**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 B₁, B₂, G₁, G₂, ochratoxin A, fumonisins B₁ and B₂, 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, [¹³C]-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 B₁ and B₂, (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 determine mycotoxins 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 B₁, ochratoxin A and zearalenone. Results of this study suggest that cereals intended for infants should be routinely monitored for mycotoxin content.

KEYWORDS: multi-mycotoxin; infant cereals; internal standard; LC-MS/MS; stable isotope dilution analysis

1. Introduction

Mycotoxins are toxic secondary metabolites produced by a variety of filamentous fungi (Van Egmond & Jonker, 2004). Fungi belonging to the genera *Aspergillus*, *Penicillium*, 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 the finished food (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 (Etzet, 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 (Newberne & Butler, 1969; Marasas et al., 1988; Peraica, Radić, Lucić, & Pavlović, 1999). Based on their carcinogenic potential to humans, the International Agency for Research on Cancer (IARC) has classified aflatoxins B₁, B₂, G₁ and G₂ (AFB₁, AFB₂, AFG₁, AFG₂) as known 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 to 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, Medina & Aldred, 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 (Commission, 2004, 2006, 2007, 2010). In the European Union (EU) limits have been set at 0.1 ng/g for aflatoxin B₁ (AFB₁), 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 also have been proposed by Health 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 AFB₁, AFB₂, AFG₁, AFG₂, OTA, FB₁ and FB₂, 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): AFG₁, 5 mg; AFG₂, 5 mg; AFB₁, 5 mg; AFB₂, 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 FB₁ and FB₂) in acetonitrile:water. The following mycotoxin internal standard mixes were purchased from Romer Labs (Vienna, Austria): Mix-11 (¹³C aflatoxins) consisting of 500 ng/mL each of

[¹³C₁₇]- AFB₁, [¹³C₁₇]- AFB₂, [¹³C₁₇]- AFG₁, and [¹³C₁₇]- AFG₂; Mix-12 (¹³C fumonisins) consisting of 5 µg/mL each of [¹³C₂₄]- FB₁ and [¹³C₂₄]- FB₂; Mix-10 (¹³C Fusarium toxins) consisting of 10 µg/mL of [¹³C₁₅]- DON, 10 µg/mL of [¹³C₂₂]- HT-2 toxin, 1 µg/mL of [¹³C₂₄]- T-2 toxin, and 3 µg/mL of [¹³C₁₈]- ZEN; and [¹³C₂₀]-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 [¹³C]-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 [¹³C]-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: [¹³C₁₇]-AFB₁, 1 ng/mL; [¹³C₁₇]-AFB₂, 1.03 ng/mL; [¹³C₁₇]-AFG₁, 1.03 ng/mL; [¹³C₁₇]-AFG₂, 1.02 ng/mL; [¹³C₃₄]-FB₁, 10.04 ng/mL; [¹³C₃₄]-FB₂, 10.02 ng/mL; [¹³C₁₅]-DON, 20.2 ng/mL; [¹³C₂₂]-HT-2 toxin, 20.6 ng/mL; [¹³C₂₄]-T-2 toxin, 2 ng/mL; [¹³C₂₀]-OTA, 20 ng/mL; [¹³C₁₈]-ZEN, 6.14 ng/mL. All stock and working standard solutions were stored in amber vials at -20 °C.

2.3. Sample preparation

Infant cereals including rice ($n = 20$), barley ($n = 8$), oats ($n = 20$), and mixed-grain ($n = 16$) were collected in supermarkets throughout the U.S. from March to June 2012. A modified method developed by Varga, et al. (2012) was employed to extract mycotoxin from the cereal samples. Infant cereals (5.00 ± 0.01 g) were weighed into 50 mL polypropylene tubes (Fisher Scientific; Hanover, IL, USA). Twenty mL of acidified acetonitrile (acetonitrile/water/formic acid, 80:19.9:0.1, v/v/v) was added, and the tubes were then shaken on a horizontal Burrell wrist action shaker (Burrell Scientific; Pittsburg, PA, USA) for 60 min at room temperature. After extraction, the tubes were centrifuged for 5 min (3500 rpm) with an Eppendorf AG Centrifuge (Hamburg, Germany). An aliquot (80 μ L) of the centrifuged extract was transferred into an HPLC vial with a microinsert (VWR International) and 20 μ L of the [13 C]-labeled working solution was added. Each cereal sample was analyzed in triplicate. A 10 μ L injection of the sample extract was made into the UHPLC-MS/MS system.

