Mycotoxin Research (2018) 34:173–178
https://doi.org/10.1007/s12550-018-0311-8

ORIGINAL ARTICLE

Occurrence of deoxynivalenol and zearalenone in brewing barley grains from Brazil

Karim C. Piacentini[1] · L. O. Rocha[2] · G. D. Savi[3] · L. Carnielli-Queiroz[4] · F. G. Almeida[5] · E. Minella[6] · B. Corrêa[1,4]

Received: 12 July 2017 / Revised: 27 February 2018 / Accepted: 2 March 2018 / Published online: 9 March 2018

Abstract

Barley (*Hordeum vulgare* L.) is an important cereal crop for food and represents one of the main ingredients in beer production. Considering the importance of barley and its derived products, the knowledge about the mycotoxin contamination in the barley production is essential in order to assess its safety. In this study, the levels of deoxynivalenol (DON) and zearalenone (ZEN) in brewing barley were determined using a LC-MS/MS method. A survey was conducted in 2015 to estimate the mycotoxin levels in these products ($n = 76$) from four crop regions in Brazil. The results showed high levels of DON and ZEN in the analyzed samples, with contamination levels of 94 and 73.6%, respectively. The mean levels of DON and ZEN ranged from 1700 to 7500 μg/kg and from 300 to 630 μg/kg, respectively. Barley samples from regions 1 and 2 presented higher levels of ZEN and DON, respectively, and those from region 4 presented lower levels of both. Co-occurrence of DON and ZEN was seen in the majority of the barley grain samples, and the mycotoxin content was above the maximum levels established by the Brazilian and European regulations.

Keywords Mycotoxins · *Fusarium* · Trichothecenes · Cereals · LC-MS/MS · Contamination

Introduction

Barley (*Hordeum vulgare* L.) is one of the most important cereals in the world, along with maize, wheat, and rice. This cereal is cultivated as a winter crop in tropical areas for malt production and has significant economic importance. Therefore, efforts have been made to improve barley grains for the global brewing industry. The research in this area includes not only the study of the grain composition but also the resistance of the grains to insects and fungi (Ryan 1990; Kumaraswamy et al. 2011).

Damage caused by fungi is responsible for losses of more than 20% of the harvested grain. Tropical conditions, such as those in Brazil, may contribute to fungal dissemination, and consequently, mycotoxin production (Bergvinson and García-Lara 2004).

The genus *Fusarium* is composed of several species that can produce a wide range of mycotoxins, including fumonisins, fusarins, moniliformin, trichothecenes, and zearalenone (ZEN) (O'Donnell et al. 2013).

Trichothecenes are products of the sesquiterpenoid metabolism of some genera in the order Hypocreales (Rocha et al. 2015), and among them, deoxynivalenol (DON) is notorious for its worldwide occurrence. This mycotoxin in mainly produced by phylogenetic species within the *Fusarium graminearum* species complex, which includes the *F. graminearum sensustrictu*. This species is encountered on barley, maize, and wheat causing *Fusarium* head blight, and important disease that leads to yield losses in small grain cereals (Tralamazza et al. 2016).

✉ Karim C. Piacentini
karim.piacentini@hotmail.com

1 Biotecnology Department, Biomedics Science Institute, ICB III, University of Sao Paulo, Av. Professor Lineu Prestes, 2415, Sao Paulo, SP CEP 05508-900, Brazil

2 Food Engineering Faculty, University of Campinas, Av. Monteiro Lobato, 80, Campinas, Brazil

3 University of the Extreme Southern Santa Catarina, Av. Universitaria, 1105, Criciuma, Santa Catarina, Brazil

4 Microbiology Department, University of Sao Paulo, Av. Professor Lineu Prestes, 1374, Sao Paulo, Brazil

5 Facility Center for Research Biomedical Science Institute, Av. Prof. Lineu Prestes 1730, Sao Paulo, Brazil

6 Embrapa Trigo, Brazilian Agricultural Research Corporation, Rodovia BR-285, Km 294, Passo Fundo, Rio Grande do Sul, Brazil

Deoxynivalenol, also known as vomitoxin, is a commonly encountered trichothecene type-B mycotoxin. Laboratory and farm animal studies have shown that DON elicits a complex spectrum of toxic effects. Chronic exposure to low doses of DON can lead to anorexia, impaired weight gain, and immunotoxicity. Acute exposure to high doses can cause diarrhea, vomiting, leukocytosis, circulatory shock, and ultimately death (Pestka and Smolinski 2005; JECFA 2011).

