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Note

Survey of mycotoxins in Southern Brazilian wheat and evaluation of immunoassay methods

Maíra Rodrigues Duffeck[1], Casiane Salete Tibola[2]*, Eliana Maria Guarienti[2], Emerson Medeiros Del Ponte[1]

[1]Federal University of Viçosa – Dept. of Plant Pathology, Campus Universitário, s/n – 36570-000 – Viçosa, MG – Brazil.
[2]Embrapa Wheat, Rod. BR-285, km 294 – 99050-970 – Passo Fundo, RS – Brazil.
*Corresponding author <casiane.tibola@embrapa.br>

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ABSTRACT: One hundred commercial wheat grain samples were collected during the 2015 season across 78 municipalities in the states of Paraná (PR), Rio Grande do Sul (RS), and São Paulo (SP), Brazil. Separate subsamples were analyzed for the concentration of deoxynivalenol (DON), zearalenona (ZEA) and ochratoxin A (OTA) mycotoxins using two methods: UHPLC-MS/MS (reference method) and a commercial enzyme-linked immunosorbent assay (ELISA) (AgraQuant®). The OTA mycotoxin was not found in the samples by both methods. DON and ZEA were detected in 55 % and 39 % of the samples by the reference method, with overall mean levels of 795.2 µg kg^{-1} and 79.78 µg kg^{-1}, respectively. There was a significant and positive correlation (Spearman rank) between DON and ZEA estimates by the reference method ($r = 0.77$, $p < 0.001$). The DON levels estimated by the immunoassay agreed poorly with the reference, being largely overestimated. Based on a cut-off level of 1000 µg kg^{-1}, the immunoassay correctly classified 57 samples as true negatives and 15 as true positives. Only 28 were classified as false positives. For ZEA, the levels estimated by the two methods were in better agreement than for DON. Using the cut-off level of 200 µg kg^{-1}, 96 % of the samples were classified correctly as true positives and only one sample was classified as false positive. The levels for both mycotoxins were mostly acceptable for human consumption. Further studies should focus on multi-toxin methods compared with immunoassays to understand the reasons of overestimation and the role of immunoassays as a cost-effective solution for fast screening of mycotoxins in the food chain.
Keywords: *Triticum aestivum*, UHPLC, ELISA, trichothecenes, ochratoxin

Introduction

Fusarium head blight (FHB) is a fungal disease of major concern to wheat production. In Brazil, the disease is caused by members of the *Fusarium graminearum* species complex (FGSC), which infect wheat florets and contaminate grains with dangerous mycotoxins (Del Ponte et al., 2015). These fungi are able to synthesize a range of mycotoxins such as trichothecenes, mainly deoxynivalenol (DON), and zearalenone (ZEA) (Bryden, 2012). Ochratoxin A (OTA) is not a *Fusarium* mycotoxin, but it is found in grain stored under inadequate conditions and infected by *Aspergillus* and *Penicillium* species (Ghali et al., 2008).

In order to mitigate the harmful effects of mycotoxins on public health, maximum limits have been established to reduce *Fusarium* mycotoxins around the world (Cheli et al., 2014), including Brazil (ANVISA, 2011). Surveys on mycotoxins in commercial Brazilian wheat grain and byproducts provide critical information for growers to assess the impact of control measures as well as to inform consumers and policy makers on the mycotoxin risks (Furlong et al., 1995; Oliveira et al., 2002; Calori-Domingues et al., 2007; Del Ponte et al., 2012; Tralamazza et al., 2016). Among the quantitative methods for mycotoxin analyses, the most specific and sensitive ones include the ultra-high performance liquid chromatography-tandem with triple quadruple mass spectrometry (UHPLC-MS/MS), liquid chromatography coupled to mass spectrometry (LC-MS) and gas chromatography coupled to mass spectrometry (GC-MS) (Shephard et al., 2011). However, high costs are limiting factors for their use in routine for processing large number of samples (Lattanzio et al., 2009; Xu et al., 2010). Commercial kits based on enzyme-linked immunosorbent assays (ELISAs) are widely used for screening commodities and foods, given their low cost and easy operation (Lattanzio et al., 2009). Although, these assays allow high throughput, disadvantages related to antibody cross-reactivity can lead to overestimation of the targeted mycotoxin compared to a reference (chromatographic) method (Berthiller et al., 2013; Liu et al. 2012). The main objective of this study was to quantify and assess the spatial variability of DON, ZEA and OTA in commercial grain harvested in southern Brazil, in the 2015 season, using UHPLC-MS/MS. A secondary objective was to evaluate commercial immunoassay kits targeting the three mycotoxins based on the accuracy, precision and validity of the estimates compared to the reference method.

