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Short communication: Analysis of mycotoxins in Spanish milk

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ABSTRACT

We surveyed the presence of 22 mycotoxins in 191 Spanish cow milk samples. Mycotoxins could be carried over from diet into animal milk and have toxic effects on human and animal health. The interaction of different mycotoxins may be additive or synergetic. Therefore, surveillance of mycotoxin co-occurrence in milk is recommended. Aflatoxins M1, B1, B2, G1, and G2, ochratoxins A and B, nivalenol, deoxynivalenol, deepoxy-deoxynivalenol, 3- and 15-acetyldeoxynivalenol, diacetoxyscirpenol, neosolaniol, fusarenon X, T-2 and HT-2 toxins, fumonisins B1, B2, and B3, sterigmatocystin, and zearalenone were analyzed. Samples were treated by liquid-liquid extraction with acidified acetonitrile, followed by an acetonitrile-water phase separation using sodium acetate. The analysis was carried out by HPLC coupled to a triple quadrupole mass spectrometer. None of the analyzed mycotoxins had a concentration level higher than their detection limit (0.05–10.1 μg/L). The aflatoxin M1 in the samples never exceeded the level established by the European Union.

Key words: milk, mycotoxin, co-occurrence

Short Communication

Foods of animal origin may be contaminated with mycotoxins when they are based on or prepared with products derived from animals whose diet contained mycotoxins (Capriotti et al., 2012). Mycotoxins can appear in animal feed due to the contamination of agricultural commodities by filamentous fungi, especially those belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium* (Binder, 2007; Rodrigues and Naehrer, 2012); therefore, the European Union has recommended limits for some of the mycotoxins and established legal limits for aflatoxins in products for animal consumption (European Commission 2006, 2013). Ruminant metabolism usually degrades mycotoxins into less toxic compounds; however, some of them can remain unaltered and they can be absorbed and accumulated in animal tissues or biological fluids, including milk (Flores-Flores et al., 2015). Special attention has been paid to aflatoxin M1 (**AFM1**), which is formed as a degradation product in the hepatic metabolism of aflatoxin B1 (**AFB1**) in ruminants (Wu et al., 2009) and is excreted into milk. Aflatoxin M1 has been classified as probably carcinogenic for humans (group 2B; IARC, 2002); accordingly, the European Community has set a maximum allowable limit of AFM1 in milk (0.05 μg/kg; European Commission, 2010), whereas the levels of other mycotoxins in milk are not regulated. Approximately 9.8% of the milk samples analyzed worldwide exceeded the maximum limit set in the European Union for AFM1, and the presence of low levels of other mycotoxins in milk have also been reported (Flores-Flores et al., 2015). Huang et al. (2014), detected the simultaneous presence of up to 4 mycotoxins in milk samples: 15% were contaminated with 2 mycotoxins, 45% with 3 mycotoxins, and 22% with 4 mycotoxins. This multiexposure can change the toxic effects of mycotoxins on human and animal health due to additive, synergistic, or even antagonistic phenomena (Smith et al., 2016), even when levels considered to be nontoxic of individual mycotoxins are present (Wan et al., 2013). Therefore, the continuous surveillance of mycotoxin co-occurrence in milk is needed to obtain data for better risk assessment and to protect consumer and animal health. This paper shows a survey on the presence of 22 mycotoxins in 191 Spanish cow milk samples.

Methanol (LC-MS grade), formic acid (MS grade, purity >98%), ammonium formate (analytical grade), and sodium acetate (anhydrous, HPLC grade >99.0%) were purchased from Sigma-Aldrich (St. Louis, MO) and acetonitrile (HPLC grade) from Merck (Darmstadt, Germany). Deionized water (>18 MΩ/cm resistivity) was purified in an Ultramatic Type I system from Wasserlab (Navarra, Spain). All mycotoxins (purity ≥98%) were obtained from Sigma-Aldrich in solution, except for ochratoxin A, which was purchased in powder form.

Three mixed stock solutions (1, 2, and 3) were prepared by dilution of appropriate volumes of each mycotoxin standard solution in 10 mL of acetonitrile, as pre-

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viously described (Flores-Flores and González-Peñas, 2015, 2017). Mixed stock solution 1 contained nivalenol, deoxynivalenol, deepoxy-deoxynivalenol, fusarenon X, neosolaniol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, diacetoxyscirpenol, HT-2 toxin, and T-2 toxin. Mixed stock solution 2 contained aflatoxin B1, aflatoxin B2, aflatoxin G1, aflatoxin G2, AFM1, ochratoxin A, ochratoxin B, zearalenone, and sterigmatocystin. Mixed stock solution 3 contained fumonisin B1, fumonisin B2, and fumonisin B3. Mixed stock solutions 1 and 2 were stored at −20°C. Mixed stock solution 3 was prepared and used daily due to the instability of fumonisins in acetonitrile. For clarity, the mycotoxins contained in mixed stock solution 1 were referred to as mycotoxin group 1 and mycotoxins from mixed stock solutions 2 and 3 were referred to as mycotoxin group 2. Due to the toxicity of these compounds, all of them were handled in solution using gloves and a face shield. In addition, low-light conditions were established during handling so as to prevent photoinstability.