Zearalenone is another mycotoxin frequently found in grains and is defined as a non-steroidal estrogenic compound produced by *Fusarium*. ZEN is structurally similar to the hormone estrogen, and it competes with 17β-estradiol for binding to the estrogen receptor, resulting in infertility and reproductive problems (Takemura et al. 2007; EFSA (European Food Safety Authority) 2011). It has also been classified as group 3 (not classifiable as to its carcinogenicity to humans) by the International Agency for Research on Cancer (IARC 2002).

Many countries have implemented regulations on the mycotoxin levels in food and feed to protect human and animal health and in the economic interest of producers and traders (Ibáñez-Vea et al. 2012). The Brazilian regulation has established maximum permitted levels for the mycotoxins of concern in unprocessed cereals. For example, the maximum permitted levels for DON and ZEN in brewing barley are 1000 and 100 μg/kg, respectively (Brasil 2017). Similarly, the limits for DON and ZEN fixed by the European Commission (2006) are 1250 and 100 μg/kg, respectively, for unprocessed cereals.

The detection of multiple toxins present in food can be challenging due to the chemical difference between the different groups of mycotoxins and the complexity of food matrices. High-performance liquid chromatography/electrospray tandem mass spectrometry is the method of choice for the separation and detection of mycotoxins in food as it is a highly sensitive, specific, and reliable tool, particularly for the development of multi-mycotoxin detection methods (Zöllner and Mayer-Helm 2006).

Matrix effects due to food components such as carbohydrates, proteins, or fats can be minimized by using internal or matrix-matched standards during the LC-MS/MS analysis of mycotoxins. The matrix effects can be compensated by stable isotope-labeled internal standards because their chemical and chromatographic properties are identical to those of the target toxins. The stable isotope dilution assay LC-MS/MS method has been successfully used in mycotoxin analysis (Varga et al. 2012; Habler and Rychlik 2016; Al-Taher et al. 2017; Habler et al. 2017).

For the reasons stated above, the aim of the current study is to use an LC-MS/MS system for evaluating the presence of DON and ZEN in brewing barley cultivated in different regions of Brazil. The data from this research is significant due to the increasing production of the cereal in the country and its widespread application in the beer industry.

Materials and methods

Chemical and reagents

Both standards (DON and ZEN) were purchased from Sigma Aldrich Chemicals (St. Louis, MO, USA). Stock solution standards were prepared in methanol at concentrations of 1 mg/ml for DON and ZEN. From the individual stock standard solutions, a standard mixture was prepared at the following concentrations: 0.025, 0.0375, 0.0625, 0.125, 0.375, and 0.500 μg/ml. The standard mixture was prepared in methanol and stored at − 18 °C.

Methanol and acetonitrile (LC-MS/MS grade) were supplied by J.T Baker (Sao Paulo, SP, Brazil). Acetic and formic acids were obtained from Biotec (Pinhais, PR, Brazil). High-purity Milli-Q water (18.2 MΩ/cm) was obtained from a Millipore Synergy system (MA, USA).

Brewing barley samples

A total of 76 brewing barley samples were collected by the Brazilian Agricultural Research Corporation (EMBRAPA) from four regions of the Rio Grande do Sul State (Region 1:Vacaria, Region 2: Santo Augusto, Region 3: Passo Fundo, and Region 4:Não-me-Toque), the largest brewing barley producer in Brazil. The regions were differentiated by the preferred periods for sowing and according to the climatic risk zoning of the map of southern Brazil (Brasil 2014). The grains were gathered from the 2015 harvests and were obtained after the cleaning and drying stages (up to a maximum of 60 °C) in the storage unit. Collection was performed using a grain auger from different points of the bulk batches with a minimum final weight of ca 10 kg. Each sample was homogenized and reduced in portions varying around 2 kg. The grains were packed in polyethylene bags and stored at 4 °C for immediate mycotoxin analysis.

