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Quality and occurrence of deoxynivalenol and fumonisins in craft beer

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ABSTRACT

Beer is an alcoholic beverage consumed on a regular basis by many people around the world. Consequently, beer quality and, specifically, its impact on the future health of the consumer must be considered seriously. One issue is the action of mycotoxins and their impact on the beverage. In this sense, the objective of the present study was to determine the occurrence of Deoxynivalenol (DON) and Fumonisin B_1 (FB_1) in many artisanal beers from southern Brazil and, additionally, to evaluate their physico-chemical properties.

The methods applied for physic-chemical characteristics were from the AOAC and Adolfo Lutz Institute. The analyses for mycotoxins were conducted using high performance liquid chromatography with fluorescence detection for fumonisin B_1 and ultraviolet detection for deoxynivalenol. The physic-chemical results were in agreement with some studies and with Brazilian regulations. DON and FB_1, were present in 32 and 15.09% of the samples, respectively. The concentrations found in craft beer from southern Brazil were probably caused by the widespread and high occurrence of these toxins in barley. Furthermore, the level of mycotoxins seem to be very stable during the brewing process.

1. Introduction

Beer is an alcoholic beverage consumed on a regular basis by many people around the world. In Brazil, for instance, the average beer consumption recorded in 2008 was around 57 l per inhabitant (Sindicerv, 2014). Nowadays, consumption of craft beer has increased due to the different types available. Together with being smaller scale and independent, the main characteristic of the craft breweries is to put the emphasis on the flavor and brewing techniques (Oliver, 2011, pp. 270–271). Craft beer is a non-filtered and unpasteurized product that maintains unaltered sensorial characteristics. When compared to industrial beers, craft beer is more subject to microbial contamination which may result in spoilage (turbidity, acidification and the production of undesired aromatic compounds) (Giovenzana, Beghi, & Guidetti, 2014).

Moreover, due to the absence of pasteurization and microfiltration in craft beer brewing, the need is much greater for quality measurements during the entire process and not only in finished beer. Monitoring must be performed on raw materials and at individual process stages. This also differs from industrial beers (Bamforth, 2003).

Beer quality is extremely important as problems with this may result in future diseases in the consumer population. In this sense, it is possible to highlight the action of mycotoxins and their impact on the beverage.

All mycotoxins are naturally occurring secondary metabolites of filamentous fungi and they can be produced in a large range of agricultural commodities, (Bertuzzi, Rastelli, Mulazzi, Donadini, & Pietri, 2011) mainly associated with cereal crops, in particular corn, wheat, barley, rye, rice and oats (Goyarts, Dänicke, Valenta, & Ueberschär, 2007; Omurtag, Yazıcıoglu, Beyoglu, Tozan, & Atak, 2006).

As beer production requires the use of barley grains and these may have been exposed to mycotoxins, a number of studies have been carried out to detect these in commercially available beers (Benesova, Belakova, Mikulíková, & Svoboda, 2012; Kawashima, Vieira, & Valente Soares, 2007; Scott, 1996). In summary, results showed that the deoxynivalenol (DON), nivalenol, T-2, HT-2, diacetoxyscirpenol, zearalenone, aflatoxins, ochratoxin A, and fumonisins have been detected in beers at trace (ppb) levels.

DON, known colloquially as “vomitoxin” (Canady et al., 2001), is one of the mycotoxins most found in barley and it is produced mainly by *Fusarium graminearum* (Pestka, 2007). The exposure of DON in human and animal bodies through ingestion of contaminated food can cause acute and chronic effects such as

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immunosuppression, neurotoxicity, embryotoxicity and teratogenicity (Pestka, 2007; Rotter, Prelusky, & Pestka, 1996; Wijnands & Van Leusden, 2000).

Furthermore, another important problem found concerning DON presence in beer is "gushing", i.e. excessive foaming and overflowing on opening a bottle. This has been reported frequently in the last few years and can seriously damage the beer quality and the reputation of the brewery.

Another relevant mycotoxin group that can be found in barley and, consequently, in beer are the FBs (FB_1 and FB_2). These are produced by a number of *Fusarium* species, notably *Fusarium verticillioides*, *Fusarium proliferatum*, *Fusarium anthophilum*, *Fusarium nygamai* as well as *Alternaria alternata f.* sp. *lycopersici* (Bennett and Klich, 2003). *F. verticillioides* produces several mycotoxins however, the most prominent is fumonisin B_1 (FB_1) (Ciacci-Zanella, Merrill, Wang, & Jones, 1998). This mycotoxin can be introduced in beer, either from contaminated input materials or from adjuncts added during the brewing process. Generally, corn starch and corn syrup are among the adjuncts alternatively used for beer production (Hlywka & Bullerman 1999).

