Fumonisin B_1 and ochratoxin A in beers made in Brazil

Fumonisina B_1 e ocratoxina A em cervejas fabricadas no Brasil

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Abstract

Samples of beer made in Brazil were analyzed for the presence of fumonisin B_1 (FB_1) and ochratoxin A (OTA). FB_1 was searched for in 58 beer samples from 30 plants located in nine states. The samples were concentrated and cleaned up with strong ion exchange column, derivatized with OPA and analyzed by HPLC with fluorescence detection. The limit of detection was 0.26 ng.mL^{-1} and the average recovery was 98%. Twenty-five samples contained FB_1 ranging from 1 to 40 ng.mL^{-1}. Beer (123 samples) from 36 plants located in 5 states were analyzed for OTA by means of immunoaffinity column cleanup followed by liquid chromatography associated with fluorescence. The detection limit was 0.1 ng.mL^{-1} and the average recovery was 92%. Five samples contained OTA in concentrations from 1 to 18 ng.mL^{-1}. The results indicate that FB_1 and OTA contamination in Brazilian beer is not geographically limited and that beer does not contribute significantly to FB_1 intake by consumers. In the case of regular high ingestion, beer could contribute sizably to OTA, intake although still below the maximum considered tolerable for the toxin.

Keywords: mycotoxins; fumonisin B_1; ochratoxin A; beer.

Resumo

A presença de fumonisina B_1 (FB_1) e de ocratoxina A (OTA) foi investigada em amostras de cerveja fabricada no Brasil. FB_1 foi pesquisada em 58 amostras de cerveja provenientes de 30 fábricas localizadas em nove Estados. As amostras foram concentradas, e a toxina isolada através de coluna de troca iônica forte, derivação com OPA e análise por CLAE com fluorescência associada. O limite de detecção foi 0,26 ng.mL^{-1} e a recuperação média foi de 98%. Vinte e cinco amostras continham FB_1 em concentrações de 1 a 40 ng.mL^{-1}. Cerveja (123 amostras) proveniente de 36 fábricas localizadas em 5 Estados foi analisada para OTA através de coluna de imunoafinidade seguida de CLAE com detector de fluorescência. O limite de detecção foi 0,1 ng.mL^{-1} e a recuperação média foi de 92%. Cinco amostras continham OTA em concentrações de 1 a 18 ng.mL^{-1}. Os resultados indicam que a contaminação da cerveja brasileira por FB_1 e por OTA não é geograficamente limitada e que não contribui significativamente para a ingestão de FB_1 por consumidores. Por outro lado, no caso de consumo alto e regular, esta pode contribuir substancialmente na ingestão de OTA, porém ainda abaixo do máximo considerado tolerável para a toxina.

Palavras-chave: micotoxinas; fumonisina B_1; ocratoxina A; cerveja.

1 Introduction

Fumonisins consist of a group of mycotoxins produced mainly by strains of *Fusarium verticilloides* and *Fusarium proliferatum*[5]. Within this group of toxins, Fumonisins B_1 (FB_1), B_2, and B_3 are the most commonly found in food and feed. FB_1 represents up to 70% of the fumonisins produced in laboratory conditions or found in naturally contaminated food or feed. It is also the most toxic of the group[9]. In rats, FB_1 causes cancer in liver, is toxic to kidneys and liver and weakens the immune system[22]. FB_1 brings about leukoencephalomalacia in horses[22,49] and pulmonary edema in swine[11]. There is no direct evidence of FB_1 carcinogenic effect in humans, but an increase of oesophageal cancer is observed in areas where maize, either contaminated by *Fusarium* or containing high levels of FB_1, is used as the main staple[5,50]. Fumonisins, mainly FB_1, have been detected in a large array of maize products in all areas of the world where analyses of such commodities have been conducted [4,6,7,16,17,25,40,45].

Fumonisins may be introduced when making beer when maize is used as an adjunct to fermentation[18]. Corn starch and corn syrup are among the adjuncts alternatively used for beer production, but no contamination by fumonisins has been found in these materials[4,28]. FB_1 is stable to heat[1,19,20] and to conditions found during fermentation of corn to produce ethanol[37].