Materials and Methods

Survey area and sampling

Grain samples were collected from the harvest of commercial wheat fields in wheat-growing regions

in southern Brazil where FHB occurs frequently. The samples (10 kg) were collected in 78 municipalities randomly chosen in the main producing regions of Paraná (PR, 60 % of the samples), Rio Grande do Sul (RS, 35 %) and São Paulo (SP, 5 %) States (Figure 1B). In most cases, one sample was obtained per municipality, but in eight municipalities, two samples were collected. Municipalities that provided more than two samples were Carambei (n = 4), Imbau (n = 6) and Tibagi (n = 7), all in Paraná State. Cooperatives of local growers and private industry provided the samples, which were collected at the storage facilities. The sampling procedures followed the protocols adopted by the provider. The moisture content across the samples ranged from 12 % to 14 %.

UHPLC and immunoassay mycotoxin quantification

Two subsamples (300 g) were obtained for the analyses of DON, ZEA and OTA using two different methods. One subsample was sent for chemical analysis by a commercial and certified laboratory (Santa Maria, RS) where the three mycotoxins were analyzed by UHPLC-MS/MS with an automated extraction, clarification and derivation, as described in Varga et al. (2012). The method has the following limits of quantification and recovery ratio: 200 µg kg^{-1} and 80 % for DON, 20 µg kg^{-1} and 85 % for ZEA and 2 µg kg^{-1} and 80 % for OTA.

Another subsample was analyzed for the same three mycotoxins using a commercial direct competitive enzyme-like immunosorbent assay (ELISA) kit (AgraQuant®). The limit of detection (LOD) and the limit of quantification (LOQ) in this test were 200 µg kg^{-1} and 250-5000 µg kg^{-1}, respectively. Extraction procedure, calibration and reading were performed according to manufacturer instructions. Details were described by Zheng et al. (2004).

Statistical analysis

Descriptive statistics were used to summarize frequency and central tendency measures of the mycotoxin data from all samples. The frequency of the number of positive detections (above the detection limit) was compared between samples from RS and PR and SP combined using the χ^2 test ($p < 0.05$). Geographic maps were made to show the origin and the mean concentration levels of each mycotoxin aggregated by municipality. The correlation between levels of two mycotoxins was assessed using the Spearman rank correlation coefficient because the data normality could not be assumed. The accuracy (systematic and constant bias) and precision of estimates by immunoassay, compared to those by UHPLC, used as reference, were determined by the components of Lin's concordance correlation analysis (LCC) (Lin, 1989). Absolute errors of the estimates by the immunoassay were also calculated.

In addition, the estimates by the two methods were categorized into two classes based on a cut-off level of 1000 µg kg^{-1} and 200 µg kg^{-1} for DON and ZEA respectively, both determined based on the current legislation for each mycotoxin in wheat (ANVISA, 2011). The number of cases in each category according to the cut-off, determined by each method, was related to one another and the validity (sensitivity and specificity) of the immunoassay compared to the reference method (UHPLC) was calculated based on the false positive rate, false negative, true positive and true negatives. Non-parametric tests were used to compare the mean DON and ZEA levels between the two states (Mann-Whitney test) and between the two methods (Wilcoxon test) ($p < 0.05$) because normality could not be assumed.

Results

UHPLC analysis of mycotoxins

OTA mycotoxin was not found in the samples. DON was detected in 55 % of the samples, with levels ranging from 200 (detection limit) to 2743 µg kg^{-1} in the positive samples, with a mean (median) of 795.2 (682.8) µg kg^{-1} in these positive samples. When all samples were considered in the calculation (including negatives), the mean and median values were 437.4 and 210.4 µg kg^{-1}, respectively.

The frequency of positive DON detections varied between samples from RS (33 in 35) and PR + SP (22 in 65) States (Figure 1B). The mean (median) DON levels in the positive samples were 685.1 (557.5) µg kg^{-1} in RS (n = 33) and 960.3 (854.0) µg kg^{-1} in PR + SP States (n = 22), respectively. When all samples were used in the calculation, the mean (median) of DON levels were 646.1 (479.4) µg kg^{-1} in RS (n = 35) and 325.0 (0) µg kg^{-1} in PR + SP States (n = 65), respectively (Figure 1A).

