milling process there is no mycotoxin reduction, although mycotoxin concentrations may be redistributed and concentrate according to the milling fractions. Mycotoxins tend to be concentrated in outer fractions intended for animal feed (bran, flour shorts screenings and middlings) and lower in inner fractions intended for human consumption (flour or semolina) (Cheli, Pinotti, Rossi, & Dell'Orto, 2013). However, this mycotoxin distribution pattern is highly variable in wheat milled fractions. The main factors for these conflicting results may be due to type of mycotoxin (hydrophilic/hydrophobic), the genotype, the level and time of fungal contamination, and the milling methodology (Cheli et al., 2013).

Previous studies showed that the tricothecenes (DON and NIV) had identical patterns among milling fractions distribution, while the transfers ratios of ZON had different features (Zheng et al., 2014). In contrast to the trichothecenes, which are soluble in water, zearalenone is hydrophobic (soluble in organic solvents). Differences in the retention of DON and ZON in patent flour may be derived from the water content within the grain. The endosperm is characterized by high moisture content (over 35%), thus the DON and other trichothecenes, which are hydrophilic, presented higher recovery in patent flour (670 μg kg^{-1}), when compared with ZON (160 μg kg^{-1}) in *norin 61* Japanese cultivar (Zheng et al., 2014). Recently, Cheli et al. (2013) presented a comprehensive review about the effects of milling on mycotoxin distribution in wheat; the overall reduction of DON content commonly ranged from 50 to 70% in semolina, when compared with initial unprocessed wheat. On the other hand, in bran, mainly used for animal feed, the increase of DON levels ranged from 150 to 340%.

Therefore, the understanding of the effects of the milling process in the mycotoxin distribution is a worldwide topic of interest, due to the high economic and health impact. Considering the levels indicated for unprocessed cereals, sometimes it may not warranty the limits proposed for cereal-derived products (Cheli et al., 2013). In Brazil, the discrepancy between the acceptable levels of mycotoxin in unprocessed cereals and by-products resulted in the extension of 2012 levels till 2017, by the government control agencies (ANVISA, 2013). So, it is a priority to identify the effects of the wheat milling process on mycotoxin repartitioning, to subsidize the wheat productive chain and to meet the upcoming legislation requirements. Moreover, the results of the mycotoxin distribution in the milling stream byproducts, usually intended for animal feed, may also represent a useful support in the perspective of maximum limits settings in the future. The objective of this study was to evaluate the influence of the wheat milling process on Fusarium mycotoxin content, using wheat lots artificially contaminated with a crescent gradient of mycotoxins.

2. Methodology

2.1. Sampling characterization

Wheat samples from different cultivars, harvested in 2013 crop season from Southern Brazil, were used. The samples were obtained from field yield trials of Embrapa's breeding program. The geographical distribution included mainly Rio Grande do Sul State, and one sample from Santa Catarina state, both located in Southern Brazil. The major Fusarium disease outbreaks in the country are concentrated in that region. Nevertheless, in the 2013 season the natural levels of DON content in the selected set of samples, were below 400 μg kg^{-1} (data no shown). Therefore, these wheat samples were artificially contaminated with residues of the cleaning and pre-cleaning process, including light and shriveled grains, obtained from different wheat cultivars susceptible to FHB. These FHB-damaged kernels were obtained from wheat produced under field conditions favorable to fungal growth (nursery). In FHB nursery, mist irrigation was provided from flowering to grain filling stage, in order to favor disease development. So, the mycotoxin content of these residues was obtained by ELISA (AgraQuant® Deoxynivalenol), to establish the contamination gradient and the amount of residues necessary for the artificial contamination.

In the artificial contamination, a contamination gradient was obtained with six different levels of deoxynivalenol, ranging from <200 to >3000 μg kg^{-1}, with 6000 g each. These levels of mycotoxins were defined based on human and animal mycotoxin regulations worldwide. Each sample was thoroughly mixed before six sub-samples were collected to obtain a laboratory sample.

The moisture and protein contents were determined by Near Infrared Reflectance Spectroscopy (NIR instrument FOSS XDS – RCA, Hoganas, Sweden), in all samples. The six wheat lots showed these chemical caracteristics, i.e., protein ranging from 12.3% to 13.5% and moisture content ranging from 14.6% to 15.8%. The yield of flour obtained from wheat in the milling process were 44.7%, 43.9%, 42.0%, 43.2%, 42.6% and 40.1%, respectively, from level 1 to level 6 of initial contamination.

