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ARTICLE

Fumonisin occurrence in wheat-based products from Argentina

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ABSTRACT

In Argentina, wheat is the most consumed cereal by the human population. Since fumonisins occurence in wheat grains and wheat-based products have been reported worldwide, a survey was conducted in order to determine fumonisin contamination in 91 wheat-based products (white wheat flour samples, wheat flour used at bakery products and whole-wheat flour samples) collected from different retail stores of Rio Cuarto city in Argentina using HPLC-MS/MS. Sixty-seven samples (74%) showed contamination by fumonisins. From these samples, 16 showed fumonisin levels between LOD and LOQ (between 0.01 to 0.05 ng/g), while fumonisins (FB_1 + FB_2) in quantifiable samples ranged from 0.05 ng/g to 18.9 ng/g. Although FB_1 was more prevalent, FB_2 was foun3d in higher levels than FB_1. Overall, fumonisin prevalence was high, but concentrations were far below EU or USA limits set for maize and maize-based products.

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Fumonisins; wheat-based products; HPLC-MS/MS; Argentina; quantitative analysis

Introduction

Wheat is the most important source of calories as well as protein for humans. World wheat production in 2016 was 749.5 million tons (FAO 2017). Wheat is the most important cereal used for human consumption in Argentina. This cereal is ground into flour and semolina for consumption, being the basic ingredients for bread and other bakery products, pasta, breakfast cereals, cookies, and cupcakes (Chandrika and Shahidi 2006). Human consumption of manufactured wheat products, either semolina (*Triticum turgidum* L. var. durum) or bread (*T. aestivum*) in Argentina is much greater than products made from other cereals (Pacin et al. 2012). Wheat flour per capita consumption varies widely by region worldwide. In Argentina its consumption per capita was estimated at 84 kg/habitant/year during 2017 (FAIM 2017).

Cereals like wheat are commonly colonised by *Fusarium* species and can be contaminated with mycotoxins, which are defined as second metabolites produced by fungi that could have a major impact on health, welfare and productivity. Among all *Fusarium* species that could be present in wheat grains and wheat-based products, several fumonisin (FB) producing *Fusarium* species could be isolated. Although several *Fusarium* species are able to produce FBs, the two most important ones are *Fusarium verticillioides* and *F. proliferatum*, both associated with maize, but also isolated from wheat (Palacios et al. 2011; Chehri et al. 2010; Busman et al. 2012; Amato et al. 2015; Guo et al. 2016). Thus, FBs are usually present in maize and maize-based products, but also, in wheat and wheat-based products (Cendoya et al. 2018a).

In terms of occurrence and toxicity Fumonisin B_1 (FB_1) is the most significant, followed by Fumonisin B_2 (FB_2), which differs structurally from FB_1 in the number and placement of hydroxyl groups on the molecule's hydrocarbon (Voss et al. 2007). FB_1 can cause leukoencephalomalacia in horses (Marasas et al. 1988), pulmonary oedema syndrome and hydrothorax in pigs (Haschek et al. 1992) and nephrotoxic, hepatotoxic and hepatocarcinogenic activities in rats (Wan Norhasima et al. 2009). In human populations that consume FB-contaminated maize, this mycotoxin has been epidemiologically associated with oesophageal cancer (Marasas 2001) and neural tube defects (Missmer et al. 2006). Moreover, recent studies suggest that FB exposure could be related with stunting in children (Kimanya et al. 2010; Shirima et al. 2015). Due to the toxicological effect in animals and humans, the International Agency for Research on Cancer (IARC) designated FB_1 in Group 2B as "possible

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carcinogenic to humans" (IARC 2002) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined a provisionally maximum tolerable daily intake (PMTDI) of 2 μg/kg body weight per day for FB_1, FB_2 and fumonisin B_3 (FB_3) alone or in combination (WHO 2001). The European Union in 2007 (EC N°1126/2007) established maximum limits for FBs in cereals and cereal-based foods for human consumption, but not specifically for wheat or wheat-based foods.

In Argentina wheat contamination with FBs has been reported (Palacios et al. 2011; Cendoya et al. 2014), but there are no studies about contamination with FBs in wheat-based products yet. Thus, due to the importance of wheat in the diet of the Argentinean population and because FB intake occurs mainly via the intake of wheat and wheat-products (Bakker et al. 2003), the aim of this survey was to determine the natural occurrence of FBs in wheat-based products used for human consumption marketed in this country.

