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Occurrence of mycotoxins in refrigerated pizza dough and risk assessment of exposure for the Spanish population

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ABSTRACT

Mycotoxins are toxic metabolites produced by filamentous fungi, as *Aspergillus*, *Penicillium* and *Fusarium*. The first objective of this research was to study the presence of mycotoxins in 60 samples of refrigerated pizza dough, by extraction with methanol and determination by liquid chromatography associated with tandem mass spectrometry (LC-MS/MS). Then, the estimated dietary intakes (EDIs) of these mycotoxins, among the Spanish population, was calculated and the health risk assessment was performed, comparing the EDIs data with the tolerable daily intake values (TDIs). The mycotoxins detected in the analyzed samples were aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), zearalenone (ZEA), enniatin A (ENA), enniatin A_1 (ENA_1), enniatin (ENB), enniatin B_1 (ENB_1) and BEA (beauvericin) with average concentration of the positive samples of 4.09 μg/kg, 0.50 μg/kg, 0.79 μg/kg, 77.78 μg/kg, 14.96 μg/kg, 4.54 μg/kg, 3.37 μg/kg, 1.69 μg/kg and 22.39 μg/kg, respectively. The presence of ZEA, ENA_1, ENB and ENB_1 was detected in 100% of the samples, AFB_2 in 32%, AFB_1 in 23%, ENA in 8% and BEA in 3%. Twelve percent of the samples contaminated with AFB_1 and 12% of the doughs contaminated with ZEA exceeded the EU legislated maximum limits. The dietary intakes were estimated considering three different age groups of population, and the EDIs calculated for the mycotoxins detected in the samples were all below the established TDI.

1. Introduction

Mycotoxins are a group of secondary metabolites produced by fungi, mainly by the genera *Aspergillus*, *Penicillium*, *Alternaria*, *Fusarium*, and *Claviceps*. Mycotoxins may contaminate cereals and other food commodities at pre-harvest, harvest and post-harvest and their presence is largely dependent on environmental factors that affect fungal growth (Zain, 2011).

The risk of human exposure to mycotoxins is related to consumption of contaminated food, which may cause diseases and can also lead to death (Erdogan, 2004). A limited number of more than 400 known mycotoxins are generally considered to play important roles in food safety (Reddy et al., 2010; Streit et al., 2012) because they can be responsible for pathophysiological changes like neurotoxicity, nephrotoxicity, hepatotoxicity, neurological cardiac lesions, gastrointestinal disorders, Balkan endemic nephropathy, tubulonefritis and so forth (Marin et al., 2013). There is much concern about chronic effects of mycotoxins by low levels of exposure, and some of them have been classified by the International Agency for Research on Cancer (IARC, 2013) as human carcinogens [Group 1: aflatoxins (AFs)] or probably human carcinogens or possible carcinogen to humans [Group 2B: fumonisin B_1 (FB_1) and ochratoxin A (OTA)].

The most important mycotoxins detected in food are: AFs, OTA, trichothecenes (TCs) (type A: HT-2 and T-2 toxin, and type B: deoxynivalenol (DON)), zearalenone (ZEA), FB_1 and fumonisin B_2 (FB_2), and the emerging mycotoxins fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA), and enniatins (ENs) (Marin et al., 2013).

Maximum levels of contamination have been established by The European Commission for some mycotoxins found in cereals and derived products, in particular: 2 μg/kg for aflatoxin B_1 (AFB_1) and 4 μg/kg for total AFs (European Commission, 2010); 750 μg/kg for DON (European Commission, 2006); 1000 μg/kg for the sum of FB_1 and FB_2 in maize-based foods for direct human consumption (European Commission, 2007); 3 μg/kg for OTA (European

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Commission, 2012); 25 μg/kg for T-2 y HT-2 (Commission Recommendation, 2013); 75 μg/kg for ZEA (European Commission, 2006).

Monitoring studies of mycotoxins in several foodstuffs should be continuously conducted. They are necessary to collect and evaluate the presence of mycotoxins in food and feed and, thus, to obtain reliable information about the real exposure of human population to these toxic compounds (Rodríguez-Carrasco et al., 2013).

Pre-cooked pizza dough is considered a potential substrate for fungal development, because it is a product based on cereals with intermediary moisture content. These fungi can grow and affect nutritional and sensory properties of the food products and, above all, if the species are toxigenic, they may produce mycotoxins. Food preparation procedures could inactivate the fungi but do not guarantee the removing or reduction of the mycotoxins already produced by the fungi because they are relatively stable to cooking and processing. The contamination can also occur during packaging and inappropriate storage. Even in samples kept at refrigerated temperatures, up to the end of shelf life, mold and yeast growth was no inhibited and mycotoxins were produced (Pinho and Furlong, 2000).

The aim of this study was to: a) determine and quantify the occurrence of different legislated and no legislated mycotoxins (AFB_1, AFB_2, AFG1, AFG_2, OTA, ZEA, FB_1, FB_2, FUS, BEA and ENs) in refrigerated pizza dough samples commercialized in Spain and b) to estimate the daily intake of these mycotoxins among Spanish population to carry out the deterministic risk assessment.