Mycotoxin extraction procedure for LC-MS/MS

For DON and ZEN analysis, extraction was carried out according to the method proposed by Al-Taher et al. (2017) with some modifications. In brief, 2 g of brewing barley was ground and homogenized in 8 ml of an acetonitrile/water (80:20 *v*/*v*) mixture and shaken for 60 min. The mixture was then centrifuged for 10 min at 3500 rpm. The supernatant was transferred to an amber vessel and dried using a heating block under a nitrogen stream. The dried extract was re-suspended in 500 μl of a mobile phase comprising 70% of a water:methanol:acetic acid (94:5:1, *v*/*v*/*v*) mixture and 30% of a water/methanol/acetic acid (2:97:1, *v*/*v*/*v*) mixture. Finally, 5 μl of the solution was injected into the LC-MS/MS system for analysis.

Optimization of chromatography and mass spectrometry conditions

Detection and quantification were accomplished with an LC-MS system from Thermo Scientific® (Bremen, Germany) composed of an ACCELA 600 quaternary pump, an ACCELAAS auto-sampler, and a triple quadrupole mass spectrometer TSQ Quantum Max.

The chromatographic conditions were adopted according to the procedure developed by Njumbe Ediage et al. (2015). A triple quadrupole mass spectrometer TSQ Quantum Max was operated at positive polarity, and the following ionization conditions were used: capillary temperature, 208 °C; vaporizer temperature, 338 °C; spray voltage, 4500 V; and sheath gas pressure, 60 bar. For selectivity, the mass spectrometer was operated in MRM mode; three transitions per analyte were monitored at a collision gas pressure of 1.7 mTorr and collision energy (CE) ranging from 11 to 40 eV.

The mass spectrometry conditions were optimized by re-tuning different analytes via direct infusion of each analyte individually. The cone voltages, collision energies, and product ions were optimized and carefully chosen (Table 1).

The most abundant mass-to-charge ratio (*m*/*z*) was selected for each compound of interest. The mycotoxins gave precursor ions and product ions with reasonably high signal intensities in positive ESI mode (ESI+), and protonated molecules [M + H] were found. Table 1 shows the retention times (*t*R), MRM transitions, and optimized cone voltages and collision energies for each compound.

Separation was performed on a C8 Luna column, with a particle size of 3 μm and a length and diameter of 150 and 2.0 mm, respectively (Phenomenex, Torrance, USA). The mobile phase comprised solvent A (water/methanol/acetic acid, 94:5:1, *v*/*v*/*v*) and solvent B (water/methanol/acetic acid, 2:97:1, *v*/*v*/*v*). The gradient program was applied at a flow rate of 0.2 ml/min under the following conditions: 0–1 min 55% B; 1–3 min 55–100% B; 3.01–7 min 100% B; and 7.01–12 min 55% B. The total analytical run time was 7.5 min for both toxins (Table 1).

LC-MS/MS method validation

The method was validated for brewing barley using the Commission Regulation (2006) guideline. To determine the limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability, and selectivity/specificity, samples with non-detectable levels of mycotoxins were analyzed via spiking experiments.

Considering linearity, a six-point calibration curve was constructed with the following concentrations of the mycotoxin standard mixture (DON and ZEN): 0.025, 0.0375, 0.0625, 0.125, 0.375, and 0.500 μg/ml.

The LOD and LOQ were determined by fortifying blank samples with different concentration levels, and the experiments were repeated on three different days. The LOD and LOQ are defined as the minimum concentration of an analyte in the spiked sample with a signal noise ratio of 3 and 10, respectively.

Statistical analysis

The obtained data was analyzed by analysis of variance (ANOVA) using the Tukey test. The results are presented as mean ± standard deviation, and values of $p < 0.05$ were considered statistically significant.