Materials and methods

Sample collection

Ninety-one wheat-based products for human consumption were randomly collected from local retail stores in Rio Cuarto (Cordoba, Argentina) in order to investigate presence of FBs. Forty-six and 45 samples were collected during 2015 and 2016, respectively. These consisted of white wheat flour (76), wheat flour used at bakery products (11) and whole-wheat flour (4). To reduce the samples to analytical portions, three subsamples (200 g) were drawn, from the top, middle and bottom layers of each individual package (1 kg). Then, these three subsamples were pooled, mixed thoroughly and quartered. Opposite quarters were rejected and the remainder re-mixed thoroughly to obtain a final reduced sample of 200 g for analysis. All reduced samples were kept in ziplock plastic bags at −20 °C prior to analysis, of which a 25-g test portion was taken for FB extraction.

Extraction

FB extraction was performed according to the method originally reported by Shephard et al. (1990) and modified by Doko et al. (1995). Twenty-five grams of each sample (25 g) were shaken with 50 mL of methanol: water (3:1) for 30 min and filtered through Whatman N° 4 filter paper (Whatman Int. Ltd, Maidstone, Kent, United Kingdom). Ten millilitres of the filtered extract were applied to a Bond-Elut strong anion-exchange (SAX) cartridge (Agilent Technologies Inc., Santa Clara, CA, USA) fitted to a Supelco solid phase extraction (SPE) manifold (Supelco, Bellefonte, PA, USA), previously conditioned by the successive passage of methanol (5 mL) and methanol:water (3:1, 5 mL), while the flow rate was maintained below 2 mL/min. After that, the cartridge was washed with methanol:water (3:1, 8 mL) followed by methanol (3 mL) and finally the FBs were eluated with 0.5% acetic acid in methanol (14 mL). The eluates were evaporated to dryness at 40 °C under a moderate stream of nitrogen and stored dry at 4 °C until HPLC MS/MS analysis.

HPLC-MS/MS analysis

Analysis was performed with a Waters 2695 module equipped with an auto sampler and interfaced to a Micromass®-Quattro Ultima™ Platinum tandem quadrupole mass spectrometer with electrospray ionisation (ESI) in positive mode, after separation on a XBridge™ C18 column (3.5 μm, 2.1 x 150 mm) connected to a XBridge BEH C18 Sentry Guard Cartridge (130 Å, 3.5 μm, 2.1 x 10 mm), all from Waters (Milford, MA, USA). The mobile phase consisted of a gradient of aqueous 1% formic acid (solvent A) and methanol with 1% formic acid (solvent B) according to Cendoya et al. (2018b). The flow rate was 0.2 mL/min. Column temperature was kept at 20 °C. The nitrogen flow was adjusted to 109 and 726 L/h for cone and desolvation gases, respectively. Blank matrix extracts were investigated to confirm the specificity of the method. Data acquisition and processing were performed using Mass Lynx V.4.1, Waters INC software (Milford, MA, USA). Nebulisation and desolvation temperatures were 150 and 200 °C, respectively. The capillary voltage was 3.00 kV. Multiple-reaction monitoring (MRM) was used for FB determination. The precursor peak $[M + H]^+$ and two product peaks monitored to accomplish both quantification and qualification criteria, as well as retention times and detector settings, are given in Table 1. The most abundant transition, 722 > 352, was used for the quantification of FB_1, while 706 > 336 was used for FB_2.

Table 1. MS parameters used to investigate fumonisins by HPLC-MS/MS.

Mycotoxin	Precursorion (*m/z*)	t_R	Productions (*m/z*)	CV (V)	CE (V)
FB_1	722	13.5	334	101	38
			352		35
FB_2	706.3	15.5	318.5	106	31
			336.3		49

Precursor ion $[M + H]^+$;
t_R, retention time (min.);
CV, cone voltage;
CE, collision energy.

Aliquots of 45 µL of sample extracts were injected into the HPLC unit. Four points of identification were used to identify FB_1, i.e. retention time, the precursor ion $[M + H]^+$ and two product ions (m/z 334 and 352). FB_1 and FB_2 stock solutions in acetonitrile: water (1:1) were used (Biopure, Tull, Austria). A calibration curve was obtained by injecting 10 µL of mixed (FB_1 + FB_2) standard solutions of 0.5, 1.0 and 2.0 µg/mL. Good linearity with a correlation coefficient higher than 0.996 was obtained for the calibration range. The limit of detection (LOD) for FB_1 and FB_2 was 0.01 ng/g, calculated as $S/N = 3$ and the limit of quantification (LOQ) with $S/N = 5$ was 0.05 ng/g. Relative within day and between-day standard deviations (RSD) were 6.5%.