In only 15 samples, five from RS and ten from PR, DON levels were > 1000 µg kg^{-1} (Figure 1A). In RS, higher DON levels were found in locations from the Planalto region, while in PR State, higher contamination was found in southeastern region at levels similar to those found in RS. Most samples from western PR and SP State were not contaminated with DON (Figure 1B).

ZEA was detected in 39 % of the samples, with an overall mean (median) of 79.78 (35.90) µg kg^{-1} in the positive samples. The relative frequency of positive ZEA detections was also higher in RS (25 in 35) than in PR (14 in 65). Mean (median) ZEA levels were 60.94 (34.4) µg kg^{-1} in the 25 positive samples of RS and 113.4 (94.8) µg kg^{-1} in the 14 positive samples of PR (Figure 2A). ZEA was not detected in SP State. Only three samples (2 from PR and 1 from RS) showed levels of ZEA > 200 µg kg^{-1} (Figure 2A). The spatial variation of the ZEA levels followed that of DON levels (Figure 2B). Overall, there was a significant and positive correlation (Spearman rank) between DON and ZEA determined using UHPLC ($r = 0.77$, $S = 36728$, p-value < 0.0001).

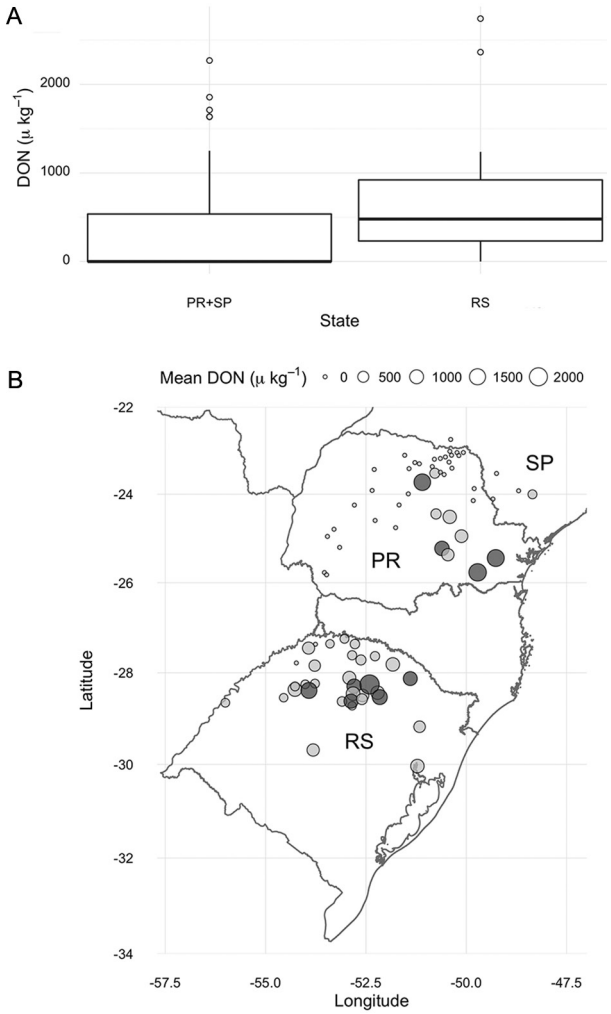

Figure 1 – Boxplot for the distribution of deoxynivalenol (DON) levels (µg kg^{-1}) estimated by UHPLC method in commercial wheat samples from Paraná and São Paulo (PR + SP) (north) States and Rio Grande do Sul (RS, south) State (A). The circle represents the geographic location of each 78 municipalities and its size is proportional to the mean DON levels for the location. The darker grey circle represent locations with mean DON above the 1,000 µg kg^{-1} threshold (B).