The presence of fumonisins in beer was described for the first time by SCOTT and LAWRENCE[36] in beverages commercialized in Canada. The levels found were low and only 4 of the 41 samples analyzed had concentrations higher than 2 ng.mL^{-1}. In a subsequent study, SCOTT et al.[38] found the toxins in 20 of 46 samples of beer. The concentrations of FB_1 + FB_2 in the positive samples ranged from 0.2 to 64 ng.mL^{-1} (average = 5.6 ± 13.6 ng.mL^{-1}). Both studies included foreign and Canadian beers. TORRES et al.[51] analyzed 32 samples of Spanish beers and found that 14 samples contained FB_1 in concentrations ranging from 4.76 to 85.53 ng.mL^{-1} (average = 28 ± 22 ng.mL^{-1}). HLYWKA and BULLERMAN[18] examined 29 samples of beers commercialized in the U.S.A. The authors detected FB_1, FB_2, and FB_3 in 25 of the samples. The contaminated samples contained FB_1 + FB_2 concentrations from 0.3 to 13.5 ng.mL^{-1}.

Ochratoxin A (OTA) is nephrotoxic to monogastric animals, especially swine, and a potent renal carcinogen in rats. It behaves as immonotoxic and teratogenic agent. It shows genotoxic properties in some tests, but not in others such as the Ames test[23,30]. Evidence gathered so far, although not conclusive, points to OTA as the possible causative agent of the endemic chronic renal disorder observed in humans in the Balkan countries[44,46]. The occurrence of OTA in food has been shown to be worldwide, especially in cereals[24,31,43]. *Penicillium ver-*

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rucosum, *Aspergillus ochraceus*, and *A. carbonarius* are OTA major producers, but their individual temperature and other growth preferences cause them to occur in different areas of the globe, as well as infecting diverse food[29,48]. OTA residues may occur in beer, if the toxin is present in barley and malt used in its making[2]. The fermentation of wort to which OTA had previously been added showed that the toxin decreased from 2 to 13%[37]. A remainder of 13 to 32% of the toxin was found in the final product when OTA contaminated malt was obtained from barley inoculated with *Penicillium verrucosum* and subsequently used for beer making[2].

Beer has been surveyed for OTA in various countries. MEDINA et al.[26] examined 88 samples of beer (domestic and imported) marketed in Spain and OTA was detected in 82.9% of them. The range for positive samples was 0.007-0.204 ng.mL^{-1}. In Hungary, VARGA et al.[52] analyzed 25 samples of beer and all but one of the samples were found to be contaminated with small amounts of OTA with a mean concentration of 0.127 ng.mL^{-1} and a range from 0.030 to 0.250 ng.mL^{-1}. DASKO et al.[12] analyzed OTA 20 samples of local and 4 samples of foreign beer commercialized in Slovenia. No positive results for OTA presence were detected in any of the beer samples. German beers (35 samples) were analyzed by DEGELMANN et al.[13] and OTA levels in the range of 0.1-0.2 ng.mL^{-1} were found in 9 beer samples, 21 samples contained trace amounts of OTA (<0.1 ng.mL^{-1}) and in 5 samples no OTA was detectable. SCOTT and KANHERE[35] found traces of OTA in 26 out of 46 samples of Canadian and imported beers. In Denmark, JORGENSEN[21] found OTA in 21 samples of beer analyzed in concentrations ranging from 0.0001 to 0.160 ng.mL^{-1}, with a mean of 0.049 ng.mL^{-1}. In Japan, 94 imported and 22 Japanese made beers were tested for OTA by NAKAJIMA et al.[27] and a mean level of 0.010 ng.mL^{-1} was determined in 86 out of the imported beers and a mean level of 0.0125 ng.mL^{-1} in 21 of the Japanese beers. VISCONTI et al.[53] analyzed Italian and imported beers (61 samples) and found that half of the samples contained OTA in concentrations from <0.01 to 0.135 ng.mL^{-1}. No correlation between the incidences of OTA and country or beverage type was found by SOLEAS et al.[42] in beer commercialized in Canada (107 samples). In Belgium, OTA was found by TANGNI et al.[47] in all samples of 62 Belgian and 20 imported beers analyzed and the highest concentration was 0.185 ng.mL^{-1}. PRADO et al.[31] investigated the presence of OTA in 26 samples of beer produced in Brazil and found traces of the toxin in 6 samples.

Brazil is the fifth world largest producer of beer after China, US, Germany, and Russia with a yearly production of 8.5 billion liters according to data collected from 2002 to 2003. The annual per capita beer intake of 47.6 liters in 2004, on the other hand, ranks ninth after countries such as the Czech Republic, Germany, the UK, Australia, the US, Spain, Japan, and Mexico[41]. So far Brazilian beers have not been examined for FB_1 and a limited number of samples have been analyzed for OTA. The present work investigated beers produced in various locations within the country for the possible presence of FB_1 and OTA.

