

Research Papers

Occurrence of fumonisins B₁, B₂ and B₃ in breakfast and infant cereals from Morocco

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Summary. A total of 68 cereal products (48 breakfast cereals and 20 infant cereals) were collected from supermarkets and pharmacies in the Rabat-Salé area of Morocco and the content of fumonisins (FB₁, FB₂ and FB₃) was analysed. Samples were extracted with a mixture of acetonitrile/water (85/15, v/v) by using an ultra-turrax homogenizer. Mycotoxins were then identified and quantified by liquid chromatography/tandem mass spectrometry. Results showed that fumonisins were detected in 20 samples (18 breakfast cereals and 2 infant cereals) with a frequency of contamination of 29.4% of total samples. The most contaminated products were cornflakes (maize) and breakfast cereals (rice, maize and cacao) with 10 and 4 positive samples respectively. The highest value was found in a breakfast cereal with 228 µg kg⁻¹ of total fumonisins.

Key words: analysis, cereals products, contamination, mycotoxins.

Introduction

Fumonisins (FBs) are a group of mycotoxins isolated initially from corn culture material of *Fusarium verticillioides* (syn. *Fusarium moniliforme*) (Gelderblom *et al.*, 1988). The elucidation of the chemical structure of the FBs was carried out by Bezuidenhout *et al.* (1988), FBs are a structurally related group of diesters of propane-1,2,3-tricarboxylic acid and various 2-amino-12, 16-dimethylpolyhydroxyeicosanes. Since 1988, FB₅ have been isolated from certain *Fusarium* species (i.e., *Fusarium verticillioides* and *Fusarium proliferatum*). Several FB₅ molecules have been isolated and characterized; fumonisin B₁ (FB₁), fumonisin B₂ (FB₂) and fumonisin B₃ (FB₃) are the major ones produced in naturally contaminated foods. Chen *et al.* (1992) reported the production of FB₁ by *Alternaria alternata* f. sp. *lycopersici*. These metabolites

that are usually found in corn have been implicated in field cases of porcine pulmonary edema (PPE) (Harrison *et al.*, 1990; Osweiler *et al.*, 1992; Colvin *et al.*, 1993) and equine leukoencephalomalacia (ELEM) (Wilson *et al.*, 1990a). Although their effect on humans is still not clearly established, consumption of corn contaminated with FBs has been statistically associated with high incidence of esophageal cancer in some regions in South Africa (Sydenham *et al.*, 1990; Rheeder *et al.*, 1992) and China (Chu *et al.*, 1994). Fumonisin B₁ (FB₁) is the major compound and has the highest toxicity amongst this class of toxins. FB₁ is classified by IARC (2002) as a “possible carcinogen” (class 2B). The mechanism of action of FB₁ seems to be correlated to the inhibition of ceramide synthetase that causes an imbalance in sphingolipid metabolism, and an altered sphinganine/sphingosine ratio is considered a reliable biomarker of the exposure to FBs (Riley and Voss, 2006; Shephard *et al.*, 2007).

Regarding this potential risk, the scientific committee for food (SCF) from the European Commission has established a tolerable daily intake of 2 µg

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kg⁻¹ bw for FB₁, FB₂, and FB₃, alone or in combination. To reduce the intake of FBs, the European Commission has set maximum limits of 4000 µg FBs kg⁻¹ for unprocessed corn, and 200 µg FBs kg⁻¹ for processed corn-based foods and baby foods for infants and young children (Commission Directive, 2007/1126/EC). FBs are polar molecules and are usually extracted with mixtures of polar solvents, such as methanol, acetonitrile, and water in different combinations and proportions (Scudamore *et al.*, 1997; Cortez-Rocha *et al.*, 2003). The aim of this study was to evaluate the presence of FBs (FB₁, FB₂ and FB₃) in some breakfast and infant cereals collected from supermarkets and pharmacies in the Rabat-Salé area, Morocco.