With respect to the mycotoxins produced by *Fusarium*, it is possible to say that they are produced mainly in the field, although some toxin synthesis may occur during storage, or also, in the case of beer, they can increase during the germination of the barley during the malting and brewing process of the beer (Beattie, Schwarz, Horsley, Barr, & Casper, 1998; Lancova et al. 2008; Pietri, Bertuzzi, Agosti, & Donaldini, 2010; Wolf-Hall, 2007). Basically, the temperature and moisture conditions are crucial factors and thereby affect the fungal infection and toxin synthesis (Doyle, 1997).

In 2012, the Brazilian regulations proposed a maximum tolerable level (MTL) of 1750 μg/kg for DON in malted barley grains. The limit will be decreased over time to allow grain producers and the industry to adapt to the legislation without causing a shortage of barley. As of January 2017, DON limits for malted barley will be set at 750 μg/kg (Brasil, 2011; Brasil, 2013).

Currently, the limit fixed by the Commission of the European Communities for DON is equal to 1750 μg/kg for cereals and sub products (European Commission, 2006). It is important to emphasize that there is no specific legislation for fumonisins neither in barley nor for the mycotoxins detected in beer around the world. Furthermore, to our knowledge no research study has examined mycotoxins in craft beer in Brazil or internationally.

For the reasons stated above, the aim of the present study was to determine the occurrence of DON and FB_1 in craft beers from southern Brazil and their quality, regarding the physic-chemical characteristics.

2. Materials and methods

2.1. Materials

2.1.1. Samples

In the first months of 2014, 53 craft beer samples (one bottle each), consisting of 14 different brands, were collected in breweries and local stores in southern Brazil and kept under refrigeration at 7° and in the dark until analysis. The samples consisted of 25 ale beers and 28 lager beers.

2.1.2. Chemicals and standards

For physical and chemical analyses, sodium hydroxide and phenolphthalein analar grade were obtained from Biotec (Pinhais, PR, Brazil). For HPLC analyses, acetonitrile, methanol and acetic acid were obtained from Vetec (Duque de Caxias, RJ, Brazil), all of which were LC grade. Sodium hydroxide was from Biotec (Pinhais, PR, Brazil) and OPA reagent (40 mg o-ftaldialdehyde in 1 ml ethanol diluted with 5 ml 0.1 M borate buffer and 50 μl 2-mercaptoethanol) was from Sigma Aldrich Chemicals (St. Louis, MO, USA). High-purity Milli-Q water (18.2 MΩ/cm) was obtained from the Millipore Synergy system (MA, USA). The standards, DON and FB_1, were obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA). A stock solution of DON was prepared by dissolving 1 mg in DON in 1 ml of acetonitrile. The standard curve solutions were prepared from appropriate dilutions of the stock solutions (200 μg/ml) with Mili-Q water (0.15–15 μg/ml). A stock solution of FB_1 was prepared by dissolving 1 mg in FB_1 in 1 ml of methanol. The standard curve solutions were prepared from appropriate dilutions of the stock solutions (50 μg/ml) with methanol (0.005–2.5 μg/ml). *Other materials used*: immunoaffinity columns from DON-test Vicam (Milford, MA, USA), strong anion exchange SPE columns (6 cm^3, 500 $mg^{-1,}$ SAX, Phenomenex, USA) and diatomaceous earth.

2.1.3. Equipments

The following equipment was required for the physical and chemical tests: pH meter (Digimed DM 20, São Paulo, SP, Brazil), water bath (Nova Técnica, Piracicaba, SP, Brazil) and a drying oven (Olided –cz, Ribeirão Preto, SP, Brazil).

The determination of DON and FBs levels was carried out by a high performance liquid chromatography (HPLC) model 321, Gilson (Middleton, WI, USA), equipped with an isocratic pump model 805, manual injector (20 μl loop) with an ultraviolet–visible (UV) detector model 118 and fluorescence detector model 121. The chromatographic columns used were C_{18} reversed-phase (Synergi 4 μm particle size, with 250 × 4.60 mm, length and diameter, respectively), model Fusion-RP 80, Phenomenex (Torrance, USA) for DON and C_{18} reversed-phase (particle size 5 μm, with 150 and 4.60 mm), model Luna (Torrance, USA), for FB_1.