Results

This method was effective in the determination of mycotoxins in barley, as displayed by the results of the study. The reported LODs were in the range of 5–10 μg/kg, and the LOQs were 25 μg/kg for both toxins (Table 2). The coefficients of correlation (R^2) of the calibration curve were 0.997 and 0.999 for DON and ZEN, respectively, confirming the method linearity. Spiking was performed in triplicate at three different levels in the barley matrix. The experiments were considered adequate with 98.6 and 98% of recovery for DON and ZEA, respectively. Each spiked level was conducted in triplicate to obtain good

Table 1 Retention time and mass spectrometric parameters used in the analysis of the mycotoxins

Mycotoxin	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	CE (V)	TubeLens
DON	2.19	297 [M + H]	203Q	17	71
			175C	18	71
			91C	39	71
ZEN	6.55	319 [M + H]	283Q	11	79
			187C	25	79
			185C	20	79

Q, quantification transition; *C*, confirmation transition

Table 2 Characteristics of the method performance for deoxynivalenol and zearalenone in brewing barley samples

Analytes	LOD (μg/kg)	LOQ (μg/kg)	Linear regression (R^2)	Spiking level (μg/ml)	Recovery (%)	RSD (%)
DON	5	25	0.997	0.0375	98	6.4
				0.375	99	5.2
				0.500	99	3.3
ZEN	10	25	0.999	0.0375	97	3.0
				0.375	98	2.3
				0.500	99	0.4

LOD, limit of detection; *LOQ*, limit of quantification

precision, and they were performed on the same day and with the same system.

Most of the evaluated samples were contaminated with high levels of both toxins. Ninety-four percent (94%, $n = 72$) of the samples, which were collected from all the analyzed regions, were contaminated with DON. Taking all samples into account, the mean levels of DON ranged from 1700 to 7500 μg/kg. ZEN had a smaller percentage of contamination (73.6%, $n = 56$), with mean levels ranging from 300 to 630 μg/kg (Table 3).

The data was compared with the maximum levels established by the Brazilian regulation (Brasil 2017); DON levels in the 66 samples (83%) were above the established limits. Fifty-four samples (68%) had ZEN content that was higher than expected based on the recognized maximum permitted levels (Brasil 2017).

DON-ZEN co-occurred in a large number of barley grain samples, with concentrations above the maximum levels established by the Brazilian regulation (Table 3), raising concerns about the toxic effects of these mycotoxins, due to human exposure.

Figure 1 shows that the regions 2 and 3 present significantly higher DON levels ($p < 0.05$) than regions 1 and 4. On the other hand, the ZEN contamination showed significant differences only between regions 1 and 4. Region 4 presented samples with the lowest DON and ZEN levels.

Discussion

Brazilian barley samples harvested in 2014 for the brewing industry were previously analyzed by Piacentini et al. (2015), and the results demonstrated contamination levels of DON between 200 and 15,000 μg/kg, which were lower than the 2015 harvest. Indeed, climatologic conditions such as temperature and precipitation increased during the last year, highlighting the importance of monitoring these conditions during plant development (CPTEC 2015; INMET 2015).

In the current study, most of the evaluated samples were contaminated with higher levels of both toxins when compared to those in other studies. Běláková et al. (2014) conducted a research on 325 barley samples collected from various regions in the Czech Republic and recorded the highest levels of DON and ZEN in 2009 (2213 and 59.4 μg/kg, respectively). Co-occurrence of *Fusarium* toxins was also recently evidenced in a study performed by Habler and Rychlik (2016), with mean DON levels ranging from 12 to 279 μg/kg in barley malt samples collected in various German federal states over 3 years (2012 to 2014). The ZEN levels varied between 1.41 and 42.4 μg/kg.

Additionally, DON and ZEN levels found in barley from Lithuania ranged from 100 to 231 μg/kg and from 10 to 41.4 μg/kg, respectively, emphasizing that a 100% of the analyzed samples were contaminated by these mycotoxins

Table 3 Occurrence and co-occurrence of mycotoxins in brewing barley from four different regions of Brazil

Regions	Mycotoxins										
	Samples	DON (μg/kg)				Samples	ZEN (μg/kg)				DON-ZEN
	Analyzed/ positive	Range of the positive samples (min-max)	Mean	SD	Median	Analyzed/ positive	Range of the positive samples (min-max)	Mean	SD	Median	Co-occurrence: positive samples
Vacaria	19/19	900–7700	3900	1700	4000	19/19	160–1500	630	380	600	19
Santo Augusto	19/18	4100–15,500	7500	2900	6800	19/17	160–1700	500	370	400	17
Passo Fundo	19/17	2800–11,100	6900	3000	6700	19/13	160–2300	500	550	300	13
Não-me-Toque	19/18	310–3800	1700	1200	1700	19/7	100–1000	350	320	200	7