2 Material and methods

2.1 Samples

Beers made in Brazil (44 Pilsner and other light colored types and 14 stout and other dark colored types) were acquired at food markets and liquor outlets during 2000 and 2001 to be analyzed for FB_1. One hundred and twenty–three samples of beer (94 light and 29 dark types) were acquired at food markets during 2003 and 2004 to be analyzed for OTA. The beers were either canned or glass bottled. A sample from each type of beer formulation was acquired whenever more than one type of beer was produced by the plant in order to cover its whole range of products.

2.2 Analytical reagents

Reagent grade solvents and salts were used for sample extraction, cleanup and derivatization. Ultra-pure water (Milli-Q Plus, Millipore, Milford, USA) and chromatographic grade acetonitrile and methanol were used for the mobile phase. Fumonisin B1 and ochratoxin A were obtained from Sigma (St Louis, USA).

2.3 Sample extraction and cleanup for fumonisin B1 determination

The AOAC Method 995.15[34] originally developed for corn and its products and modified by CAMARGOS et al.[8] was used as follows. In short, the pH of beer samples was brought to the 5.8-6.5 range with 1N NaOH and the samples were filtered through qualitative filter paper. For sample cleanup and concentration, an aliquot of 50 mL beer was applied to a strong anion exchange SPE column (10 cm^3.500 mg^{-1}, SAX, Bond Elut LRC, Varian, Walnut Creek, USA) previously conditioned with 10 mL methanol followed by 10 mL de methanol/water (3:1). The sample was followed by 10 mL methanol/water (3:1) and 6 mL methanol. FB_1 was eluted with 20 mL methanol/acetic acid (95:5). The elution was dried under nitrogen stream in a 60 °C water bath.

2.4 Fumonisin B_1 determination by liquid chromatography

The dried extract was suspended in 500 µL acetonitrile/water (1:1), an aliquot of 100 µL was transferred to a reaction vessel and 200 µL OPA reagent (40 mg o-ftaldialdehyde in 1 mL ethanol diluted with 0.1 M borate buffer and 50 µL 2-mercaptoethanol) was added. The flask was kept in an ultrasonic bath at 5-15 °C for 30 seconds. After 60 a second reaction time the derivatized sample was injected into a Model 1050 liquid chromatograph (Hewlett Packard, Palo Alto, USA), Rheodyne manual injector with a 20 µL loop, HP 1046 fluorescence detector (335 nm and 440 nm), HP 3393A integrator, Spherisorb ODS-2, 5 µm, 250 x 4.6 mm column mm (Supelco, Bellefonte, USA), guard column Varian (Walnut Creek, USA), 2 cm, filled with C18 ODS, 32 µm (Alltech, Deerfield, USA) and mobile phase water/acetonitrile/acetic acid (54:46:1) at a flow rate of 1.0 mL/minute. Identification was based on a comparison of

standard and sample retention times, and external standards were used for quantification.

2.5 Sample extraction and cleanup for ochratoxin A determination

Ochratoxin A was extracted from beer samples according to the method from R-Biopharm Rhone[33] modified as follows. The beer samples were degassed by mixing with a magnetic stirrer at 100 rpm for 60 minutes. The pH was adjusted to 7.2 with 2 M NaOH. An aliquot of 30 mL was applied to the immunoaffinity column (Ochraprep, R-Biopharm Rhone, Glasgow, Scotland). The column was subsequently washed with 20 mL ultrapure water at the flow rate of 5 mL/minute (Mili-Q Plus, Millipore, Milford, USA). The column was allowed to dry and air was flushed through it. The toxin was eluted into a vial with 3.0 mL chromatographic grade methanol and the elution was dried under nitrogen.

2.6 Ochratoxin A determination by liquid chromatography

The dried extract was suspended in a 1.0 mL mobile phase and 100 µL of it was injected into an HPLC system consisting of a Rheodyne manual injector, pump system model 1050 (Hewlett Packard, Palo Alto, USA), C18 Chromolith 100 x 4.6 mm analytical column and 5 x 4.6 mm guard column (Merck, Darmstadt, Germany), fluorescence detector model 1046A (Hewlett Packard), integrator model 3393A (Hewlett Packard) under the following analytical conditions: mobile phase acetic acid/methanol (35:65) at 1 mL/minute, excitation and emission wavelengths 333 nm and 470 nm, respectively. Calibration curves were prepared in the range of 0.027 to 0.136 µg OTA mL^{-1}.