2.2. Methods

2.2.1. Physical and chemical analyses

To accomplish the physical and chemical analyses, each bottled beer sample was gently mixed and approximately 200 ml were degassed by ultrasonic bath.

2.2.1.1. pH. The pH tests were in accordance with the AOAC Method 945.10 (2012) for beer, using a pH/reference electrode system.

2.2.1.2. Acidity. The acidity analyses were in accordance with the AOAC Official Method 950.07 (2012) for beer. In short, 10 ml of previously decarbonated beer, 50 ml of distilled water and 0.5 ml of 0.5% phenolphthalein were added to a flask. After this first step, the sample was titrated with 0.1 N NaOH to first appearance of faint pink.

2.2.1.3. Real extract. The determination of the real extract is in accordance with the Adolfo Lutz Institute method (IAL 2008) and it is based on the dry residue weight of a certain volume of sample submitted to evaporation.

2.2.2. Fumonisin (FB_1) determination

To perform the FB_1 analyses, the AOAC Official Methods of Analysis 995.15 (AOAC, 2005), originally developed for corn and its products with modifications, was used as follows: Briefly, the pH of craft beer samples was brought to a 5.8–6.5 range with 1 N NaOH and the samples were filtered through qualitative filter paper. For sample cleanup and concentration, a 50 ml aliquot of beer was applied to a strong anion exchange SPE column (6 cm^3, 500 $mg^{-1,}$ SAX, Phenomenex, USA), previously conditioned with 10 ml of methanol, followed by 10 ml of methanol:water (3:1). The sample was followed by 10 ml of methanol:water (3:1) and 6 ml of

methanol. FB_1 was eluted with 10 ml of methanol:acetic acid (95:5). The elution was dried under a nitrogen stream at 60 °C.

The dried extract was suspended in 300 μl of acetonitrile:water (1:1) and was cleaned with a syringe filter (0.45 μm, 13 mm, CA membrane) and dried under a nitrogen stream at 60 °C. The dried extract was then suspended in 100 μl aliquot of methanol and transferred to a reaction vessel and 200 μl of OPA reagent was added. After 60 s reaction time, 20 μl of the derivatized sample was injected into a LC-FLD at 335 and 440 nm for excitation and emission, respectively. The mobile phase was methanol: sodium dihydrogen phosphate (77:23, v/v) adjusted to pH 3.3 with H_3PO_4 at a flow rate of 1 ml/min.

2.2.3. Deoxynivalenol analyses

The craft beer samples were analyzed using immunoaffinity columns for the cleaning step and LC/UV for detection, according to the Vicam protocol DON test, N_o G1005 USA (Vicam 2013), with some modifications. In short, 84 ml of acetonitrile and 3.2 g of diatomaceous earth were added to 16 ml of a degassed sample and mixed for 5 min and then filtered through a Whatman no. 4 filter paper.

For sample cleanup and concentration, an aliquot of 1 ml of the extract was applied to an immunoaffinity column (DONTest HPLC) at a flow rate of one drop per second. This column was previously conditioned with 1 ml of LC grade water. The sample was followed by 2.5 ml of LC grade water to wash the column and the toxin was slowly eluted with 2 ml of 100% LC grade methanol. The eluate was evaporated using a heating block device at 40 °C in a gentle nitrogen stream and then the dry residue was redissolved in 100 μl of mobile phase acetonitrile:water (10:90, v/v). The extract (20 μl) was injected into the LC/UV System set at a wave length of 218 nm and the mobile phase was delivered at a constant flow rate of 0.8 ml/min.

3. Results

3.1. Physical and chemical characteristics

The physical and chemical analyses are extremely important to the quality of the beer and, consequently, for consumer acceptability. In this paper, the pH, the acidity and the real extract of the beers analyzed are also shown in Table 1, in terms of mean and standard deviation according to beer type.

3.2. Mycotoxins

3.2.1. Don analyses

The LC/UV method for DON chromatographic separation and the validation parameters obtained (linearity, limit of detection – LOD, limit of quantification – LOQ reproducibility, repeatability and recovery), were adequate. For instance, under the chromatographic conditions used, the DON retention time (Rt) was equal to 14 ± 0.5 min. Linearity was confirmed using the calibration curve for each DON concentration; i.e., it was linear from 0.15 to 15 μg/ml (correlation coefficient 0.996). The LOD (signal to noise ratio = 3) and LOQ (signal to noise ratio = 10) were 67 and 119 μg/l, respectively. Recovery experiments were conducted by spiking blank beer with 200 μl of DON stock solution at concentrations of 80, 160 and 250 μg/l and performed with the same HPLC system. The extraction was carried out according to the methodology previously mentioned and the eluate (2 ml) was evaporated with nitrogen at 60 °C for 10 min until dry. A duplicate analysis was conducted.