Co-occurrence was expressed as the number of samples with simultaneous contamination of both mycotoxins

SD standard deviation

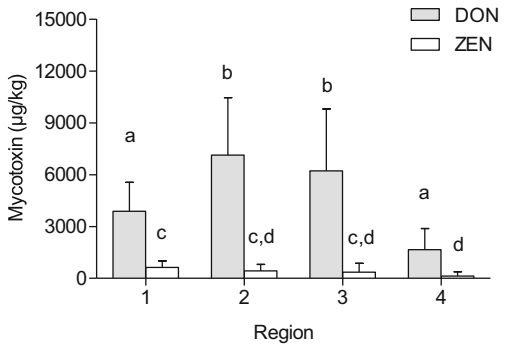

Fig. 1 DON and ZEN contamination of 19 samples for each crop region (total 76 samples). Data expressed as mean ± SD; the same letter indicates that the mycotoxin contamination are not different significantly according to Tukey test ($p < 0.05$)

(Mankeviciene et al. 2011). In 2013, 34 barley samples from Croatia were analyzed for both toxins, and the mean levels were found to be 342 μg/kg for DON and 32 μg/kg for ZEN (Pleadin et al. 2013).

Weather is one of the most important factors affecting the occurrence of *Fusarium* and its toxins. Warm and humid conditions during the flowering season are crucial for the development of some pathogenic species within the genus *Fusarium*, including *F. graminearum*, which is able to cause FHB and produce high levels of trichothecenes and ZEN, compromising the quality of small cereals, such as barley (Xu 2003).

Samples from the current study were sowed in May 2015 and harvested in December 2015. During this period, high precipitation levels were registered (mean = 95 mm), therefore, influencing the air humidity in the studied regions (CPTEC 2015), with maximum values of 70, 97, 84, and 65%, for regions 1, 2, 3, and 4 respectively (INMET 2015). Temperature may also have influenced the occurrence and levels of the studied mycotoxins in barley during this year, as the recorded average was 28 °C for all of the regions, which is considered suitable for *F. graminearum* growth and mycotoxin production.

In addition, it is important to mention that trichothecenes can resist the brewing process and persist in the final product. Studies have shown that the amount of these mycotoxins were even higher in beer than in barley due to mycotoxin carryover from the brewing process (Lancova et al. 2008). This fact raises the hypothesis that high levels of DON could possibly be recovered from Brazilian beer, as these barley samples are destined for the brewing industry.

Modified mycotoxin such as deoxynivalenol-3-glucoside (D3G) can be cleaved in human and animal gastrointestinal tracts in glucose and DON which can increase the DON levels in the samples. They can be formed by plant metabolism (Rychlik et al. 2014); however, it is not detectable using standard approaches. In the study of Habler and Rychlik (2016), DON and its modified metabolite, D3G, were found in barley malt at frequencies of up to 73 and 53%, respectively. The amount of DON ranged between 31 μg/kg and 10 mg/kg and the amount of D3G between 28 μg/kg and 19 mg/kg. DON and ZEN have derivatives that are also produced by *Fusarium*, such as the acetyl derivatives 3-acetyl-deoxynivalenol (3-Ac-DON) and 15-acetyl-deoxynivalenol (15-Ac-DON) and also glucosides of ZEN, as ZEN-14 Glc (Rychlik et al. 2014). ZEN-14-Glc may be cleaved during digestion and release its active parent compound as demonstrated in pigs (Gareis et al. 1990). These toxins are designated masked mycotoxins and are undetectable by conventional analytical techniques.

Overall, because of the high contamination levels found in brewing barley, it is possible to state that the preventive measures, such as the use of fungicides, resistant cultivars, and crop rotations, were not able to effectively reduce DON and ZEN contamination in barley samples from the south of Brazil. These findings have clearly shown that mitigation efforts are required to reduce the toxin levels to those below the Brazilian and international regulations.

Source of funding The authors thank the Research support foundation of the state of Sao Paulo (FAPESP/Project 2016/01798–4) for the financial support.

Compliance with ethical standards

Conflict of interest None.

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