A recovery experiment was performed in triplicate by spiking 25 g of FB-free wheat flour with FB_1 and FB_2 at levels of 10, 100 and 200 ng/g. Spiked samples were left overnight at room temperature to allow solvent evaporation prior to extraction. Mean FB_1 and FB_2 recoveries were 99.9% and 93.6%, respectively.

Results and discussion

FB mean values, as well as maximum amounts of FB_1 and FB_2 found in the present study and the incidence percentage of positive samples are shown in Table 2. Figure 1 resumes FB content in total wheat-based products analysed. Natural FB contamination was present in 74% of all analysed wheat-based samples (67/91). From these positive samples, 16 showed trace levels, i.e. levels between LOD and LOQ (13 white wheat flour samples, 1 wheat flour sample used at bakery and 2 whole-wheat flour samples). Total FB amounts ($FB_1 + FB_2$) reached 18.9 ng/g, with a mean level of 1.54 ng/g. FB_1 was present in 64 out of 91 positive samples, while FB_2 was present in 52 samples. Most samples were contaminated with both FB_1 and FB_2 (55%), while 14 samples only contained FB_1 and 2 samples only FB_2. Although the incidence percentage of FB_1 was higher than of FB_2, amounts of FB_2 were higher than those of FB_1 (high ratio FB_2/FB_1) when these mycotoxins were found together. Moreover, the highest amount of FB found in the present study was 17.5 ng/g of FB_2. Maximum amounts of total FBs were found in white wheat flour samples as well as in wheat flour samples used for bakery products. In both types of samples, FB_2 content was at least 8 fold higher than FB_1. Regarding whole-wheat flour samples, FB content was lower than in other analysed samples, being FB_1 content higher than FB_2.

Since FBs have become important due to their toxicity, the willingness to study their incidence has been

Table 2. Incidence rate and concentration of fumonisins in wheat-based samples collected from retail stores in Argentina.

Sample types	Mycotoxin	N_{pos}/N_{tot} [a]	Incidence (%)	Maximum (ng/g)	Mean[b] (ng/g)
Total samples	FB_1	64/91	70	2.10	0.32
	FB_2	52/91	57	17.51	1.24
	FB_1 + FB_2	67/91	74	18.94	1.54
White wheat flour	FB_1	52/76	68	2.10	0.28
	FB_2	40/76	53	17.52	0.92
	FB_1 + FB_2	52/76	68	18.94	1.19
Wheat flour used at bakery	FB_1	9/11	82	2.00	0.66
	FB_2	11/11	100	16.13	3.80
	FB_1 + FB_2	9/11	82	18.14	4.33
Whole-wheat flour	FB_1	3/4	75	1.10	0.28
	FB_2	2/4	50	0.72	0.19
	FB_1 + FB_2	3/4	75	1.82	0.47

[a]Number of positive samples/number of total samples.
[b]Mean of all samples.

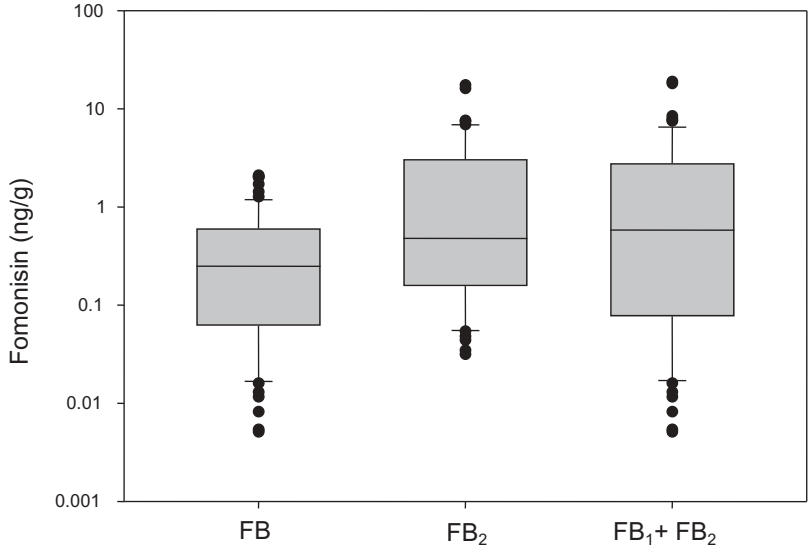

Figure 1. Box plot of FB_1, FB_2, and FB_1 + FB_2 content in wheat-based products marketed in Argentina.

increasing over the years. Together with the development of more sophisticated analytical techniques that allow the detection of small amounts of mycotoxins in different foods, this raised multiple studies related to their incidence in different foods. Thus, the occurrence of FBs in wheat and wheat-based products has been reported in many countries worldwide (Cendoya et al. 2018a).