The recovery experiments showed yields of 99.9, 99.9 and 96.0% for concentrations of 80, 160 and 250 μg/l, respectively. The mean recovery rate for the extraction method was 97.9%.

In the present study, DON was detected in approximately 32% (17 of 53 samples) of the samples, with levels ranging from 127 μg/l to 501 μg/l (mean 221 μg/l). The descriptive statistics are shown in Table 2.

Table 1
Physico-chemical characteristics of the craft beer.

Beer type	Samples	pH	Acidity (% latic acid)	Real extract
Ale	25	4.55 ± 0.27	0.26 ± 0.06	5.40 ± 1.48
Lager	28	4.74 ± 0.21	0.24 ± 0.07	5.17 ± 1.59

3.2.2. FB_1 analyses

The method applied for FB_1 was successfully validated under laboratory conditions. The validation criteria were linearity, selectively, reproducibility, limits of detection and quantification (LOD and LOQ, respectively) and recovery. The retention time (rt) of FB_1 was 5.5 min ± 0.5. Linearity was confirmed by constructing a calibration curve for FB_1 ranging from 0.005 to 2.5 μg/ml, which showed coefficients of correlation r^2 = 0.990 for FB_1. The LOD (signal noise ratio = 3) was 6.6 μg/l, and LOQ (signal noise ratio = 10) was 21.0 μg/l. Recovery experiments were conducted by spiking blank beer with 500 μl of the FB_1 stock solution at concentrations of 35 and 70 μg/l, on the same day and with the same HPLC system. The extraction was carried out according to the methodology previously mentioned and the eluate (10 ml) was evaporated with nitrogen at 60 °C for 10 min until dry. A duplicate analysis was performed. The recoveries ranged from 99.1 % to 99.7 % for 35 and 70 μg/l, with an overall mean of 99.4%.

For FB_1, 8 (15.09%) samples were contaminated with levels ranging from 29 μg/l to 285 μg/l. The descriptive statistics are detailed in Table 2.

4. Discussion

Firstly, it is important to mention that the pH for beer is essential because it has an influence on several factors such as microorganism growth, color intensity, enzymatic activity, flavor and oxy-reduction potential, as discussed in Oliveira (2011). The results of the pH analyses for both beer types are in accordance with Compton (1978) with values ranging from 3.8 to 4.7, which determine a great parameter for the beer quality. Moreover, a survey carried out by Sleiman and Venturini in 2004 also showed pH levels similar to those found in our study and those stated by Compton (1978).

In addition, the results concerning the acidity and real extract analyses are in agreement with the Brazilian regulation established by Anvisa decree n^0 2.314/1997 (Brasil, 1997).

With respect to DON, which is considered one of the mycotoxins most found in cereals in the world, especially in barley, the study was able to demonstrate that the contamination levels must be monitored especially in products made from stored cereals which are potentially contaminated.

Table 2
Descriptive statistics for concentrations of DON and FB_1 in craft beer.

Samples	Range of positive samples (μg/l)[a]	Mean of positive samples (μg/l)	Median of positive samples (μg/l)	Maximum value (μg/l)
Deoxynivalenol				
17	127–501	221	177	501
Total: 17				
Fumonisin FB_1				
8	29–285	105	90	285
Total: 8				

[a] > Method LOQ 119 μg/l for deoxynivalenol and 21.0 μg/l for fumonisin B_1.

The same idea presented in this paper was also researched by Schothorst Jekel (2003) who conducted a survey on mycotoxins in the Netherlands. On that occasion, the results showed contamination in only three of 51 analyzed industrial beer samples (approximately 6%), and the DON levels were low, varying between 26 μg/l and 41 μg/l. Additionally, no detectable levels of mycotoxins in industrial beer were found in a study conducted by Omurtag and Beyoglu (2007) in Turkey.