One hundred seven full-cream milk samples were purchased from supermarkets in Spain between September 2013 and April 2016 due to the fact that Spanish families purchase more than 50% of the liquid milk consumed in this type of establishment (MAGRAMA, 2015, 2016). Between 2010 and 2014 (INE, 2016), added together, Galicia, Castile and Leon, Andalusia, Catalonia, Asturias, Cantabria, Navarra, Castile-La Mancha, and Basque Country made up 93% of the total production of cow milk in the country in 2014. Samples were from 26 collection centers located in the high-milk-producing regions (Figure 1); samples were opened and analyzed during 1 to 2 d.

Likewise, 84 raw milk samples were collected in March 2016. All of them were from dairy farms located either in Navarra, La Rioja, Basque Country, or Catalonia. One of the raw samples was taken from a cow with signs of disease of unknown origin. None of these samples suffered any treatment procedure after collection, with the exception of the addition of azidiol (sodium azide/chloramphenicol), a preservative compound frequently used by milk testing laboratories in Spain (Llopis et al., 2013) as a preservative. Samples were analyzed within the week that they were collected and maintained at 4°C until analysis. Prior to chromatographic analysis, milk samples were treated following the procedures previously developed by our group (Flores-Flores and González-Peñas, 2015, 2017). Briefly, 1 mL of milk was poured into a tube for the analysis of mycotoxin group 1 and another 1 mL was poured into a second tube for the analysis of mycotoxin group 2. Each tube was extracted with acidified acetonitrile. After centrifugation, the upper phase of each tube was transferred to another clean tube and water-acetonitrile phase separation was induced by the addition of sodium acetate. Next, each acetonitrile phase was dried and the residue from each one of the tubes was reconstituted with LC mobile phase. In addition, the 2 groups of mycotoxins were analyzed in separate runs with different separation conditions, as explained below.

An Agilent Technologies (Santa Clara, CA) 1200 LC system was used. The chromatographic column was an Ascentis Express C18, 2.7 μm particle size, 150 × 2.1 mm from Supelco Analytical (Bellefonte, PA), maintained at 45°C. The mobile phase consisted of solution A (5 m*M* ammonium formate and 0.1% for-

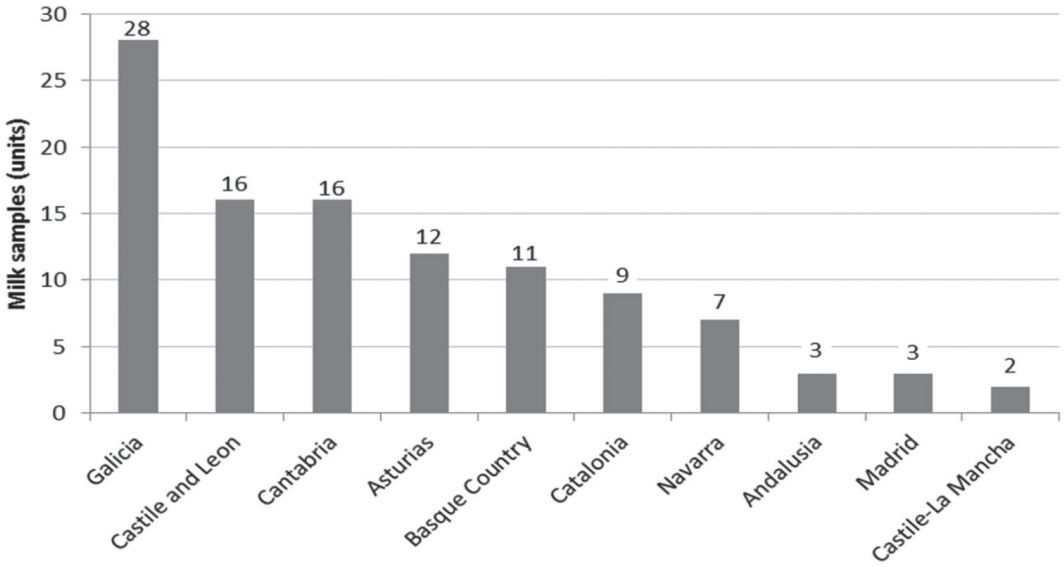

Figure 1. Distribution of analyzed commercial milk samples classified by collection center code. Color version available online.