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Risk assessment associated to the intake of the emerging *Fusarium* mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin

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ABSTRACT

Forty-five samples of Spanish follow-up infant formula with different chemical compositions were analyzed determining the emerging *Fusarium* mycotoxins beauvericin (BEA), enniatins (ENs) (A, A_1, B, B_1), and fusaproliferin (FUS). The samples were extracted three times with ethyl acetate using an Ultra-turrax homogenizer. Mycotoxins were identified and quantified using a liquid chromatography (LC) coupled to a diode array detector (DAD). Results showed that the percentage of the samples contaminated with ENs and FUS were 46.6 and 20.0% respectively, whereas all analyzed samples were free of BEA. The ENs A and B were detected only in one sample with 149.6 and 39.4 mg/kg respectively. The ENB_1 was the more detected mycotoxin with levels ranging from 11.4 to 41.9 mg/kg. The ENA_1 was detected at levels ranging from 6.3 to 101.7 mg/kg. The minor *Fusarium* mycotoxin FUS was detected in a range variable from 0.7 to 1.7 mg/kg. Finally, dietary exposure of Spanish infants (between 6 and 12 months) to ENs, BEA and FUS, was estimated through the consumption of commercial follow-up infant formula by the calculation of the estimated daily intake (EDI). Considering the sum of the mycotoxins studied, the data evidenced that the EDI was 236.2 μg/kg bw/day.

1. Introduction

Mycotoxins are naturally occurring toxic secondary metabolites produced under appropriate favorable conditions by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. Among the mycotoxins produced by *Fusarium* spp., are included the enniatins A, A_1, B and B_1 (ENA, ENA_1, ENB and ENB_1), beauvericin (BEA) and fusaproliferin (FUS), which are a group of bioactive compounds called emerging mycotoxins or minor *Fusarium* mycotoxins (Meca, Zinedine, Blesa, Font, & Mañes, 2010). FUS is a bicyclic sesterterpene formed by five isoprenoid units, whereas BEA and enniatins (ENs) are cyclic hexadepsipeptides. In particular BEA is composed by of alternated D-α-hydroxyisovaleryl-(2-hydroxy-3-methylbutanoic acid) and N-methyl-L-phenylalanyl residues, whereas ENs are of three D-α-hidroxyisovaleryl and three N-methyl-L-amino acid residues (Jestoi, 2008). BEA and ENs show a similar chemical structures, and therefore can be assumed that present the same toxic dynamic actions: has been demonstrated that have cytotoxic effects in several cell lines (Ferrer, Juan-García, Font, & Ruiz, 2009; Fornelli, Minervini, & Logrieco, 2004; Kamyar, Rawnduzi, Studenik, Kouri, & Lemmens- Gruber, 2004); whereas FUS showed causes teratogenic effects on chicken embryos (Ritieni et al., 1997). Due to these toxic effects, the contamination by mycotoxins causes severe economic losses annually, and depends of several factors including climatic conditions, genetic susceptibility of cultivars to fungal infection, soil type and nutritional factors (Bakan, Melcion, Richard-Molard, & Cahagnier, 2002).

Several analytical methods have been described in the literature for the detection of minor *Fusarium* mycotoxins in several foods. The methods include the common steps applied in mycotoxin analyses: sample preparation using extraction of the analytes and removal of impurities using different types of columns. Sample analysis is carried out using high performance liquid chromatography (LC) with ultraviolet (UV) or mass spectrometric (MS) detection (Jestoi, Rokka, Rizzo, & Peltonen, 2005; Santini, Ferracane, Meca, & Ritieni, 2009). The presence of ENs, BEA and FUS in food commodities has been recently reported during the last decade in some European countries (Finland, Norway, Spain, Slovakia, Croatia, Switzerland and Italy), in USA, in South Africa and in Australia. Recently our research group has reported the contamination of cereals (maize, wheat and barley) and cereal products (breakfast cereals) available in Morocco and Spain (Mahnine et al., 2011; Meca et al., 2010; Sifou et al., 2011; Zinedine, Meca, Mañes, & Font, 2011). Mahnine et al. (2011) studied the presence of ENs, BEA and FUS in breakfast and infant cereals from Morocco, evidencing principally the presence of the ENB_1 and ENA_1.

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Infants are considered a vulnerable group of the population considering the mycotoxins intake due to a diet rich in cereals and also to a reduced body weight (bw) compared to the adults. As consequence, maximum limits for mycotoxins in baby foods are much lower than the limits set for other cereal products (European Commission, 2006). In order to prevent infant exposure to mycotoxin contamination, dietary exposure studies are of great interest. Most evaluations have focussed in the called "traditional" mycotoxins such as fumonisins or trichothecenes (D'Arco, Fernández-Franzón, Font, Damiani, & Mañes, 2009; González-Osnaya, Cortés, Soriano, Moltó, & Mañes, 2011), and Tolerable Daily Intakes (TDIs) have been set by scientific committees such as the Scientific Committee for Food (SCF) for many of *Fusarium* mycotoxins (SCF, 2002). In contrast, no TDIs have been set for minor *Fusarium* mycotoxins, probably due to their late recognition and therefore, their limited data available on their toxicity, concentration levels and occurrence. Several studies suggest that infants up to the age of 6 months typically lose the capacity to detoxify and eliminate substances (Dourson, Charnley, Scheuplein, & Barkhurst, 2004). In this context, the aims of this study were: a) to determine the presence of the minor *Fusarium* mycotoxins BEA, ENs and FUS in different follow-up infant formula of Spanish origin and b) to evaluate the risk exposure of Spanish infants to these *Fusarium* mycotoxins present in the samples analyzed by the evaluation of the Estimated Daily Intake (EDI).