Furthermore, in order to explain the DON levels, some studies have shown that this mycotoxin seems to be very stable during the brewing process (Böhm-Schraml, Stettner, & Geiger, 1997; Wolf-Hall, 2007). Niessen (1993), found deoxynivalenol to be carried over into the final beer. In the study mentioned, they also showed a four-fold increase in deoxynivalenol concentrations during mashing. It suggested that deoxynivalenol may be released from protein conjugates during mashing.

Moreover, another study carried out by Lancova et al. (2008), showed that in malt, the content of monitored mycotoxins (tricothecenes) was higher compared with the original barley. The most significant increase was found for DON -3-Glc. Additionally, during the brewing process, significant increases in levels occurred.

According to the aspects aforementioned, chemical treatments are promising, such as ozonation, (Savi, Piacentini, Bittencourt, Scussel, 2014; Savi, Piacentini, Scussel, 2014) as this would not leave residual chemicals in the barley grain or in the beer. This means that the problem would be reduced or solved still in storage where some conditions can be monitored.

It is important to highlight that the current Brazilian and international regulations do not determine minimum levels for DON in beer. However, there are maximum values for barley and malted barley which correspond to levels of 1500 μg/kg and 1250 μg/kg, respectively. These levels can serve as a benchmark. Based on this, it is possible to conclude that beers brewed from quality, purified and well-stored raw materials do not represent any health risk of DON exposure to consumers.

Considering FB_1, the results presented previously are similar to a study carried out in Brazil in 2007, where 43.1% of the industrial beers analyzed were contaminated with levels ranging from 1 to 40 μg/l (Kawashima et al. 2007). Furthermore, the results from this survey were in agreement with previous European studies: Bertuzzi et al. (2011) detected FBs in 97% of the samples ($n = 32$) at maximum levels of 30 μg/l and Torres, Sanchis, and Ramos (1998) detected FBs in 43.8% of Spanish beer ($n = 32$) at levels of 4.8–85.5 μg/l. Also for FB_1, some studies have reported the stability of this toxin in heat and during the fermentation process (Scott, 1995).

It is necessary to highlight that most of the craft beers are made only with barley, therefore, these results may suggest that the beer contamination can be affected by the products used. An example of this, is corn starch and corn syrup which are among the adjuncts alternatively used for beer production in Brazil. These can increase the levels of FB_1 as cited by Hlywka and Bullerman (1999) and Kawashima et al. (2007).

According to these numbers of contamination in beer, many studies also documented levels of *Fusarium* mycotoxins and their pattern in barley grains (Castañares et al., 1998; Marín et al.1999; Oliveira, 2011; Rubert et al. 2012). This problem is strongly influenced by agricultural practices which, in combination with weather conditions during critical phases of plant growth, determine a number of toxigenic fungi invading the crop under field conditions (Edwards, 2004).

Table 3
Estimation of mycotoxin dietary intakes from beer.

	Mean[a] (μg/l)	Daily average exposure (μg/kg bw)	Tolerable daily intake (μg/kg bw)	% of tolerable daily intake
Deoxynivalenol	71	0.18	1	18
Fumonisin B_1	16	0.04	2	2

[a] All of 53 samples.

4.1. Estimation of mycotoxin dietary intakes by the consumption of beer

By considering the mean values of DON and FB_1 obtained from this survey, the mean Brazilian beer consumption (57/l year per capita, equivalent to 0.158/l day) and a body weight of 60 kg, it was possible to calculate the daily average exposure, as shown in Table 3. The Scientific Committee for Food (SCF) and FAO/WHO Joint Expert Committee on Food Additives (JECFA) (Bolger et al., 2001) indicated a tolerable daily intake (TDI) of 1 and 2 $\mu g/kg^{-1}$ bw for DON and FBs, respectively.

According to Table 3, it is evident that, for a moderate consumer, the daily average exposure from beer was low, in agreement with previous studies (Harcz et al., 2007; Matumba et al., 2014). Nevertheless, it is extremely important to maintain the high levels of monitoring.

5. Conclusion

This study reported the results from a survey investigating the physical and chemical characteristics of craft beer produced in southern Brazil and the presence of mycotoxins in it.

In summary, the pH, acidity and real extract were in agreement with some surveys and with the Brazilian regulation, however, DON and FB_1 were present in 32 and 15.09% of the samples, respectively. The concentrations found in craft beer from southern Brazil were probably caused by the widespread and high occurrence of these toxins in barley. Furthermore, the mycotoxins seem to be very stable during the brewing process.

The elimination of both toxins from the product should be contemplated by the industry either for quality or health reasons.

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