Materials and methods

Chemicals and reagents

Acetonitrile was purchased from Fisher Scientific (Madrid, Spain). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

FB₁ and FB₂ were purchased from Sigma (St. Louis, MO, USA); FB₃ was supplied by PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerber, South Africa). A stock standard solution for each FB was prepared at 1000 µg mL⁻¹ in 1 mL acetonitrile/water (50/50, v/v) and stored at 4°C. Working standard solutions containing all compounds were obtained by further dilution of stock individual solutions with acetonitrile/water (50/50, v/v).

Sampling

Sixty eight samples of breakfast and infant cereals were collected from different supermarkets and pharmacies in Rabat (Morocco). Breakfast cereal (n=48) samples included the following major ingredients, alone or mixed: maize, sugar, malt extract, salt, chocolate, cacao, cereals (wheat, rice and/or barley), vitamins, vegetable oil and dried fruits (raisins, bananas, nuts, apple, apricots, and prune). Infant cereals (n=20) included the following major ingredients, alone or mixed: cereals (wheat flour, rice flour and/or rye), sugar, vegetable oil, lecithin of soy, vitamins, minerals, milk traces, dried fruits (apricots, apple, orange, pear), skimmed milk, honey, carrot and/or

iron. All the samples were kept in their original bottles and stored in a dark and dry place until analysis.

Mycotoxin extraction procedure

The extraction and determination of FBs was based on the method reported by D'Arco *et al.* (2008). Briefly, 3 g of cereal samples were extracted with 20 mL of a mixture of acetonitrile / water (85/15, v/v) using an Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The extract was centrifuged at 4500 g for 5 min and then the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland) and then redissolved in 2 mL of extraction solvent. This final solution was filtered through a 25 mm/0.45 µm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before the injection into the LC-MS/MS.

LC MS/MS

LC separation was carried out on a Luna C18 analytical column (150 mm×4.6 mm I.D., 5 µm) preceded by a C₁₈ security guard cartridge (4 mm×2 mm I.D., 5 µm), both from Phenomenex (Madrid, Spain). The analytical separation was performed using gradient elution with water as mobile phase A and methanol as mobile phase B, both containing 0.5% formic acid. After an isocratic step of 65% B for 3 min, it was gradually increased to 95% B in 4 min and held constant for 3 min. Afterwards, the initial conditions were maintained for 10 min. Flow rate was maintained at 0.3 mL min⁻¹.

A TQ mass spectrometer Quattro LC from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA), with an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and Mass Lynx NT software 4.1 was used for data acquisition and processing. Parameters were optimized by continuous infusion of a standard solution (10 µg mL⁻¹) via a syringe pump at a flow rate of 10 µL min⁻¹. The analysis was performed in positive ion mode. The electrospray ionization (ESI) source values were as follows: capillary voltage, 3.20 kV; cone, 50 V; extractor, 3 V; RF lens, 0.2 V; source temperature, 125°C; desolvation temperature, 300°C; desolvation gas (nitrogen, 99.99% purity) flow, 500 l h⁻¹; cone (gas flow) 50 l h⁻¹. The analyzer settings were: resolution, 12.0 (unit resolution) for the first

and third quadrupoles; ion energy, 0.5; entrance and exit energies, -3 and 1; multiplier, 650 V; collision gas (argon, 99.995%) pressure 3.74×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s. The mass spectrometer was operated in scan, product-ion scan, and MRM modes. All the measurements were carried out in triplicate.