2.2. Milling procedures

The samples were milled according to the mycotoxin concentration, from low to high content, to minimize cross-contamination in the milling process. Cleaned wheat (1000 g) was milled in the Laboratory Mill 3100® (Perten, Sweden), to obtain the milled whole wheat fraction. The same set of samples, composed of 5000 g, were conditioned to 14% moisture content (weight and moisture content measured) and milled using a pilot-scale mill Quadrumat Senior® (Brabender, Germany), with a standard setting for hard wheat (AACC, 2000). This mill simulates the industrial milling process, and recovers approximately 98% of wheat fractions. The mill produced the following fractions: one breaking flour and one reduction flour and two outer layer fractions (bran and shorts). All milling fractions were kept at 8 °C prior to use.

The following fractions were collect for mycotoxin analysis: finished flour (reduction and break flour) and bran (shorts and bran). Finished flour (patent flour or semolina) was obtained directly from the mill and is the sole component of commercial flour. The shorts and bran were mixed to obtain a single sample. Shorts, is a mixture of germ and fine bran, separated from reduction flour after reduction rollers. Finally, bran, the outer layers of wheat kernel, is coarse, separated from break flour after the break rollers and used mainly to animal feed.

Each fraction was weighted and mixed, before the separation of 200 g for mycotoxin analysis. All the collected set of samples were sent for mycotoxin quantification at reference laboratory (Samitec, Santa Maria/RS).

2.3. Mycotoxin and ergosterol analysis

DON and its acetylated derivatives (15-ADON and 3-ADON), nivalenol and ZON contents were determined by ultra high-performance liquid chromatography-tandem with triple quadrupole mass spectrometry (UHPLC-MS/MS). The extraction, clarification and derivatization methodology is fully automatized, developed and validated in house, using the method described in Varga et al. (2012). The laboratory presents the following limits of quantification and recovery: for DON 200 μg kg^{-1} and 80%; for 15-ADON, 3-ADON and NIV 100 μg kg^{-1} and 91%, 87% and 74%, respectively; finally, for ZON, the limits of quantification/recovery were 20 μg kg^{-1}/85%.

Ergosterol, a fungal marker was analyzed, to elucidate the relationship between toxin accumulation and fungal dispersion in wheat layers. Ergosterol was measured by high-performance liquid chromatography (HPLC), as described in Moraes et al. (2003). The limits of quantification and recovery for ergosterol were 110 μg kg^{-1} and 99.6%, respectively.

2.4. Statistical analysis

The samples within the six initial levels of DON, were homogenized and quadruplicates (n = 4) were collected for mycotoxin analysis in milled wheat and in milled fractions.

Lme4 package developed by Bates, Maechler, Bolker, and Walker (2014), was used to fit a linear mixed effects model to the data. Prior the fitting process, the raw data were linearized using a logarithmic transformation. Then a varying-intercept model was fitted to data corresponding to each stratum represented by type variable. A Likelihood Ratio Test (LRT) procedure was used for model selection. R Code Team Software (2014) was used for analyzing the data.

Results regarding average DON in unprocessed wheat and in milling fractions, for each mycotoxin level, were reported as the Mean ± Standard Deviation (SD). The parameter Distribution Factor, previously reported in Cheli et al. (2010), was adopted to express the overall reduction or increase in DON content in each milled fraction compared to their respective contents measured in milled wheat. This parameter was expressed as a percentage and it was calculated as the ratio between mycotoxin content in processed fractions and its content in unprocessed whole wheat.

3. Results

3.1. Ergosterol content

Ergosterol is a metabolite used as an indicator of fungal colonization in grains. The distribution of ergosterol in the wheat milled fractions was similar to deoxynivalenol. The concentration of ergosterol was always lower in finished flour and higher in bran, indicating that most of the fungi were retained in the outer layers of grain in all levels of mycotoxins (Table 1).

Table 1
Mean of ergosterol (μg kg^{-1}), in milled wheat and in milling fractions.