2.7 Analytical quality control

The recovery tests for FB_1 were conducted with samples spiked with standard to achieve concentrations of 5, 10, 20 or 50 $ng.mL^{-1}$. A reagent blank and a spiked sample with 8 $ng.mL^{-1}$ FB1 were added to each series of 8-12 samples analyzed. A sample spiked with 3 ng OTA mL^{-1} was analyzed in triplicate and accompanied each series of 8 – 12 samples during the OTA survey. The results of these spiked samples were used to calculate recovery. All positive samples were analyzed twice, each duplicate analyzed on a different day. The detection limit for FB_1 was based on 3 times the average SD response for 3 injections from 8 different contaminated samples containing the toxin standard at concentrations close to 5 $ng.mL^{-1}$. The detection limit for OTA was taken as 3 times the SD derived from the area of 7 injections of a spiked beer sample (3 $ng.mL^{-1}$). The quantification limit was taken as 5 times the detection limit in both cases.

3 Results and discussion

The average recovery was 98 ± 17% (n = 12) for FB_1 concentrations in beer between 5 and 50 $ng.mL^{-1}$ (Table 1). No interfering compound was observed in the chromatograms (Figure 1). The detection limit for FB_1 in beer samples was 0.26 $ng.mL^{-1}$.

Table 1. Recovery of fumonisin B_1 (FB_1) added to beer.

FB_1 added ($ng.mL^{-1}$)	Recovery (%)
5	118.3
5	121.2
5	78.0
10	74.7
10	105.9
10	71.6
20	99.0
20	90.1
20	100.6
50	87.5
50	104.8
50	120.4

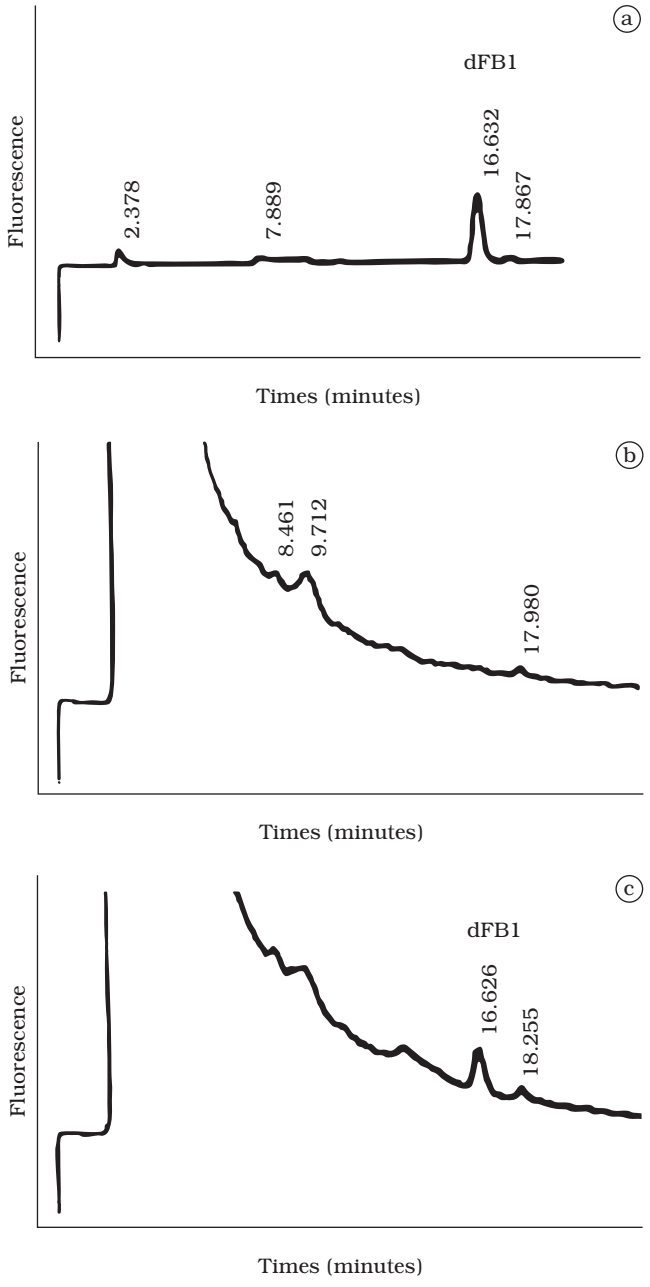

Figure 1. a) Chromatogram of fumonisin B_1 standard (4 $ng.mL^{-1}$); b) chromatogram of uncontaminated beer sample; and c) chromatogram of naturally contaminated beer sample (14.3 ng FB_1 mL^{-1}).

