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Short communication

Occurrence of fumonisins B_1 and B_2 in *broa*, typical Portuguese maize bread

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Abstract

Fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) are mycotoxins mainly produced by *Fusarium verticillioides*, and *Fusarium proliferatum*, fungi species most commonly isolated from maize. The natural occurrence of FB_1 and FB_2 in *broa*, typical Portuguese maize bread, was evaluated in 30 samples. Twenty five were found positive with levels ranging from 142 to 550 μg kg^{-1}. The limit established by the European regulations was exceeded by 27% of the samples. The tolerable daily intake for fumonisin B_1, and B_2, alone or in combination, for all of the analysed samples, was lower than 2 μg kg^{-1} body weight per day established by the European Commission.

Keywords: Fumonisins B_1 and B_2; Maize bread; HPLC

1. Introduction

Fumonisin mycotoxins are produced by a limited number of morphologically related *Fusarium* species, of which *Fusarium verticillioides* (*F. moniliforme*) and *F. proliferatum* are the most important as they infect maize crops around the world. Of the currently identified fumonisins (FBs), fumonisin B_1 (FB_1), and B_2 (FB_2) are the most abundant in foods and feeds (Shephard et al., 1996).

Due to its resemblance with sphingoid bases they produce a wide range of biological effects including, leucoencephalomalacia in horses (ELEM), pulmonary oedema in pigs (PPE), and nephrotoxicity and liver cancer in rats. Besides they have been associated with human oesophageal cancer in the Transkei region of South Africa and China, and with neural tube defects (NTD) in South Texas, USA (Lino et al., 2004, 2006; Lerda et al., 2005; Shephard et al., 1996; Stack, 1998). On the basis of toxicological evidence, the International Agency for Research on Cancer (IARC) classified FB_1 as a possible carcinogen to humans, Group 2B (IARC, 2002).

Given the widespread of these mycotoxins, reliable data on the dietary exposure of populations must be obtained (Shephard et al., 1996). High FBs levels are frequently reported in maize or maize-based foods from many countries, such as Benin (Fandohan et al., 2005), Korea (Park et al., 2005), China (Liu et al., 2005), Brazil (Bittencourt et al., 2005), Nigeria (Bankole and Mabekoje, 2004), South Africa (Sydenham et al., 1990), United Kingdom (Scudamore and Patel, 2000), France (Molinié et al., 2005), and Italy (Cirillo et al., 2003).

In Portugal maize area and maize production are 126,000 ha and 665,000 Mt, respectively (FAO, 2003). Even though, only two investigations of FB_1 and FB_2 in maize and maize-based products have been reported. The first, which studied naturally contaminated maize hybrids from the 1992 crop of Agricultural School of Coimbra (Doko et al., 1995), revealed a frequency of contamination of 100%. Lino et al. (2006) concluded that 45% of the maize and maize-based samples of the central zone of Portugal were contaminated with FB_1 and FB_2.

Broa, a traditional maize bread highly consumed, especially in the north and central zones of Portugal, has never been studied. Conversely, there are several studies on the FBs content in other traditional foods, such as *tortillas*, and *polenta*, typical maize-based products from Mexico and northern Italy, respectively (Stack, 1998; Dombrink-Kurtzman and Dvorak, 1999; De La Campa et al., 2004; Palencia et al., 2003).

Being traditional products produced mainly from maize from different countries, the cooking processes of *tortilla*, *polenta*,

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and *broa* are very different. *Tortilla* preparation involves alkaline cooking using lime, $Ca(OH)_2$ (Stack, 1998; Dombrink-Kurtzman and Dvorak, 1999; Shephard et al., 1996). *Polenta* is a boiled corn meal made from milled yellow maize (www.initaly.com). The traditional processing of *broa* (Fig. 1) consists of adding sieved corn flour, wheat flour, hot water, yeast and leavened dough from the late *broa*. After mixing, working up and leavening, the dough is baked in a wood-fired oven (www.gastronomias.com).

The aim of the present study, carried out in the central zone of Portugal, was to evaluate the FB_1 and FB_2 contamination in *broa* samples, using liquid chromatography with spectrofluorimetric detection (LC-FD), with pre-column derivatization, and confirmation by liquid chromatography with mass detection (LC-MS).