The results presented above are in agreement with similar surveys performed with HLPC-MS/MS for FB detection and quantitation in different wheat-based samples. In most studies the incidence and concentration of FBs found were low. In China, Liu et al. (2012) detected total FBs (FB_1 + FB_2 + FB_3) in 7% of 30 wheat flour samples, with a mean level of 0.1 ng/g. In the same way, also in China Li et al. (2015) detected FB_1 incidence of 6.2% in 369 wheat flour samples with levels ranging from 0.3 to 34.6 ng/g. In the same country Sun et al. (2011) analysed 16 wheat flour samples and observed 81% of FB_1 incidence with a mean level of 200 ng/g. Similar results were found in other wheat-based products for human consumption. In France, Sirot et al. (2013) analysed FB_1 and FB_2 occurrence in 14 bread and dried bread products and 8 sweet or savoury biscuits and bars, detecting mean levels of 1.4 and 0.3 ng/g and 35 and 75 ng/g for FB_1/FB_2, respectively. Also in France, Rubert et al. (2013) studied FB_1 and FB_2 incidence in 188 wheat-based products. These authors observed 27 positive samples (FB_1 and FB_2 contamination) with levels ranging from 20.2 to 125.8 ng/g and from 21.7 to 101.1 ng/g for FB_1 and FB_2, respectively. Cirillo et al. (2003a and 2003b) performed two different surveys in Italy in order to analyse FBs occurrence in different wheat-based products, obtaining similar results regarding incidence percentages and quantities. These authors also observed a higher incidence of FB_2 than FB_1 in both studies for all analysed samples and in some cases higher amounts of FB_2 than FB_1. Although those results are in agreement with the results obtained in the present study, they are different to most results reported in the literature, where commonly FB_1 is the most frequent FB found in different foods and also commonly higher amounts of FB_1 than FB_2 or FB_3 are found. In Tunisia, 2 wheat-based products showed FB_1 contamination with levels ranging from 88.3 to 184 ng/g (Serrano et al. 2012). In Canada, Roscoe et al. (2008) studied FB incidence in 166 breakfast cereals (maize-, oat-, wheat- and rice-based cereals, as well as mixed-grain cereals) samples collected from the Canadian retail market. As a result total FBs (FB_1 + FB_2) were found in 17% of wheat-based samples with a mean level of 3 ng/g and in one buckwheat cereal sample 5 ng/g. Although FB levels found in that study were low, the authors concluded that the risk of human exposure to these mycotoxins is present. Just 2 studies showed high amounts of FBs when wheat-based samples were analysed by HLPC-MS/MS. Mashinini and Dutton (2006) analysed 210 wheat-based products from South Africa and observed FB_1 occurrence in four samples, but with FB levels ranging from 1000 to 30,000 ng/g. In Spain Rubert et al. (2013) studied FB_1 and FB_2 incidence in 35 organic Gofio wheat samples and observed both 77% incidence of FBs (FB_1 + FB_2) and high amounts.

On the other hand there are studies that report higher amounts of FBs in wheat-based products than those observed in the present study, but the method used for detection and quantification was ELISA. In Iran Roohi et al. (2012) studied total FB (FB_1 + FB_2 + FB_3) occurrence in different types of wheat flour by ELISA and observed percentages of incidence higher than 80% and FB levels that reached 4500 ng/g. A worldwide survey was conducted by Rodrigues and Naehrer (2012), who analysed mycotoxins by ELISA and HPLC in 7049 samples between 2009 and 2011 from America (North and South), Europe, Oceania and Asia. Regarding FB presence in wheat and wheat bran samples no positive samples out of 7 analysed samples were found in North America, while 2 out of 40 samples were contaminated with FBs (average: 1407 ng/g) in South America. No FBs were detected in a single sample analysed from Northern Europe, while, respectively, 33% and 30% of wheat and wheat bran samples from Central and Southern Europe were contaminated with this mycotoxin (mean levels: 268 and 386 ng/g, respectively). In North Asia, South-East Asia and Oceania 11, 5 and 12% of wheat-based samples were contaminated with FBs, respectively, with mean levels of 371, 172 and 269 ng/g, respectively.