The beers used in the FB_1 survey were produced at 30 plants placed at 23 locations mostly located in the south-east (states of São Paulo, Minas Gerais, and Rio de Janeiro, 45 samples), and a few in the south (states of Paraná and Rio Grande do Sul, 6 samples), the north-east (states of Sergipe, Pernambuco, and Paraíba, 6 samples), and the north (state of Pará, 1 sample). The south-eastern region of the country besides being the most industrialized also concentrates 43% of the population.

FB_1 was detected in 25 (21 light and 4 dark) of the 58 samples of beer analyzed (43.1%) in concentrations ranging from 1 to 40 ng.mL^{-1} (Table 2). The average concentration FB_1 for contaminated samples was 9.6 ± 10.2 ng.mL^{-1}. The contaminated samples were produced in plants at 16 locations, of which 11 were located in the southeast, 3 in the northeast, and 2 in the south. The distribution of the contaminated samples shows that the contamination is not confined to a single area of the country and it is probably connected with the use of maize as an adjunct. The levels found are within the range and the magnitude found in other countries where toxin has been searched for in beer[18,38,51].

Table 2. Concentration of fumonisin B_1 in positive samples of commercial beer produced in Brazil during 2000 and 2001[a].

Sample no.	FB_1 conc. [b] (ng.mL^{-1}) ± SD
1	4.5 ± 0.3
2	15 ± 1
3	14 ± 1
6	7.3 ± 0.5
8	14.7 ± 0.2
14	2.9 ± 0.3
15	39 ± 2
25	8 ± 2
27	17 ± 1
28	11 ± 2
29	3.0 ± 0.6
30	3.0 ± 0.7
34	10.7 ± 0.1
36	1.2 ± 0.2
37	12 ± 1
38	40 ± 4
39	2.3 ± 0.3
40	2.2 ± 0.4
42	10 ± 1
43	1.4 ± 0.2
45	4 ± 1
46	3.0 ± 0.3
51	4.3 ± 0.6
54	4.3 ± 0.3
56	4.0 ± 0.2

[a]Total number of samples analysed = 58; and [b]Mean of duplicate results.

The average per capita intake of beer in Brazil is 130 mL.day^{-1} [40]. The Provisional Maximum Tolerable Daily Intake (PMTDI) by the Joint FAO/WHO Expert Committee on Food Additives[5] is 2000 ng.kg^{-1} bw for fumonisins B_1, B_2, and B_3, alone or in group. In the present survey, the average concentration of FB_1 in beer was 4.1 ng.mL^{-1}. The intake of FB_1 due to beer of a consumer who ingested 1300 mL of the beverage daily (10 times the national average) and that had a body weight of 70 kg would be 3.8% of the PMTDI.

The average recovery of OTA for a concentration of 3 ng.mL^{-1} of the toxin was 93% (n = 16) for beers of light color such as Pilsner and 87% (n = 6) for dark beers such as stout and Malzbier (Table 3). The detection and quantification limits were 0.1 and 0.5 ng.mL^{-1}, respectively, for light and dark beers. The RSD was 1.5% for the recovery tests (n = 18) for light beers and 1.2% (n = 6) for dark beers. No interfering compound was observed in the chromatograms (Figure 2).

Table 3. Recovery of ochratoxin A added to beer[a].

Beer type	Recovery (%) ± SD[b]
Pilsner	79.7 ± 5.7
	107.3 ± 5.5
	82.3 ± 5.1
	101.3 ± 1.1
	79.3 ± 7.1
	93.0 ± 2.6
	95.3 ± 6.8
	93.3 ± 5.9
	108.7 ± 3.0
	101.7 ± 8.6
	89.3 ± 2.5
	92.0 ± 1.7
	89.7 ± 2.1
	98.7 ± 1.5
	95.3 ± 6.4
	95.0 ± 4.0
	83.0 ± 14.0
	89.0 ± 8.5
Dark	85.0 ± 2.6
	65.3 ± 4.1
	114.0 ± 2.6
	62.0 ± 3.6
	88.3 ± 0.6
	109.0 ± 8.2

[a]Uncontaminated beer samples spiked with OTA (3 ng.mL^{-1}); and [b]Mean of triplicate results.