2.4. Method validation

Each of the infant cereal matrices (5.00 ± 0.01 g) was weighed into 50 mL polypropylene tubes (Fisher Scientific; Hanover, IL, USA) and then tubes were spiked with unlabeled mycotoxins at three levels: 12.5 ng/g, 25 ng/g and 50 ng/g. They were then extracted with acidified acetonitrile, shaken and centrifuged as indicated in the previous section. The spiked samples were then prepared for analysis by mixing 20 μ L of [13 C]-labeled working solution, 20 μ L of the spiked sample, and 60 μ L of the solvent mixture (30:70 acetonitrile/water, v/v) in a HPLC vial containing a microinsert. Triplicates were prepared for each of the spike levels and matrices. An internal standard calibration curve containing unlabeled mycotoxins at 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 was prepared with [¹³C]-labeled internal standards at various concentrations as discussed in the Standard Preparation section.

One mL portions of a solvent mixture (30:70 acetonitrile/water, v/v) were spiked with different concentrations of unlabeled mycotoxins (*n* = 5) ranging from 0.01 ng/mL to 50 ng/mL for the determination of limits of detection (LODs) and limits of quantitation (LOQs) for each of the mycotoxins in solvent solutions. Similarly, infant cereals were spiked with different concentrations of unlabeled mycotoxins (*n* = 5) also ranging from 0.01 ng/g to 50 ng/g for the determination of limits of detection (LOD) and limits of quantitation (LOQ) for each of the mycotoxins in the four infant cereal matrices (rice, barley, oatmeal and mixed-grain).

2.5. LC-MS/MS conditions

An Agilent 1290 Infinity UHPLC system equipped with an Agilent Zorbax Eclipse Plus C18 (100 mm × 2.1 mm, 1.8 μm) column was used for chromatographic separation of the mycotoxins. Mobile phase A was composed of 0.1% formic acid in water and mobile phase B was composed of 0.1% formic acid in methanol, and both eluents contained 5 mM ammonium formate. The column temperature was kept at 35°C and the flow rate was 0.3 mL/min. The gradient conditions were as follows: initial time, 30% B; 0.5 min, 30% B; 8 min, 100% B; 10 min, 100% B and re-equilibration to 12 min.

Mycotoxins in sample extracts were detected and quantified with an Agilent 6460 Triple Quadrupole LC-MS/MS with Jet Stream Technology. They were identified and quantified using two or three transitions for each mycotoxin by dynamic multi-reaction monitoring (dMRM) in positive electrospray ionization mode (ESI+). The gas temperature was 300°C and the flow rate was 10 L/min. The nebulizer was set at 45 psi, and sheath gas temperature and flow rate were

350°C and 11 L/min, respectively. The capillary and nozzle voltages were 3500 V and 0 V, respectively. The MS/MS acquisition parameters that were used for the analysis of mycotoxins in infant cereals are shown in Table 1.

2.6. Data analysis

Mycotoxin identification and quantitation analyses in infant cereals were performed using Agilent's Mass Hunter Quantitative Analysis Software (version B.04.00). Microsoft Excel 2010 (Redmond, WA, USA) was used to calculate the means, standard deviations and relative standard deviations.

3. Results and discussion

3.1. Determination by LC-MS/MS

An initial investigation was performed to optimize the MS conditions for mycotoxin detection. The Mass Hunter Acquisition optimizer software was used to determine LC-MS/MS MRM transitions, fragmentor voltages and collision energies for 11 mycotoxins evaluated in this study. For each mycotoxin, two mass transitions with the highest abundances were selected. One quantifier ion and one qualifier transition were monitored for each of the unlabeled mycotoxins, whereas only one transition was chosen for each of the [¹³C]-labeled internal standards. An overlay of the MRM transitions for the 11 unlabeled mycotoxins (25 ng/g) spiked into an oat cereal before extraction and the isotopically labeled internal standards of the mycotoxins is shown in Figure 1.