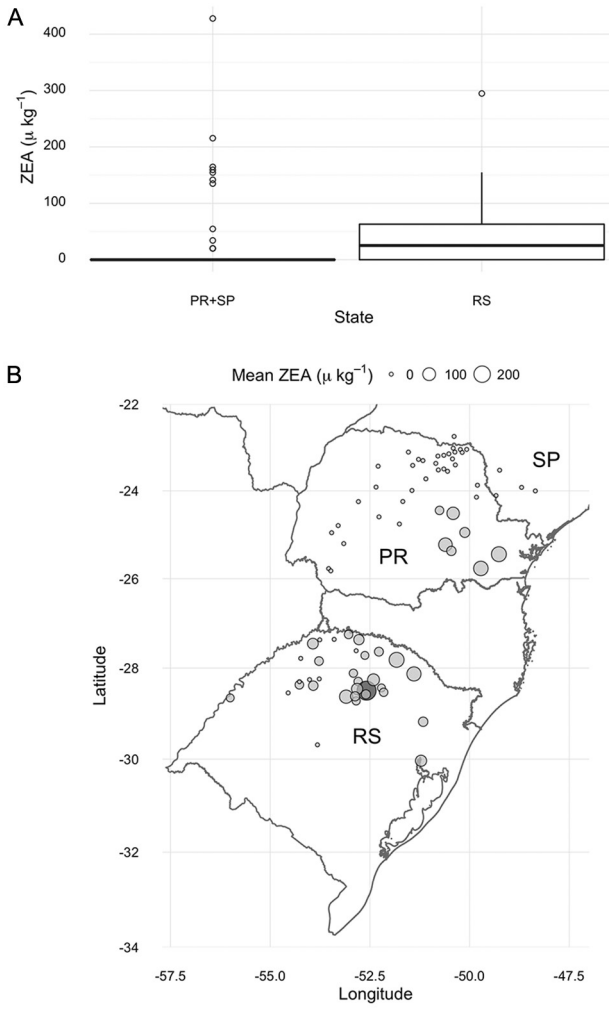

Figure 2 – Boxplot for the distribution of zearalenona (ZEA) levels (µg kg^{-1}) estimated by the UHPLC method in commercial wheat samples from Paraná and São Paulo (PR + SP) (north) States and Rio Grande do Sul (RS, south) State (A). The circle represents the geographic location of each 78 municipalities and its size is proportional to the mean ZEA levels for the location. The darker circle represents locations with mean ZEA above the 200 µg kg^{-1} threshold (B).

Immunoassay analysis of mycotoxins

OTA was not found with the direct competitive immunoassay, agreeing with results of the UHPLC analysis. Conversely, DON was detected in all samples when the immunoassay was used, differing from UHPLC. There was only one sample in which DON was not detected by both methods. In 44 samples where DON levels were below the detection limit based on UHPLC analysis, DON levels determined by immunoassay averaged 690 µg kg^{-1} (190 to 1490 µg kg^{-1}); eight samples showed DON levels > 1000 µg kg^{-1}.

The mean (median) of DON estimated by the immunoassay for all samples was 1274.5 (800) µg kg^{-1}, which was significantly higher than the mean (including all samples) levels measured by UHPLC (437.3 µg kg^{-1}) (Figure 3A). In 43 % of the samples, DON was greater than 1000 µg kg^{-1}. There was no difference ($p > 0.05$) in the mean DON levels in the samples from RS (1792.4 µg kg^{-1}) and PR + SP States (1824.54 µg kg^{-1}).

ZEA levels were similar between the two methods, although the frequency of positive detection was higher using UHPLC (39 %) when compared with the immunoassay (27 %). The mean estimated ZEA levels were 31.11 and 35.94 µg kg^{-1} for UHPLC and the immunoassay, respectively (Figure 3B), and did not differ statistically ($p > 0.05$).

Accuracy and validity of immunoassay

The estimates of DON levels by immunoassay showed good precision (r = 0.87). However, there were large positive deviations from the UHPLC estimates in 54 % of the samples, which resulted in low accuracy (C_b = 0.57) and moderate overall concordance between the two methods (ρc = 0.5; 95 % CI: 0.42 - 0.58) (Figure 3C).

For samples classified as accepted or rejected based on the cut-off level of 1000 µg kg^{-1}, immunoassay correctly classified 57 samples as true negative (correctly accepted) and 15 as true positives (correctly rejected). There were 28 false positives (incorrectly rejected) and no false negative (incorrectly accepted) (Figure 3C). The immunoassay test showed 72 % of accuracy, 35 % of sensitivity and 100 % of specificity.

ZEA levels estimated by immunoassay were less precise than those estimated for DON (r = 0.79), but they were more accurate (C_b = 0.91), resulting in a higher overall concordance among results by the two methods compared to DON (ρc = 0.72; 95 % CI: 0.67 - 0.77) (Figure 3D). Based on a 200 µg kg^{-1} threshold, 96 % of the samples were classified correctly as true positive and only one sample was classified as false positive (Figure 3D). False negative values were not found either. The immunoassay presented 99 % of accuracy, 75 % of sensitivity and 100 % of specificity. The errors of the estimated DON by immunoassay and UHPLC were mostly positives in high magnitude > 3000 µg kg^{-1} (Figure 3E). On the other hand, ZEA errors by immunoassay were both negative and positive < 150 µg kg^{-1} (Figure 3F).