Beers used in the survey for OTA were produced at 36 plants corresponding to 15 locations in the states of Paraná (southern region, 115 samples), São Paulo, Rio de Janeiro (south-eastern region, 3 samples), Paraíba (north-eastern region, 2 samples), and Pará (northern region, 3 samples). OTA was found in 5 samples from the 123 beers analyzed. All were light colored beers, with OTA concentrations varying from 1 to 18 ng.mL^{-1} (Table 4). Four of the samples were from 3 plants belonging to the same beer company. One of the samples was from a plant located in the south of the country, 3 from the south-east, and one from the north. Although the number of contaminated samples is small, it shows the contamination is not restricted in terms of region.

Incidences of 83 to 100% of OTA in beer have been reported in all countries where the toxin has been searched for, except for Slovenia where no contamination has been found by OTA

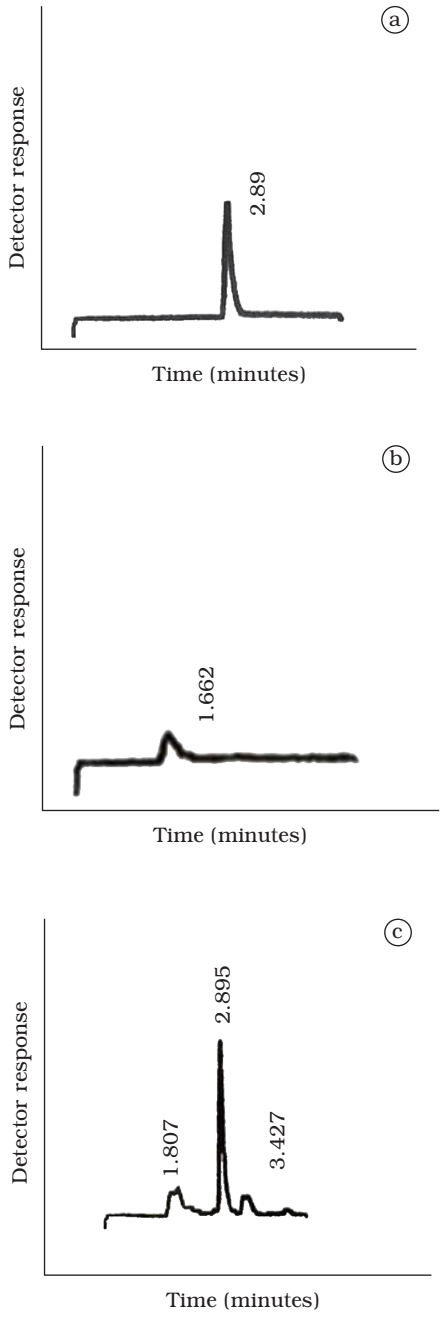

Figure 2. a) Chromatogram of ochratoxin A standard (0.091 ng.mL^{-1}); b) chromatogram of uncontaminated beer sample; and c) chromatogram of naturally contaminated beer sample (7 ng OTA mL^{-1}).

Table 4. Concentration of ochratoxin A in positive samples of commercial beer produced in Brazil during 2003 and 2004[a].

Sample nº	OTA conc.[b] (ng mL^{-1}) ± SD
48	18.0 ± 0.08
85	7.56 ± 0.01
113	11.66 ± 0.004
115	1.03 ± 0.03
116	3.52 ± 0.20

[a]Total number of samples analysed = 123; and [b]Mean of duplicate results.

in beer. The incidence found in Brazilian beer in the present survey (4.8%) is therefore low. The levels found, on the other hand are higher then the levels reported by other countries by various orders of magnitude[12,13,21,26,27,35,47,52,53].

The Provisional Tolerable Weekly Intake (PTWI) by JECFA[3] for OTA is 100 ng.kg^{-1} bw. Considering that the average contamination of beer by OTA found in the present work was 0.3 ng.mL^{-1}, a consumer of 1,300 mL beer per day (10 times the national average) would be ingesting 2,730 ng OTA weekly and that would amount to 39 % of the PWTI. The incidence of OTA in beer was low but its high toxicity turns it into a significant contributor at an OTA intake in case of regular high ingestion of the beverage.

4 Conclusions

The survey conducted in Brazilian beer found FB_1 and OTA to be present in 43% and 4.8% of the samples, respectively. The toxicity of FB_1 combined with the low levels present in the samples examined, indicate that the presence of this toxin in Brazilian beer is more of a quality issue for the industry than a reason for public health concern. On the other hand, the high levels of OTA found and its high toxicity are cause for public health concern even with the low incidence observed. The elimination of both toxins from the product should be contemplated by the industry either for quality or health reasons.

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