Levels of DON (μg kg^{-1})	Milled wheat (μg kg^{-1})	Finished flour (μg kg^{-1})	Bran (μg kg^{-1})
(1) 0–200	664.4	ND[a]	1596.0
(2) 201–500	3322.3	748.7	3959.6
(3) 501–1000	3239.0	578.9	3463.3
(4) 1001–1500	2101.1	745.2	5951.7
(5) 1501–3000	4649.2	837.1	9687.4
(6) >3000	6939.5	2864.0	10,306.0

[a] ND: Below the limit of quantification (<110 μg kg^{-1}).

3.2. Deoxynivalenol content

The DON content in wheat milled fractions (finished flour and bran), increased exponentially in relation to the initial levels of mycotoxin in the milled wheat (Fig. 1).

In the lower gradient levels, from <200 to 1000 μg kg^{-1}, the DON concentration is significatively different in bran compared with milled wheat and finished flour. When the initial toxin concentration gradient increased, all milled fractions increased similarly. Thus, the higher level of DON observed in bran (8000 μg kg^{-1}), was not significantly different from milled wheat and finished flour (6000 μg kg^{-1}).

The mean DON concentrations in cleaned milled wheat and wheat milled fractions are presented in Table 2. DON was present in all samples, except level 1 (0–200 μg kg^{-1}). The DON content in samples 2 through 6 ranged from <200 to 6150 μg kg^{-1} in milled wheat. DON contamination in finished flour ranged from <200 to 5830 μg kg^{-1}. The maximum DON concentration was observed in the bran, regardless of initial contamination level (Table 2).

Overall, using the five contrasting levels of mycotoxins (from >200 to >3000 μg kg^{-1}), DON concentrations in finished flour were reduced by 19%, although in bran it increased by 125%, when compared with milled wheat using pilot-scale milling process (Table 2). The distribution patterns among all fractions were variable, particularly for finished flour, where the retention of DON was reduced by approximately 43% in level 2 (201–500 μg kg^{-1}) and only 11% in the highest level of contamination (>3000 μg kg^{-1}), when compared with milled wheat.

3.3. Zearalenone, nivalenol and DON acetylates levels

ZON was detected only in the two higher levels of initial contamination of mycotoxins (Table 3). In the finished flour fraction, ZON was not detected in any analyzed sample.

The DON acetylated derivatives (15-ADON and 3-ADON) and nivalenol were not detected in any sample (data no shown).

4. Discussion

The higher levels of mycotoxin were more likely to occur due to the fungal growth in the inner grain layers, promoted by the ideal conditions in the FHB nursery, than mycotoxin diffusion among grain layers (Table 1). Our results are in agreement with other studies conducted by Young, Fulcher, Hayhoe, Scott, and Dexter (1984), Trigo-Stocki (2002) and Pinson-Gadais et al. (2007), which indicated that the effectiveness of the milling process, in reducing the level of DON in semolina, depends on the extent of the fungi penetration in the wheat kernels. According to Young et al. (1984), the mycotoxin is produced at the site of fungal growth, rather than transported from the kernel surface to the interior. A more recent study indicated that the DON concentration in flour fractions was equivalent to that of the milled wheat, with a lower concentration in outer layers (bran), in the wheat from a season characterized by high pre-harvest rainfall, which resulted in movement of DON to inner layers due to the DON highly solubility in water (Edwards et al., 2011). In our study, the DON and ZON distribution in wheat fractions also resulted in movement of DON inside the grains, although, there was no significant distribution of ZON within wheat inner layers. Although, ZON were quantifiable only in a small number of samples. A study conducted by Pinson-Gadais et al.

