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Detection and quantitation of mycotoxins in infant cereals in the U.S. market by LC-MS/MS using a stable isotope dilution assay

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ABSTRACT

The aim of this study was to develop an analytical method for the simultaneous determination of aflatoxins B_1, B_2, G_1, G_2, ochratoxin A, fumonisins B_1 and B_2, zearalenone, deoxynivalenol, T-2 toxin, and HT-2 toxin in infant cereals using rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS). Another goal was to survey infant cereals purchased in the United States for the presence and amounts of 11 mycotoxins. Separation of the mycotoxins was accomplished using ultra-high performance liquid chromatography (UHPLC) with <10 min analysis time. The toxins were then detected by dynamic multiple reaction monitoring (dMRM) in positive electrospray ionization mode. Due to matrix effects, [^{13}C]-uniformly labeled mycotoxins were added to the sample extracts prior to LC-MS/MS analysis. Overall recoveries of mycotoxins were 81–130% in rice, 70–119% in barley, 87–123% in oat, and 82–127% in mixed-grain cereals with lower recoveries for fumonisins B_1 and B_2, (33–67%) at three spiking levels (12.5, 25 and 50 ng/g). The relative standard deviation was <20% for all analytes in the infant cereals. When this method was applied to measure mycotoxin concentrations in infant cereals (n = 64) purchased in the U.S. in 2012, 78% of infant cereals were found to be contaminated with at least one of these mycotoxins. None of the infant cereals exceeded the established regulatory limits in the United States for any of the analyzed mycotoxins. However, a total of 21 samples exceeded the European Union maximum limits for three of the mycotoxins including aflatoxin B_1, ochratoxin A and zearalenone. Results of this study suggest that cereals intended for infants should be routinely monitored for mycotoxin content.
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1. Introduction

Mycotoxins are toxic secondary metabolites produced by a variety of filamentous fungi (Van Egmond & Jonker, 2004). Fungi belonging to the genera *Aspergillus*, *Pencillium*, and *Fusarium* are the major contributors to mycotoxin contamination in food crops (Magan, Medina, & Aldred, 2011). Fungal infection and mycotoxin production may occur during growth, harvest, and storage of agricultural commodities. Although food processing operations (e.g. milling, thermal processing) may reduce mycotoxin levels in agricultural commodities, they typically are not able to completely eliminate them from finished foods (Bullerman & Bianchini, 2007). Aflatoxins (AFs), fumonisins, trichothecenes (deoxynivalenol, HT-2 and T-2 toxin), zearalenone (ZEN) and ochratoxin A (OTA) are mycotoxins of primary importance in human foods (Etzel, 2002; Sherif, Salama, & Abdel-Wahhab, 2009). Contamination of mycotoxins in foods is of significant concern as they can cause immune system suppression, cancer, birth defects, and damage to organs in animals and humans (Bennett & Klich, 2003; D'Mello & MacDonald, 1997). Several reports have indicated that exposure to high levels of exposure to mycotoxins can result in death to domestic and laboratory animals, and to humans (Marasas et al., 1988; Newberne & Butler, 1969; Peraica, Radić, Lucić, & Pavlović, 1999). Based on their carcinogenic potential to humans, the International Agency for Research on Cancer (IARC) has classified aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1, AFG_2) as known

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carcinogens (Group 1), and OTA and fumonisins as possible carcinogens (Group 2B) (IARC, 1993).

During the first several months of life, breast milk or formula is the major food source to the growing infant. However, by ages 4–6 months, infants require the gradual replacement of breast milk or formula feeding with solid or pureed foods, because their nutritional needs become greater than breast milk or formula can provide (Devaney et al., 2004; Forrest & Riley, 2004). Infant cereal products are one of the most common foods provided to the infant since they are highly digestible and an excellent source of iron, an essential mineral for proper infant growth. However, cereal-based products are one of the main sources of mycotoxins in the diet, and estimates have been made that approximately 25% of the world's cereal supply is contaminated with these natural toxins (Charmley, Trenholm, Prelusky, & Rosenberg, 1995). Moreover, factors such as increased environmental temperatures and decreased water availability may result in increased fungal infection and mycotoxin contamination of food and feed (Magan et al., 2011).

Infants are believed to be more susceptible to the toxicological effects of mycotoxins than adults as they have a lower body weight, higher metabolic rate, and reduced ability to detoxify toxins than adults (Sherif et al., 2009). Due to the risks associated with mycotoxin intake by infants, nearly 20 countries have set regulations for permissible levels of mycotoxins in baby foods (European Commission, 2004, 2006, 2007, 2010). In the European Union (EU) limits have been set at 0.1 ng/g for aflatoxin B_1 (AFB_1), 200 ng/g for deoxynivalenol (DON), 20 ng/g for ZEN, 200 ng/g for fumonisins and 0.5 ng/g for OTA in infant and baby foods (European Commission, 2004, 2006, 2007, 2010). Regulatory limits exist in some of the Asian countries (e.g. China, South Korea, Sri Lanka, Taiwan, etc.) for mycotoxins (aflatoxins, OTA, ZEN) in infant foods (Anukul, Vangnai, & Mahakarnchanakul, 2013). The U.S. Food and Drug Administration (FDA) has established guidance or action levels for aflatoxins, DON and fumonisins in unprocessed and processed foods; however, no limits have been established for OTA. In addition, the FDA has not established specific limits for mycotoxins in baby foods (FDA, 2016), such as infant cereals. In Canada, maximum limits for OTA (0.5 ng/g) in baby foods and DON (1 μg/g) in uncleaned soft wheat for use in baby food have been proposed by Heath Canada (HC, 2009, 2012). Overall, regulatory limits for mycotoxins in infant cereals are lacking in many countries of the world.