Results and discussion

Method validation

Fumonisin were determined in all samples by the method previously optimized for these toxins (D'Arco *et al.*, 2008). Precision was calculated in terms of run to-run ($n=45$) and day-to-day precision (five different days) on a standard of 0.5 mg L^{-1} . The run-to-run precision led to RSD values ranging from 1.5% to 6.1%. The day-to-day precision was better than 9.4% for all instances. Calibration graphs were prepared by spiking blank cereal sample extracts and were linear from the limit of quantification

(LOQ) to 100 times the LOQ with correlation coefficients (r^2) greater than 0.9774. The extraction method showed mean recoveries from spiked cereal samples of 68%, 82% and 83% for FB₃, FB₁ and FB₂, respectively, with relative standard deviation (RSDs) below 12%. The limits of quantification obtained were $0.1 \text{ } \mu\text{g kg}^{-1}$ for FB₁ and FB₂, and $0.5 \text{ } \mu\text{g kg}^{-1}$ for FB₃. Fumonisin in contaminated samples were quantified and identified by the acquisition of two transitions for each compound and confirmed with the ratio of both transition areas. Samples with a concentration of fumonisin higher than the LOQ were considered positive. Results obtained from the monitoring programme were corrected with the recoveries obtained.

Fumonisin occurrence in samples

The levels of FB₁, FB₂, FB₃ and total FBs (FB₁ + FB₂ + FB₃) are given in Table 1. FBs were found in 37.5% of total breakfast samples (18/48) with levels that ranged from 6.2 to $228 \text{ } \mu\text{g kg}^{-1}$. The maximum value

Table 1. Levels of FBs in breakfast and infant cereals from Morocco.

Cereal product	Major composition subsample	Number of samples	FB ₁ ($\mu\text{g kg}^{-1}$)		FB ₂ ($\mu\text{g kg}^{-1}$)		FB ₃ ($\mu\text{g kg}^{-1}$)		FB ₁ +FB ₂ +FB ₃ ($\mu\text{g kg}^{-1}$)	
			Mean*	Max**	Mean	Max	Mean	Max	Range	Mean
Breakfast cereals (n=48)	Maize (Cornflakes and other)	17(10)	44	116	133	29.5	2.6	7	6.2–152	69
	Wheat (other)	7(1)	20	20	14.6	14.6	2.3	2.3	37	37
	Rice (other)	9(4)	70.5	152	39.2	62.3	7.4	13.9	8–228	116
	Other (fruits rings)	2(1)	24.8	24.8	20.4	20.4	2.7	2.7	48	48
	Other (muesli)	10(1)	14.2	14.2	7.4	7.4	1.1	1.1	22.7	22.7
	Other (fitness)	2(1)	nd***	nd	8	8	nd	nd	8	8
	Oats	1(0)	nd	nd	nd	nd	nd	nd	-	-
Infant cereals (n=20)	Wheat flour	14(1)	nd	nd	2.3	2.3	nd	nd	2.3	2.3
	Rice flour	6(1)	2	2	1.2	1.2	nd	nd	3.1	3.1

* Mean value on positive samples, ** Maximum value, *** not detected

(228 $\mu\text{g kg}^{-1}$) was found in a cereal with chocolate (rice, maize, cacao); this sample was co-contaminated with FB₁ (151.9 $\mu\text{g kg}^{-1}$); FB₂ (62.3 $\mu\text{g kg}^{-1}$) and FB₃ (13.9 $\mu\text{g kg}^{-1}$). The most contaminated products were cornflakes (maize) and breakfast cereals (rice, maize, cacao) with 10 and 4 positive samples, respectively. Figure 1 shows results from a positive sample contaminated with fumonisins B1, B2 and B3.

In cornflake samples, FBs were detected in 55.5% of samples (10/18) at concentrations ranging from 6.2 to 152.4 $\mu\text{g kg}^{-1}$. The maximum value of FBs found in cornflakes was 152.4 $\mu\text{g kg}^{-1}$. This sample was co-contaminated with FB₁ (116.4 $\mu\text{g kg}^{-1}$), FB₂ (29.5 $\mu\text{g kg}^{-1}$) and FB₃ (6.4 $\mu\text{g kg}^{-1}$). Four breakfast samples (rice, maize and cacao) were found to be contaminated with FBs. The frequency of contamination was 22.2% (4/18) of total breakfast samples. The highest value was found in a cereal with chocolate (rice, maize and cacao) with 152, 62.3 and 14 $\mu\text{g kg}^{-1}$ for FB₁, FB₂ and FB₃ respectively. One muesli sample was found contaminated with three FBs with about 14.2, 7.4 and 1.1 $\mu\text{g kg}^{-1}$ for FB₁, FB₂ and FB₃ respectively. One breakfast sample (wheat) was co-contaminated with 20, 14.6, 2.3 $\mu\text{g kg}^{-1}$ for FB₁, FB₂ and FB₃ respectively. One fitness sample was contaminated with total FBs at a level of about 48 $\mu\text{g kg}^{-1}$. One fruit sample was contaminated only with FB₂ (8 $\mu\text{g kg}^{-1}$).