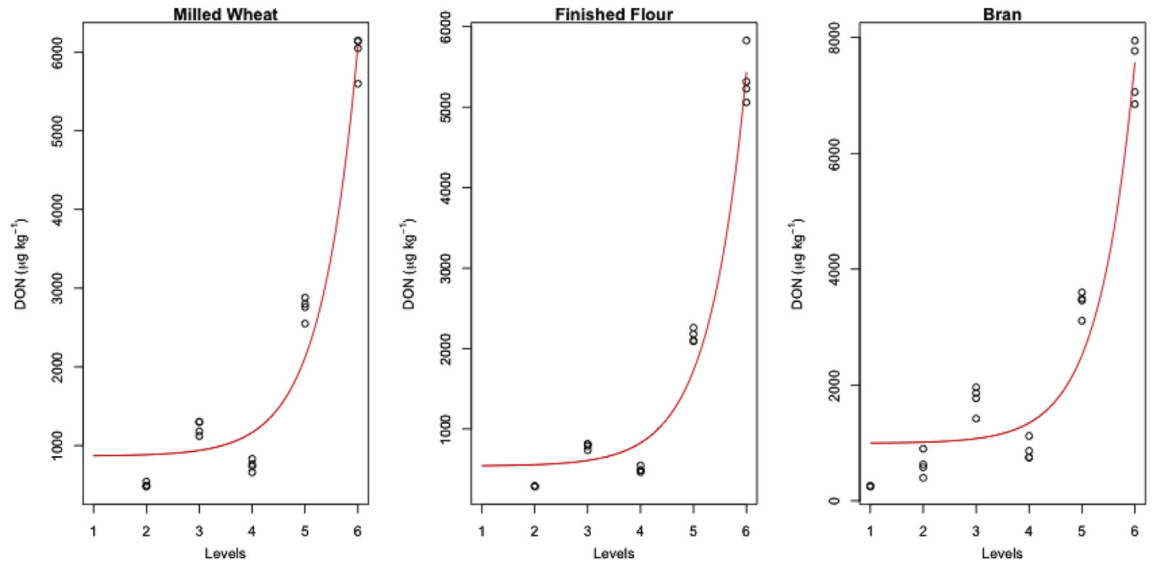

Fig. 1. Relationship of DON content (µg kg^{-1}) in wheat milled fractions and levels of initial contamination. The dots represent the observed values and the continuous line is an exponential curve fitted to the data.

(2007), reported the penetration of toxigenic Fusarium fungi into the interior of durum wheat kernel and indicated that none of the tissue structures within the wheat kernel acted as an effective barrier to fungal invasion. Moreover, after inoculation by toxigenic Fusarium strains, semolina was shown to allow high yields of trichothecenes, while bran was demonstrated to contain biochemical inhibitors able to significantly reduce trichothecene production.

Table 2
Average DON levels and distribution factors in wheat and milling fractions.

Levels of DON (µg kg^{-1})	Fractions	Mean ± standard deviation of DON (µg kg^{-1})	Distribution factor[a] (%)
(1) 0–200	Milled wheat	ND[b]	–
	Finished flour	ND	–
	Bran	252.67 ± 7.09	–
(2) 201–500	Milled wheat	498.75 ± 29.30	100.00
	Finished flour	288.67 ± 4.16	57.87
	Bran	626.25 ± 207.00	125.56
(3) 501–1000	Milled wheat	746.50 ± 70.71	100.00
	Finished flour	495.75 ± 36.10	66.40
	Bran	870.00 ± 175.21	116.54
(4) 1001–1500	Milled wheat	1225.00 ± 90.00	100.00
	Finished flour	787.00 ± 36.08	64.24
	Bran	1752.50 ± 234.8	143.06
(5) 1501–3000	Milled wheat	2747.50 ± 140.80	100.00
	Finished flour	2157.50 ± 79.32	78.525
	Bran	3415.00 ± 212.05	124.29
(6) >3000	Milled wheat	5985.00 ± 260.57	100.00
	Finished flour	5360.00 ± 331.36	89.55
	Bran	7407.50 ± 534.56	123.76

[a] Distribution factor: percentage of overall reduction or increase in DON content for each milling fraction, in comparison with initial level in unprocessed wheat.
[b] ND: Below the limit of quantification (<200 µg kg^{-1}).

Different from our results, Thammawong et al. (2011) reported that DON and NIV distributions were different from ergosterol, indicating that the diffusion of toxins inside the grain is independent of fungal biomass in wheat artificially inoculated with *Fusarium graminearum*. We considered that this difference might be due to the heavily Fusarium-damaged kernels used in this study, where the artificial contamination was performed by mixing Fusarium damaged wheat kernels from FHB susceptible cultivars produced in the Fusarium nursery (highly favorable environment).