2. Materials and methods

2.1. Chemical and reagents

Methanol was purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Molsheim, Francia). Chromatographic solvents and water were filtered through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain). Formic acid (HCOOH) and ammonium formate were obtained from Sigma–Aldrich (St Louis MO., USA).

2.2. Analytical standard

AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEA, FB_1, FB_2, FUS, BEA, ENB, ENB_1, ENA and ENA_1, were obtained from Sigma–Aldrich (St Louis MO., USA). All stock solutions were prepared by dissolving 1 mg of the mycotoxin in 1 mL of pure methanol, obtaining a 1 mg/mL solution. These stock solutions were then diluted with pure methanol in order to obtain the appropriated work solutions. All solutions were stored in darkness at −20 °C.

2.3. Sampling

A total of 60 refrigerated pizza dough samples of eight brands and all from different lots were randomly purchased from different supermarkets located in Valencia (Spain) from March to June 2015. All the samples were stored at −20 °C before sample extraction. Refrigerated pizza dough ingredients were: wheat flour, water, vegetable fat (palm, rape), salt, wheat starch, alcohol and yeast.

2.4. Mycotoxin extraction

The method used for mycotoxins analysis is based on that described by Serrano et al. (2013) with some modifications. The pizza doughs were finely ground with an Oster Classic grinder (220–240 V, 50/60 Hz, 600 W; Madrid, Spain). Five grams of each homogenized sample were weighed in a 50 mL plastic tube and 25 mL of methanol were added. The extraction was carried out using an Ultra Ika T18 basic Ultra-turrax (Staufen, Germany) for 3 min. The extract was centrifuged at 4000 rpm for 5 min at 5 °C and the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The residue was dissolved with 5 mL of methanol and was evaporated to dryness by nitrogen gas at 35 °C using a multi-sample Turbovap LV Evaporator (Zymark, Hopti-kinton, USA). After solvent evaporation, the extract was resuspended with 1 mL of methanol, vortexed, filtered through 13mm/0.22 μm nylon filters and injected into liquid chromatography associated with tandem mass spectrometry (LC-MS/MS). All the extractions were carried out in triplicate.

2.5. LC-MS/MS analysis

The liquid-chromatography analysis system was an Agilent 1200 Chromatograph (Agilent Technologies, Palo Alto, CA, USA) which consisted of a binary LC-20AD pump, a SIL-20AC homoeothermic auto sampler and a CMB-20A controller Analyst Software 1.5.2 was used for data acquisition and processing. The separation of mycotoxins was performed on a Gemini NX C18 column (150 × 2.0 mm I.D, 3.0 μm, Phenomenex, Palo Alto, CA) at room temperature (20 °C). The mobile phase was composed of solvents A (5 mM ammonium formate and 0.1% formic acid in water) and B (5 mM ammonium formate and 0.1% formic acid in methanol) at a flow rate of 0.25 mL/min. The elution gradient was established initially with 10% eluent B, increased to 80% in 1.5 min, then kept constant from 1.5 to 4 min, increased to 90% from 4 to 10 min, increased again to 100% from 10 to 14 min and finally return to the initial conditions and requilibrate during 10 min. The injection volume was 20 μL. A 3200QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an ESI interface in positive mode was used for detection in multiple reactions monitoring (MRM) mode. The main MS parameters were optimized and finally set as follows: nebulizer gas (GS1), 55 psi; auxiliary gas (GS2), 50 psi; curtain gas (CUR) 15 psi; capillary temperature 550 °C; ion spray voltage (IS) 5500 V. Nitrogen was used as the nebulizer, heater, curtain and collision gas. The precursor-to-product ion transitions were *m/z* 313.3/241.3–284.9 for AFB_1, *m/z* 315.3/259.0–288.4 for AFB_2, *m/z* 329.7/243.3–311.1 for AFG_1, *m/z* 331.1/313.0–245.0 for AFG_2, *m/z* 801.2/784.1–244.1 for BEA, *m/z* 657.3/196.1–214.0 for ENB, m/z 671.2/214.2–228.1 for ENB_1, *m/z* 699.4/210.2–228.2 for ENA, *m/z* 685.4/214.2–210.2 for ENA_1, *m/z* 722.4/334.3–352.3 for FB_1, *m/z* 706.4/336.2–318.3 for FB_2, *m/z* 404.0/102.0–239.0 for OTA, *m/z* 319.0/282.9–301.0 for ZEA, *m/z* 355.0/175.0–246.7 for FUS. Analytical parameters of the validation method are showed in Table 1.

Table 1
Analytical parameters of the validation method.

Mycotoxin	LOD (μg/Kg)	LOQ (μg/Kg)	Recovery (%)	SSE (%)
AFB_1	0.08	0.2	70 ± 13	37
AFB_2	0.08	0.2	64 ± 12	29
AFG_1	0.16	0.5	62 ± 16	27
AFG_2	0.3	0.9	66 ± 15	34
ZEA	7.8	26.1	55 ± 7	106
ENA	2.5	7.5	66 ± 8	14
ENA_1	0.5	1.5	65 ± 5	21
ENB	0.03	0.1	72 ± 12	49
ENB_1	0.06	0.2	75 ± 5	49
BEA	7	20	50 ± 11	32

- LOD = limit of detection
- LOQ = limit of quantification
- SSE = Signal Suppression − Enhancer