It is remarkable that in the present study most wheat-based samples were contaminated with FBs at levels comparable to those found in the studies described above when the analytical method used was the same: HPLC-MS/MS. This is an important point as other techniques such as ELISA demonstrated to be able to produce false positive results in wheat and wheat-based samples (Shephard et al. 2005). These authors reviewed several reports on FBs in wheat and concluded that careful evaluation of the analytical method and possible source of contamination, as well as confirmation by an appropriate and valid analytical method is required in order to prevent the report of false positive results.

The high incidence of FBs in wheat-based samples found in the present study is important as it has been demonstrated by other studies, using biomarkers

(Szabo-Fodor et al. 2016), that in global areas where maize is contaminated with FBs, the local population is exposed to a higher risk when compared to consider only the individual mycotoxin. Therefore, in those regions where FB-contaminated wheat and wheat-based products consumption is high, the same exposure risk could occur. Bakker et al. (2003) estimated FB_1 intake of the Dutch population by comparing FB_1 concentrations in different food products with consumption rates of those products and reported wheat to be the main contributor (73%) to the total FB_1 intake. Thus, although FB_1 concentration in maize was higher than that in wheat, it appeared that wheat and not maize was the main source of FB intake in the Netherlands.

Similar situations were reported in other countries without specific FB legislation, where a common staple food was reported to be contaminated with FBs. In some occasions at low contamination incidence/levels, in which case most authors concluded that daily intake of low doses of mycotoxin-contaminated food stuff over a period of time could lead to chronic mycotoxicosis. For example, Garcia et al. (2016) studied FBs presence in dry soybean samples sold for human consumption in Brazil and found that 10% of all samples were contaminated with FBs, with levels up to 1495 ng/g. Therefore, the authors emphasised the need for frequent monitoring of FBs in soybeans. Sorghum is a main staple cereal in some districts of Ethiopia and its consumption per day per person is high. Therefore, Taye et al. (2016) studied AFB_1 and FBs presence in sorghum grains they and found all 90 sorghum samples to be contaminated with *Aspergillus* and *Fusarium* species, as well as with AFB_1 and FBs. Aflatoxin B_1 was determined at levels ranging from < LOD to 33.10 µg/kg and total FB levels varied between 907 and 2041 µg /kg. This leads to the conclusion their survey could serve as a starting point to create awareness on toxicogenic fungi and associated mycotoxins in the studied area of the country. Lui et al. (2017) analysed FBs in maize kernel samples from 8 provinces of China and found that 28 samples exhibited higher levels than set by the Food and Drug Administration and 12 samples exhibited higher levels as set by the European Commission. Although the average exposure to FBs which they calculated to be 0.12 µg body weight/day, was within the provisional maximum tolerable daily intake as set by the Joint FAO/WHO Expert Committee on Food Additives.

Another important item to consider is the fact that FB exposure determined by routine analysis could lead to underestimate the actual FB intake by consumers, generally astimated at 40%, as bio-accessibility is not taken into consideration (Szabo-Fodor et al. 2016). Conventional analytical methods fail to take into account the mycotoxin content bound to the food matrix, which is bio-accessible at consumption as it can be absorbed from the gastrointestinal tract (Versantvoort et al. 2005). Thus, mycotoxin burden in cereals could be significantly higher if the "modified" forms of mycotoxins are considered as well. In agreement with that, Dall'Asta and Battilani (2016) concluded that modified FB should be included in food monitoring plans in order to have an overview of the possible contribution to human exposure. Moreover, in the present study we did not consider co-occurrence of FBs with other mycotoxins like trichothecenes and zearalenone, which are known to occur in wheat and wheat-based products as well, raising the question of possible interactions, synergistic or antagonistic actions in the manifestation of toxicity (Stanciu et al. 2015). Simultaneous exposure to more than only one mycotoxin is of increasing concern and requires more study.

Conclusion

From the findings as established in this survey can be concluded that more studies of FB presence in wheat and wheat-based products as well as co-occurrence with other mycotoxins are needed in order to determine the actual FB intake of the population of Argentina. Also these studies could result in regulatory limits for cereals for human consumption, as nowadays legislation for FB levels in wheat and wheat-based products does not exist in Argentina. Thus, in the near future we are planning to perform further analyses, including more wheat-based products intended for human consumption, especially those consumed by infants and babies, as well as determination of FBs bound to the food matrix and co-occurrence with other mycotoxins, especially deoxynivalenol. These considerations are in agreement with the views of the Joint FAO/WHO Expert Committee on Food Additives, who expressed the need to analyse FB content in food samples using appropriate analytical methods. Moreover, this Committee highlighted the need to perform studies on bound mycotoxins as well (JECFA 2017).

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Disclosure statement

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