Edwards et al. (2011) stated that the proportion of DON in flour increased as the concentration of DON in original wheat lot increased. In our study, DON content was significantly higher in bran, when compared to milled wheat and finished flour, in the lower contamination levels (<200–1000 µg kg^{-1}). However, similar levels of DON among finished flour fractions and whole milled wheat were observed in all contamination levels, indicating that toxins were not removed by the milling process. These results are in agreement with previous studies, that used heavily Fusarium-infected wheat grains. Thammawong et al. (2011) examined the milling effect in wheat with low, medium and high DON contamination levels. In the medium (900 µg kg^{-1}) and higher levels (5270 µg kg^{-1}) DON accumulation in patent flour was similar to the levels obtained in the original grains. According to their results,

Table 3
Average ZON levels and distribution factors in wheat and milling fractions.

Levels of DON (µg kg^{-1})	Fractions	Mean ± standard deviation of ZON (µg kg^{-1})	Distribution factor[a] (%)
(5) 1501–3000	Milled wheat	27.55 ± 1.84	100.00
	Finished flour	ND[b]	
	Bran	62.60 ± 42.80	227.22
(6) >3000	Milled wheat	38.65 ± 6.52	100.00
	Finished flour	ND	
	Bran	61.15 ± 158.21	158.21

[a] Distribution factor: percentage of overall reduction or increase in ZON content for each milling fraction in comparison with initial level in unprocessed wheat.
[b] ND: Below the limit of quantification (<20 µg kg^{-1}).

only the bran differed among fractions and presented significantly higher DON levels.

Other studies, with lower levels of initial mycotoxin contamination, reported higher DON reduction in semolina during the milling process. Cheli et al. (2010), reported that DON concentrations in semolina reduced by 60%, although in shorts (middling) it increased by 152%, when compared with unprocessed wheat (68.58 μg kg^{-1}), using conventional milling. The relative concentration of DON in semolina declined 40.7% and increased by 162.4% in bran, in comparison to the initial levels obtained in whole milled wheat (1890 μg kg^{-1}) (Zheng et al., 2014). Similar to our findings, Thammawong et al. (2011) reported that DON concentration decreased 11.2% in patent flour and increased 137% in bran, when compared with milled wheat, in the higher level of initial contamination (5270 μg kg^{-1}). Zhang and Wang (2014) reported contrasting results showing DON reductions of 79–90% in the finished flour, when compared with milled wheat, with initial levels of contamination ranging from 4680 to 36,719 μg kg^{-1}. These great differences in results might be accounted by the cultivar effect and the degree of fungal penetration in wheat kernel (Pinson-Gadais et al., 2007; Young et al., 1984; Zhang & Wang, 2014). Fungal colonization limited to outer layers results in higher toxins concentration in the grain surface. To summarize, all studies reported similar trends, with more toxins being accumulated in the surface of wheat kernels and the main sources of difference in mycotoxin distribution were: cultivar effect, degree and time of fungal infection, the weather conditions and the milling process.

According to our study, when DON contaminated unprocessed wheat enters the milling process with maximum level (3000 μg kg^{-1}), there are concerns raised in flour production. In both maximum contamination levels 5 and 6 (1501–3000 and >3000 μg kg^{-1}), the content of DON in finished flour was higher than the upcoming upper limit settled at 750 μg kg^{-1}, for 2017 (ANVISA, 2013). The DON content in wheat milled fractions (finished flour and bran), presented an exponential growth pattern, thus requiring lower initial contamination in raw grain, to guarantee food safety in by-products. In turn, level 4 was the closest to the levels foreseen in Brazilian mycotoxin regulations in 2017; the mean of DON in milled wheat was 1225 μg kg^{-1} and in the finished flour was 787 μg kg^{-1}. Below this level, would be the maximum DON content tolerable, to meet the upcoming regulation criteria.

More studies are necessary to evaluate the seasonal climate variation, which is the major factor to promote Fusarium outbreaks. Further investigations, with broad samples set under natural contamination conditions, will contribute to understanding the fate of these contaminants in the primary processing.

5. Conclusion

We conducted a study concerning the impact of milling processes on the Fusarium mycotoxin content in wheat artificially contaminated and displaying contrasting levels of mycotoxins (<200 to >3000 μg kg^{-1}). In the lower gradient levels (<1000 μg kg^{-1}), DON concentration is significatively different in bran compared with milled wheat and finished flour. The milling process cannot be solely used as an effective tool for DON reduction in the finished flour, especially in the higher contaminated wheat lots. The mycotoxin concentration, in the inner grain layers, must be due to the fungal growth within the grain, rather than the toxin diffusion.

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