2. Materials and methods

2.1. Chemicals

FB_1 and FB_2 standards and naphthalene-2,3-dicarboxaldehyde (NDA) were commercially obtained from Sigma Chemicals Co (St. Louis, USA). FumoniTest™ immunoaffinity columns (IAC) were from Vicam (Watertown, USA). HPLC grade solvents and analytical grade reagents were used for all purposes.

Stock solutions, prepared in the FB_1 and FB_2 vials, were made in 1 mL acetonitrile–water (50:50, v:v) at 1000 μg mL^{-1}. Intermediate solutions were prepared at 50 μg mL^{-1}. For fortification assays, one work solution was prepared with acetonitrile–water (50:50, v:v) at 10 μg mL^{-1} for both fumonisins. All solutions were kept in amber flasks to protect from light.

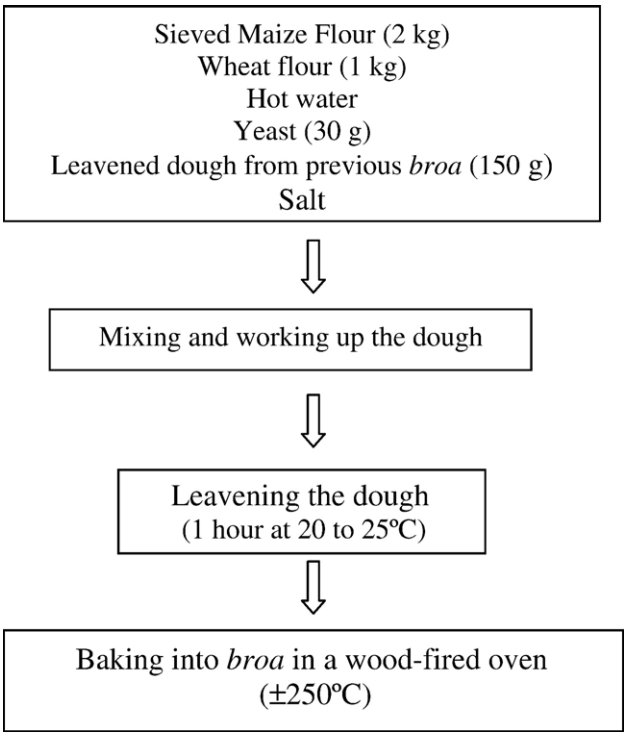

Fig. 1. Traditional production of *broa*.

2.2. Samples

Thirty samples were purchased in commercially available size during September 2005 from bakeries, confectionery's shops and supermarkets located in the city of Coimbra and its countryside- central zone of Portugal.

2.3. Recoveries

The recoveries of fumonisins from *broa* were determined by spiking the ground sample with a standard solution of 10 μg mL^{-1} of each FB_1 and FB_2, for three replications, with known amounts of fumonisins at final concentrations of 250 μg kg^{-1} for FB_1 and 200 μg kg^{-1} for FB_2.

2.4. Fumonisins analysis

Extraction, clean-up, and LC-FD determination were performed according to Lino et al. (2006). Briefly, ground samples were extracted with methanol:water (80:20, v:v), and centrifuged for 15 min at 2500 ×*g*. The extract was filtrated, diluted with phosphate-buffered saline (PBS), and filtrated once more. An aliquot was added to a FumoniTest™ IAC attached onto a vacuum manifold. The column was washed with PBS, and fumonisins were eluted with methanol.

For LC-FD analysis, the residue was reconstituted with methanol:water and derivatization was carried out on the NDA-derivatives of fumonisins. The liquid chromatography (LC) apparatus used consisted of a 307 Gilson (Gilson Medical Electronics, Villiers-le-Bel, France) pump model, one 50 μL Rheodyne 7125 injector (Cotati, CA, USA), a C_{18} −5 μm Nucleosil 120 KS (30 mm×4 mm i. d.) guard column, and a C_{18} −5 μm Nucleosil 120 (250 mm×4.6 mm i. d.) column. A Perkin Elmer LS45 spectrofluorimeter (Perkin Elmer, Beaconsfield, UK) operated at an excitation wavelength of 420 nm and an emission wavelength of 500 nm was used. The results were recorded on a 3390 integrator (Hewllet-Packard, Philadelphia, PA). The mobile phase (acetronitrile:water:acetic acid, 61:38:1, v:v:v) was maintained at a flow rate of 1 mL min^{-1}.