3.2. Investigation of matrix effects

Mycotoxin concentrations and the recoveries for mycotoxins spiked into infant cereals before extraction were calculated using 1/x weighted calibration curves for each analyte by plotting the relative response versus the analyte concentration. The relative response was the peak area of the quantifier transition of the analyte signal divided by the peak area of the corresponding internal standard transition. In these experiments, responses for the target analytes in the spiked sample were reduced or enhanced compared to those spiked into solvent. Ion suppression can reduce the ion intensity as well as affect the reproducibility and accuracy of the assay. The chemical properties of the target compound, matrix type, and the matrix-to-analyte concentration ratio are factors affecting mass spectrometric detection and quantitation (Varga et al., 2012). Also, sample preparation (extraction and cleanup procedures), chromatographic conditions, mass spectrometric instrumentation (i.e design of the ion source) and ionization conditions influence the extent and nature of matrix effects (Gosetti, Mazzucco, Zampieri, & Gennaro, 2010).

In this study, ^{13}C -labeled internal standards were used to compensate for matrix effects. Table 2 compares the recoveries and standard deviations for each of the 11 mycotoxins spiked at the three levels (12.5, 25 and 50 ng/g) into the four infant cereal matrices (rice, barley, oat, mixed-grain). Average recoveries of 51 – 130% were obtained for mycotoxins spiked into rice-based infant cereal, while recoveries for the mycotoxins in barley, oat and mixed-grain cereals were 33 – 119%, 54 – 123%, and 67 – 127%, respectively. In all four types of cereal matrices, the lower recoveries (33 – 67%) were obtained for FB₁ and FB₂, at the three spike levels. If one excludes fumonisins from the calculations, spike recoveries for the remaining mycotoxins ranged from 81 – 130%.

Use of the extraction solvent consisting of acetonitrile/water/formic acid (80:19.9:0.1, v/v/v) resulted in high extraction recoveries (> 81%) for all target analyses, except fumonisins (i.e. FB₁ and FB₂). The fumonisins were not as efficiently extracted as the other mycotoxins because they are more polar, and hence require extraction with a more polar or aqueous solvent. Similar results were reported by Varga, et al. (2012), who reported that fumonisins were less efficiently extracted with acidified acetonitrile from a corn matrix than other mycotoxins (aflatoxins, DON, T-2 toxin, HT-2 toxin, OTA, ZEN). Use of a second extraction step with a more polar solvent would have enabled greater recoveries of fumonisins but this would add complexity and time to the analysis. Although the extraction efficiencies for fumonisins were less than optimal, the method is still useful for quantifying fumonisins in cereal samples.

3.3. Validation of method

The LOD for each mycotoxin analyzed by LC-MS/MS was determined to be the amount of mycotoxin that would produce at least 3:1 signal-to-noise in the standards for the quantifier ion. As the quantifier ion is the most abundant fragment, the qualifier ion fragments cannot be detected at the LOD and the identity of the analyte cannot be confirmed. Most of the LOD values for mycotoxins in solvent solutions ranged from 0.01 ng/mL to 1.0 ng/mL, compared with 0.05 to 10.0 ng/g for mycotoxins in rice, 0.05 to 5.0 ng/g in barley, 0.05 to 10.0 ng/g in oats and 0.1 to 5.0 ng/g in mixed-grain cereal (Table 3). The LOQ for each of the mycotoxins analyzed by LC-MS/MS method was defined as the amount of mycotoxin that would produce at least 10:1 signal-to-noise in the standards for the quantifier ion. Most of the mycotoxins were confirmed by the qualifier ion, where the intensity was at least 30% of the quantifier ion. For the mycotoxins in solution, most of the LOQ values ranged from 0.05 to 1.5 ng/mL, compared with