Discussion

This study updated critical data on the occurrence and spatial distribution of two important *Fusarium* mycotoxins in commercial wheat grain produced in the main growing regions in Brazil. Results suggested that the DON and ZEA levels in commercial wheat were gener-

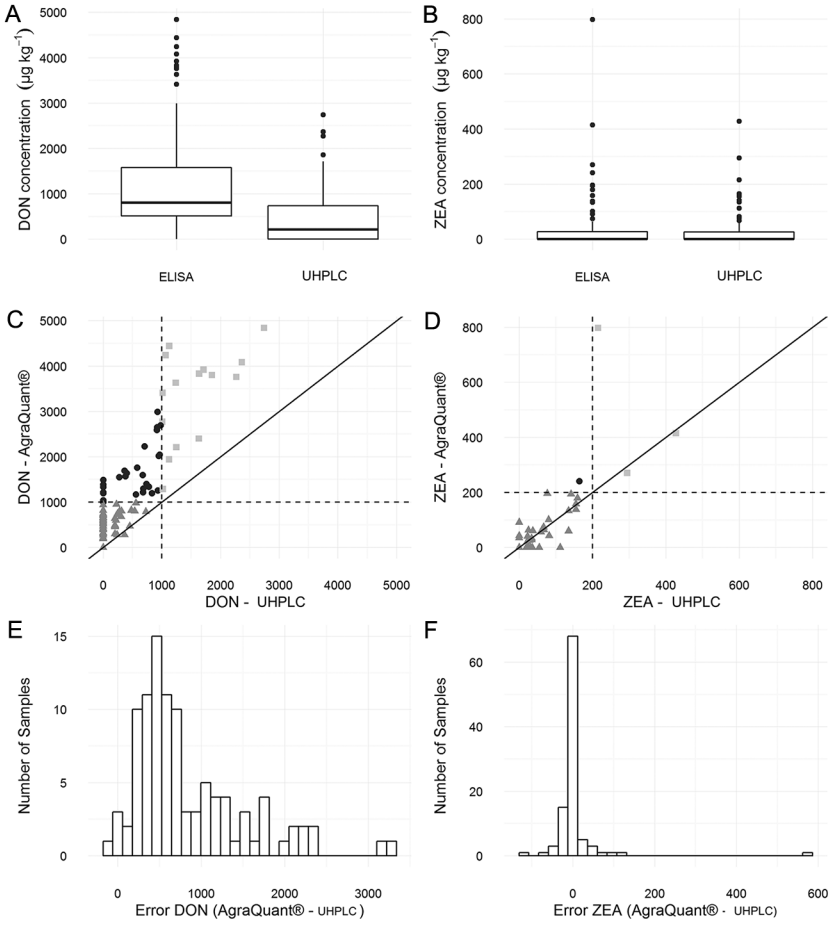

Figure 3 – Boxplot for the distribution of deoxynivalenol (DON) (A) and zearalenone (ZEA) (B) levels (µg kg^{-1}) obtained by the reference method (UHPLC) and commercial immunoassay (ELISA) methods (AgraQuant®). Relationship between concentration of DON (C) and ZEA (D) estimated by the UHPLC and immunoassay methods. In C and D, the samples were classified as true negative (triangle), true positives (square) and false positives (circle) considering the threshold of 1000 µg kg^{-1} for DON and 200 µg kg^{-1} for ZEA. Frequency of samples by ranges of absolute errors (µg kg^{-1}) of the estimates to DON (E) and ZEA (F) by immunoassay in relation to the concentration determined by the reference method.