For confirmation of fumonisins with liquid chromatography with electrospray ionization and mass detection (LC-ESI–MS), the residue was reconstituted with 50 μL methanol–water (50:50, v:v). A Hewlett Packard (Palo Alto, CA, USA) HP-1100 Series LC-MS system equipped with a binary solvent pump, an autosampler, and a MS detector coupled with an analytical workstation was used. The MS detector consisted of a standard API (atmospheric pressure ionization) source that can be configured as APCI (atmospheric pressure chemical ionization) or ESI. A C_{18} −5 μm (150 mm×4.6 mm i. d.) (Phenomenex, USA) stainless steel column and a guard column LiChrosorb RP-8 (10×4.6 mm, 5 μm) were used. The analytical separation for LC-ESI–MS was performed using the following gradient: (a) methanol: 0.5% formic acid 75% and (b) water: 0.5% formic acid 25%, isocratic for 4 min, then increased to 95% (a) and

25% (b) in 4 min and held for 7 min. The flow rate was maintained at 0.5 mL min^{-1}.

The ESI–MS interface was operated in positive mode under the conditions of 350 °C gas temperature, 13.0 L min^{-1} drying gas flow, 30 psi nebulizer gas pressure and 4000 V of capillary voltage. Mass spectra were obtained by scanning from *m*/*z* 300 to 800.

FB_1 (positive ion *m*/*z*=722) and FB_2 (positive ion *m*/*z*=706) were monitored by selected ion monitoring (SIM) with the cone voltages ranging from 60 to 180 V. The highest responses were obtained at 160 V for both compounds, so this value was chosen for identification and quantification purposes. The above described conditions allowed the elution of FB_1 and FB_2 with a retention time of 6 min and 10 min, respectively.

3. Results and discussion

FB_1 and FB_2 linearity in the working standard solutions at four determinations of five concentration levels, between 0.25 and 5.0 μg mL^{-1}, which corresponds to 0.52 ng and 10.4 ng of injected quantity, was good as shown by the fact that the correlation coefficients (r^2) were 0.984 and 0.994 for FB_1 and FB_2, respectively.

The limit of detection obtained by the NDA derivatization procedure, at a signal-to-noise of 3:1, was 20 μg kg^{-1} for FB_1 and 15 μg kg^{-1} for FB_2.

Accuracy was determined by calculating the mean recovery values for each fortification level. For FB_1 was 99.7% for a fortification level at 250 μg kg^{-1}, for FB_2 was 74.8% for a fortification level at 200 μg kg^{-1}. Precision was calculated by intraday repeatability (*n*=3) and interday repeatability (3 days). The intraday repeatability obtained for FB_1 was 6.5% for a fortification level at 250 μg kg^{-1}, and for FB_2 was 8.6% at 200 μg kg^{-1}. The interday repeatability (*n*=9), for FB_1 and FB_2 was 7.5% and 15.2% at fortification levels of 250 and 200 μg kg^{-1}, respectively. Accuracy and precision results are in accordance with the performance characteristics for FB_1 and FB_2 established by CE 2005/38/EC of 6 June 2005 (Commission Directive, 2005).

The analytical methodology was successfully applied to 30 *broa* samples. In this study, 24 (80%) were found contaminated with FB_1, and 25 (83%) presented FB_2. The analysed samples revealed contamination levels of FB_1 between nd and 448 μg kg^{-1}, and of FB_2 between nd and 207 μg kg^{-1}. Mean concentrations of 197 μg kg^{-1} for FB_1, and 77 μg kg^{-1} for FB_2 were observed. The mean contamination of the combined FB_1 and FB_2 was 274 μg kg^{-1}, having the most contaminated sample 550 μg kg^{-1} of FB_1 and FB_2 (Table 1). The mean concentration of FB_2 is lower than FB_1.

Table 1
Prevalence and levels of fumonisins in *broa* from Portugal

	No (%) of positive samples	Contamination range (μg kg^{-1})	Mean±SD (μg kg^{-1})	Median (μg kg^{-1})
FB_1	24 (80)	nd–448	197±130	205
FB_2	25 (83)	nd–207	77±60	70
FBs	25 (83)	nd–550	274±267	266

The detected levels for both fumonisins in Portuguese contaminated samples of *broa* suggests that the fumonisin content in flour maize was very high, what is confirmed by an investigation conducted in maize, maize flour and other maize-based foods for the determination of FB_1 and FB_2. In this study, it was observed that maize flour, the main ingredient of *broa*, presented the highest mean concentration of FB_1, 822 μg kg^{-1}, while FB_2 presented a mean value of 173 μg kg^{-1}, and one sample presented a total FB_1+FB_2 contamination of 2026 μg kg^{-1} (Lino et al., 2006).