2. Materials and methods

2.1. Chemical and reagents

Acetonitrile and ethyl acetate, all of HPLC grade, were purchased from Merck (Whitehouse Station, N.J., U.S.A.). 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. The stock standard solutions of BEA and ENA, ENA_1, ENB and ENB_1 were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Standard solution of FUS was gently given by Professor Alberto Ritieni, Department of Food Science, University of Naples "Federico II", Italy. All stock solutions were prepared by dissolving 1 mg of the mycotoxin (BEA, FUS or ENs) in 1 mL of pure methanol, obtaining a 1 mg/ml solution. These stocks solutions were then diluted with pure methanol in order to obtain the appropriated work solutions.

All solutions were stored in darkness at 4 °C until the LC analysis.

2.2. Samples description

A total of forty-five samples of infant formula were studied and divided in: nine follow-up infant formulas composed with eight different flours (wheat, corn, rice, oat, barley, rye, sorghum, and millet), seven follow-up infant formulas composed with enriched with fruit juice (8–25%) and eight different cereal flours (wheat, corn, rice, oat, barley, rye, sorghum, and millet), seven follow-up infant formulas composed with enriched with honey (5%) and eight different flours (wheat, corn, rice, oat, barley, rye, sorghum, and millet), five follow-up infant formulas composed with six different flours (wheat, corn, rice, oat, barley, rye), ten follow-up infant formulas whole cereals (corn, wheat and rice), and seven follow-up infant formulas composed with two different flours (rice and corn). A 20–40 g of sample (according to the indication of the package) was diluted in 200 ml of milk infant formulas. A 100 ml subsample was collected in a plastic bag and kept at −20 °C until analysis.

2.3. Mycotoxin extraction procedure

The method used for the analysis of the mycotoxins (BEA, ENs and FUS) was reported by Jestoi (2008). Briefly, 3 g of reconstituted infant formula were extracted with 30 ml of ethyl acetate using an Ultra Ika T18 basic Ultra-turrax (Staufen, Germany) for 5 min. The extract was centrifuged at 4500×*g* for 15 min and then the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland) and then re-dissolved 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-DAD system for analysis.

2.4. LC-DAD analysis

LC analyses of BEA, ENs and FUS were performed using a Shimadzu LC system equipped with LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Gemini (150 × 4.6 mm, 5 μm) Phenomenex column was used. LC conditions were set up using a constant flow at 1.0 mL/min and acetonitrile-water (70:30 v/v) as starting eluent system. The starting ratio was kept constant for 5 min and then linearly modified to 90% acetonitrile in 10 min. After 1 min at 90% acetonitrile, the mobile phase was taken back to the starting conditions in 4 min. BEA and ENs were detected at 205 nm, while FUS was detected at 261 nm. All samples were filtered through a 0.22 μm syringe filter Phenomenex prior to injection (20 μl) onto the column. Mycotoxin identification was performed by comparing retention times and UV spectra of purified extracted samples to pure standards. Quantification of mycotoxins was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

2.5. LC-MS/MS confirmation

The presence of ENs, FUS, and BEA in positive samples of cereals was confirmed by LC-MS/MS according to the method described by Sørensen, Nielsen, Rasmussen, and Thrane (2008). Briefly, analysis of BEA, FUS and ENs was carried out with a TQ mass spectrometer Quattro LC from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters, Milford, MA) consisted of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and a Mass Lynx NT software 4.1 were used for data acquisition and processing.

The auto injector was programmed to inject 20 μl into the Luna C18 column (150 × 4.6 mm, 5 mm) Phenomenex maintained at 30 °C.

The analytical separation for LC-MS/MS was performed using gradient elution with water as mobile phase A, and acetonitrile as mobile phase B, both containing 0.5% formic acid. After an isocratic step of 65% B for 3 min, it was linearly increased to 75% B in 4 min and held constantly for 3 min. Flow rate was maintained at 0.3 ml/min.

Analysis was performed in positive ion modes. The ESI source values were as follows: capillary voltage, 3.20 kV; source temperature, 125 °C; desolvation temperature, 350 °C; desolvation gas (nitrogen, 99.95% purity) flow, 700 l/h. Ideal fragmentation conditions were accomplished varying the cone voltage and collision energies for each compound. For the detection of FUS and BEA the precursor ion were *m*/*z* 445 and 801 being the product ions selected were *m*/*z* 427–409 and 784–756 specifically. For ENB the precursor ion was *m*/*z* 640, and the product ions were *m*/*z* 196 and 527. For ENB_1 the precursor ion was *m*/*z* 654 and the product ions *m*/*z* were 196 and 228, for ENA the precursor ions was *m*/*z* 682, and the product ions were *m*/*z* 210 and 555, for ENA_1 the precursor ion was *m*/*z* 668, and the product ions were *m*/*z* 210 and 541.