ally safe. Only a relative small percentage showed contamination levels above the threshold, considering the reference (UHPLC) method. The DON levels determined in our study are in agreement with previous reports in the country, using chromatography methods. However, year-to-year (or decades) and region-to-region variations are expected, given the strong dependence on seasonal weather conditions as well as management practices that contribute to suppress the disease and mycotoxin levels (Del Ponte et al., 2009). For example, Furlong et al. (1995), two decades earlier, analyzed 38 samples of commercial wheat grain and found DON in 55 % of the samples, but at lower mean and range (400 to 590 µg kg^{-1}) than those found in our study. The analyses of 50 Brazilian and 50 imported wheat samples showed mean DON levels of 332 µg kg^{-1} and 90 µg kg^{-1}, in 94 % and 88 % of the samples, respectively (Calori-Domingues et al., 2007). More recently, using LC-MS/MS, 12 in 64 commercial wheat samples showed DON >1000 µg kg^{-1} and an average of 540 µg kg^{-1} was found across three growing seasons (2009 to 2011) (Del Ponte et al., 2012). These results were similar to the median value (437.3 µg kg^{-1}) found in this study, including all samples. We found that DON was significantly higher in samples from RS compared to those from PR, when all samples are taken into account. This is in agreement with a previous study that reported mean DON ranging from 426 to 453 µg kg^{-1} in samples from PR and SP States, and 1200 µg kg^{-1} in samples from RS, all from the 2012 season (Tralamazza et al., 2016).

We found ZEA at lower levels than DON levels and only in samples where DON was also present, being 14 samples from PR and 25 samples from RS. The co-occurrence of both toxins has been reported previously in the country (Tralamazza et al., 2016). Although ZEA is an important mycotoxin produced by members of the FGSC, few studies report its occurrence in Brazilian wheat (Tibola et al., 2015; Tibola et al., 2016). It is known that ZEA co-occur with DON especially when produced by FGSC isolates (Tangni et al., 2010), which is indeed the main pathogen in Brazil and a known ZEA producer (Geraldo et al., 2006). The ZEA levels found in our study are similar to those reported in previous studies on commercial wheat from Brazil (RS, 70.9 µg kg^{-1} and PR, 57.9 µg kg^{-1}) (Tralamazza et al., 2016).

OTA is produced by species of *Aspergillus* and *Penicillium*, which commonly grow during the grain storage (Duarte et al., 2010). The absence of OTA in our study may be due to short storage time and good conditions, which may not have favored growth of these fungi. OTA is an important target due to its frequent report in cereal food and feed products such as rice and wheat (González et al., 2006; Ghali et al., 2008). In Brazil, OTA has been reported in corn and rice samples (Machinski et al., 2001).

The DON levels estimated by immunoassay were generally higher than those estimated by chromatography, which would result in considerable increase in the rejections based on the current regulation. Overestimation of *Fusarium* mycotoxins by immunoassays was known from previous studies targeting DON in other countries (Meneely et al., 2011; Lattanzio et al., 2009). An essential requirement for an immunoassay is the antibody specificity. Many studies described the successful production of monoclonal or polyclonal antibodies against the trichothecenes, but often the cross-reactivity profile is not ideal and can lead to overestimation and increased uncertainty in the measurements (Meneely et al., 2011). This is true for DON where most literature highlights that antibodies raised against deoxynivalenol show strong cross-reactivity to 3-acetyldeooxynivalenol, 15-acetyldeoxynivalenol or both (Meneely et al., 2011). The antibodies designed for DON may also cross-react against DON-3βglucoside (DON-3G) and monoacetylated derivatives (3-ADON) (Tangni et al., 2010). The presence of acetylated DON derivatives (15-ADON and 3-ADON) has been generally reported together with DON, especially in cereals, although at relatively lower level than DON levels (Berthiller et al., 2013).

Using an immunoassay in Brazil, Santos et al. (2013) quantified DON levels in wheat samples from Paraná State and reported 66 % of positive samples at levels ranging from 206.3 to 4732.3 µg kg^{-1} (mean 1894.9 µg kg^{-1}). These levels are similar to those found in our study using immunoassay, but no comparison was made with a reference method in that study. In southern Brazil, FHB is caused by *F. graminearum* strains that potentially produce 15-ADON, but also *F. meridionale* that produces nivalenol, and a few other species that produce 3-ADON (Del Ponte et al., 2015; Nicolli et al., 2015). Further studies should focus on the analysis of acetylates, glucosilates and other masked forms, which are known to be related to mechanisms of plant resistance. Considering that acetylates and conjugates are also toxicologically important, immunoassay results are useful for fast screening of DON in commodities. On the other hand, the ZEA levels estimated by immunoassay were comparable to those determined by UHPLC, agreeing with previous findings (Shephard et al., 2011).

In summary, the results of this study suggested that Brazilian wheat is mostly acceptable for human consumption. However, it is important to increase vigilance of mycotoxins in wheat because of yearly fluctuations due to climate variability and crop management practices. Future studies should focus on multi-toxin methods combined with immunoassays to improve the accuracy of the screening methods in routine analyses.

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