The comparison with other European countries is somehow difficult regarding the few reports of contamination by fumonisins in this kind of goods. Cirillo et al. (2003) found FB_1 in bread ranging between 30 and 150 μg kg^{-1}, with a mean value of 50 μg kg^{-1}, while FB_2 appeared in a range of 56 to 400 μg kg^{-1}, with a mean value of 118 μg kg^{-1}. Comparison between Portuguese and Italian surveillance shows that FB_1 levels in *broa* are higher than those found in Italian bread. However FB_2 levels are lower.

As *broa* is a typical Portuguese maize-based food, the comparison with other similar products all over the world is complex. However, though the cooking processes of *tortilla* and *polenta* are completely different from *broa*, these are also typical maize-based foods, from Mexico and northern Italy, respectively.

A surveillance of fumonisins during 1990 and 1991 in *tortillas* from USA, showed that FB_1 and FB_2 were present in an average concentration of 60 μg kg^{-1} and 50 μg kg^{-1}, respectively (Sydenham et al., 1991). In the Texas–Mexico border, the mean level of FB_1 found in *masa* was 262 μg kg^{-1}, while in *tortillas* was found a mean level of 187 μg kg^{-1} (Stack, 1998).

In what concerns *polenta*, Doko and Visconti (1994) analysed home made *polenta* from Italy and concluded that it was contaminated with 2880 μg kg^{-1} of FB_1, and 620 μg kg^{-1} of FB_2. The same authors have also analysed the contamination levels of *polenta* corn flour and observed that FB_1 and FB_2 were present in a range of 420 to 3730 μg kg^{-1}, and 80 to 840 μg kg^{-1}, respectively.

The processing of *broa* implies two main procedures, fermentation and baking. Regarding fermentation, Fandohan et al. (2005) concluded that it did not appear to have a significant impact on the levels of mycotoxins. Only 13% reduction of fumonisin levels were observed during fermentation of *ogi*, a maize product from Benin.

Jackson et al. (1996a,b) suggested that foods heated at temperatures between 100–125 °C did not present substantial reduction in FBs levels. However, when temperatures above 150 °C were applied, substantial losses of fumonisins may occur.

For FB_1 and for the sum of FB_1 and FB_2, two and eight samples, respectively, exceeded the maximum limit proposed by the European Commission (Commission Regulation EC N°856/2005), 400 μg kg^{-1}. For *broa* samples collected at central zone of Portugal, the average sample contamination of FB_1+FB_2 was 274 μg kg^{-1}. Assuming that the estimation of average daily intake of bread, in 1994, of Portuguese population was 32 kg per person per year (Instituto Português do Consumidor, 2005) and considering that the consumption of

broa represents a quarter of the total consumption of bread, *broa* consumption was, in 1994, 21.9 g per person per day. Therefore, the estimated daily intake (EDI) of both fumonisins for an adult whose body weight is 60 kg reached, in average, 0.10 μg kg^{-1} body weight/day or 0.70 μg kg^{-1} bw/week. Relatively to FB_1, the intake was in average 0.072 μg kg^{-1} bw/day or 0.504 μg kg^{-1} bw/week, a distant value (36%) from the estimated total intake of FB_1 in the European diet, 1.4 μg kg^{-1} bw/week (Soriano and Dragacci, 2004).

The tolerable daily intake (TDI) proposed by the European Commission for FB_1+FB_2 is 2 μg kg^{-1} bw/day. This data was not overlapped by any of the contaminated sample, representing 5% of theTDI.

4. Conclusions

The analytical methodology provides good results in terms of accuracy, repeatability, intermediate precision and sensitivity for determination of FB_1 and FB_2 in *broa*.

In the 25 contaminated samples (83%), FB_1 contamination levels were higher than FB_2. Twenty seven per cent of the samples exceeded the recommended limits.

None of the analysed samples exceeded the tolerable daily intake, appearing that FBs contamination does not present a hazard to Portuguese population.

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