In general, levels found in our study are below the limit set by the European Commission. In this work, it was demonstrated that contamination by FBs was not only found in maize-based cereals but also in those containing wheat or rice.

The occurrence of FBs in breakfast cereals or cornflakes has been reported by several authors from different countries. For example in France, FBs were analyzed in 32 samples of breakfast cereals containing maize, oats and rice by the method described by Visconti *et al.*, 2001. FB₁ was detected in 94% of samples containing maize, oats or rice at concentrations from 1 to 1110 $\mu\text{g kg}^{-1}$ (Molinié *et al.*, 2005). In another study, Castells *et al.*, 2007 reported that 21% of cornflakes imported from Argentina contained up to 67 $\mu\text{g kg}^{-1}$ of FBs. In a survey from Spain and Italy, D'Arco *et al.* (2009) reported the contamination of corn based samples including baby food and cornflakes with FB₁, FB₂ and FB₃. Results from the occurrence of the FBs in analysed samples of infant's cereals are summarized in Table 1. As shown, there were two contaminated samples. One infant cereal (rice based products) was contaminated with 2 and 1.2 $\mu\text{g kg}^{-1}$ of FB₁ and FB₂ respectively, and the other sample (wheat based product) was contaminated with FB₂ only (2.3 $\mu\text{g kg}^{-1}$).

The presence of FBs in breakfast cereals and infant cereals from Morocco was described herein. Results

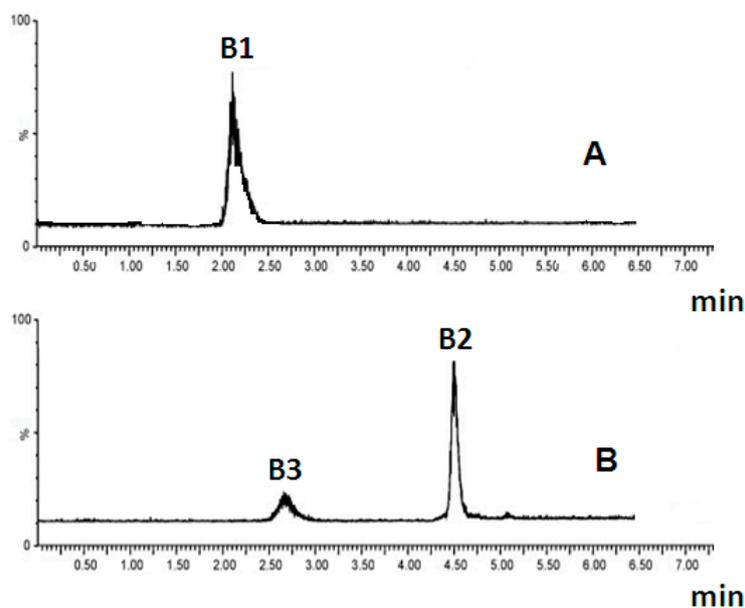


Figure 1. LC-MS/MS chromatogram of a naturally contaminated sample with FB1, FB2 and FB3.

showed that FBs were detected with a frequency of contamination of 29.4% of total samples. The most contaminated products were cornflakes (maize) and breakfast cereals (rice, maize and cacao). The highest value was found in breakfast cereal (rice, maize and cacao) with 228 $\mu\text{g kg}^{-1}$ for total FBs.

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