To protect vulnerable groups in the population such as infants, surveillance studies are needed to determine the types and levels of mycotoxins in foods fed to babies and young children. Over the past decade, there have been a number of studies that have evaluated the mycotoxins content of infant foods (cereals, infant formula, cookies, jarred foods, etc) purchased in Canada, Europe, Malaysia and elsewhere (Aksenov, Eller, & Tutel'ian, 2005; Alvito, Sizoo, Almeida, & van Egmond, 2010; Baydar, Erkekoglu, Sipahi, & Sahin, 2007; Hernández-Martínez & Navarro-Blasco, 2010; Lombaert et al., 2003; Soleimany, Jinap, & Abas, 2012), but relatively few studies have been published on the levels and types of mycotoxins found in infant foods purchased in the U.S. (Zhang, Wong, Krynitsky, & Trucksess, 2014).

The concentrations and types of mycotoxins present in food can vary considerably as fungal and toxin contamination depends on geographical location and environmental conditions during growth, harvest and storage. In addition, many fungi are capable of producing more than one mycotoxin. Consequently, multi-analyte detection methods are needed by regulatory agencies to evaluate the mycotoxin content of food to assess compliance with regulatory limits and for enforcement purposes (Njumbe Ediage, Diana Di Mavungu, Monbaliu, Van Peteghem, & De Saeger, 2011; Ren et al., 2007). The detection of multiple mycotoxins can be challenging due to the chemical differences between groups of mycotoxins, the variability and complexity of food matrices and the presence of these chemical contaminants at trace levels in food.

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS) is a highly sensitive, specific and reliable tool for detecting contaminants in food and has become the most universal approach for multi-analyte analysis (Krska et al., 2008; Zöllner & Mayer-Helm, 2006). This analytical tool also has an increasing impact on the expanding field of mycotoxin analysis, particularly in the development of multi-mycotoxin detection methods (Zöllner & Mayer-Helm, 2006). Food components (such as carbohydrates, proteins or fats) can cause suppression or enhancement of the target analysis and may hamper accurate mass spectrometric quantification. Matrix effects encountered with LC-MS/MS analysis of mycotoxins can be minimized by applying internal or matrix-matched standards. The stable isotope dilution approach in mycotoxin analysis of food has been successfully used to overcome the problem of ion suppression or enhancement (Rychlik & Asam, 2008; Sulyok, Krska, & Schuhmacher, 2007; Varga et al., 2012). Stable isotope labeled internal standards compensate for matrix effects due to their identical chemical and chromatographic properties compared to the target toxins.

The aim of the present study was to develop a rapid LC-MS/MS method for the simultaneous determination of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1 and FB_2, ZEN, DON, T-2 toxin, and HT-2 toxin in infant cereal products using a stable isotope dilution assay. A secondary goal was to apply the method to determine the presence and quantity of the 11 mycotoxins in 64 grain-based infant cereals purchased in the U.S.

2. Material and methods

2.1. Standards

The following unlabeled mycotoxins standards were purchased from Sigma-Aldrich (St. Louis, MO, USA): AFG_1, 5 mg; AFG_2, 5 mg; AFB_1, 5 mg; AFB_2, 5 mg; OTA, 5 mg; ZEN, 5 mg; DON, 5 mg; T-2 toxin, 5 mg; HT-2 toxin, 5 mg; fumonisin mix, 50 μg/mL (each of FB_1 and FB_2) in acetonitrile:water. The following mycotoxin internal standard mixes were purchased from Romer Labs (Vienna, Austria): Mix-11 (^{13}C aflatoxins) consisting of 500 ng/mL each of [$^{13}C_{17}$]-AFB_1, [$^{13}C_{17}$]- AFB_2, [$^{13}C_{17}$]- AFG_1, and [$^{13}C_{17}$]- AFG_2; Mix-12 (^{13}C fumonisins) consisting of 5 μg/mL each of [$^{13}C_{24}$]- FB_1 and [$^{13}C_{24}$]-FB_2; Mix-10 (^{13}C Fusarium toxins) consisting of 10 μg/mL of [$^{13}C_{15}$]-DON, 10 μg/mL of [$^{13}C_{22}$]- HT-2 toxin, 1 μg/mL of [$^{13}C_{24}$]- T-2 toxin, and 3 μg/mL of [$^{13}C_{18}$]- ZEN; and [$^{13}C_{20}$]-OTA at 10 μg/mL.

2.2. Working standard solution preparation

A 10 μg/mL stock solution of the unlabeled mycotoxin stock solution was prepared in 5 mL acetonitrile (Fisher Scientific, Hanover Park, IL, USA). The [^{13}C]-labeled analogues were prepared as another stock solution in 1 mL of acetonitrile/water (30:70, v/v). A 1250 ng/mL working solution of the unlabeled mycotoxins was made, and dilutions were prepared at the following concentrations: 0.125 ng/mL, 0.625 ng/mL, 1.25 ng/mL, 6.25 ng/mL, 12.5 ng/mL, 62.5 ng/mL and 125 ng/mL. For the preparation of the calibration standards, 80 μL of the standards solutions was transferred into HPLC vials with microinserts (VWR International, Batavia, IL, USA) and 20 μL of the [^{13}C]-labeled stock solution mixture was added. This resulted in concentrations of 0.1 ng/mL, 0.5 ng/mL, 1 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL and 100 ng/mL for the mycotoxin calibration curve with the internal standards. The internal standard solutions had various final concentrations as follows: [$^{13}C_{17}$]-AFB_1,