0.1 to 50.0 ng/g for mycotoxins in rice, 0.1 to 10.0 ng/g for mycotoxins in barley, 0.1 to 50.0 ng/g for mycotoxins in oats, and 0.5 to 10.0 ng/g in mixed-grain cereal (Table 3). Figure 2 shows the chromatograms for DON and OTA spiked into rice cereal at concentrations of 10 ng/g and 0.5 ng/g, respectively, and the corresponding signal-to-noise ratios for the mass transitions. Figure 3 shows the overlay of the MRM transitions for a naturally incurred barley infant cereal containing 5.03 ng/g DON and 1.05 ng/g AFB₂, and the isotopically labeled internal standards. The validation study indicates that the method is sensitive enough to detect mycotoxins in cereal foods at levels far below the regulatory limits set by the EC and the FDA.

3.4. Application to commercial infant cereal samples

Infants are a vulnerable group within the population with respect to mycotoxin exposure since a large portion of their diet is comprised of cereal grains, agricultural commodities known to potentially have a high occurrence of mycotoxin contamination (Duarte, Pena, & Lino, 2010). To obtain a better understanding of the types and concentrations of mycotoxins in different grain-based infant cereals purchased in the U.S., we conducted a survey to determine the concentration of 11 mycotoxins in 64 infant cereals of which 20 were rice-based, 8 were barley-based, 20 were oat-based, and 16 contained a mixture of grains (wheat, oats, barley, and rye). The stable isotope dilution assay LC-MS/MS method developed and validated as described previously (Varga et al., 2012), was applied to the analysis of the infant cereals.

Table 4 lists all of the results obtained from the analysis of the commercial infant cereal samples. Of the 64 infant cereals, 56 of them (78%) were contaminated with at least one mycotoxin. For individual types of cereals, at least one mycotoxin was detected in 11 out of 20 rice samples (55%), 6 out of 8 barley samples (75%), 16 out of 20 oat samples (80%), and 15 out

of 16 mixed-grain samples (94%). It should be noted that, of 56 positive samples, 29 (52%) were contaminated with more than two mycotoxins. Such co-contamination was most evident among oat-based cereals as 2-8 different mycotoxins were detected simultaneously from 12 out of 20 samples. Co-contamination was also commonly observed (9 out of 16 samples) in mixed grain cereals with 2-5 different mycotoxins found in each of these samples (results not shown).

None of the infant cereal samples analyzed in this survey exceeded the established regulatory limits (action or guidance levels) in the U.S. for aflatoxins, DON, and fumonisins; at present, no limits have been established for the other mycotoxins (OTA, ZEN, T-2 toxin, HT-2 toxin) evaluated in this survey (FDA, 2011). In contrast, a total of 21 samples exceeded the EU maximum limits for mycotoxins in baby foods; three cereals exceeded the EU limits for AFB₁ (0.1 ng/g), 16 samples were greater than the limit for OTA (0.5 ng/g), and one sample exceeded the EU limit for ZEN (20 ng/g). Among them, two mixed-grain infant cereal samples exceeded the EU maximum limits for both AF₁ and OTA.

Three out of 64 samples (5%) were contaminated with AFB₁ (one rice and two mixed-grain cereals) at concentrations ranging from 2.4 to 5.9 ng/g. AFB₂ was detected in 14 out of 64 samples (22%) in the range of 1.1 to 5.0 ng/g. While nine samples (15%) were contaminated with AFG₂ in the range of 0.4 – 1.7 ng/g, AFG₁ was not detected in these samples. The results of our study are similar to those obtained in a survey of infant cereals purchased in Spain for aflatoxins (Hernández-Martínez & Navarro-Blasco, 2010). In this study, five of 91 infant cereal products contained AFB₁ in concentrations higher than the EU limit of 0.1 ng/g, with two of these products containing 1.98 and 3.11 ng/g AFB₁. Our results and those reported by Hernández-Martínez and Navarro-Blasco (2010) differ somewhat from the results of other investigations of aflatoxin levels in infant cereal products. Tam, et al. (2006) found that 50% of

177 infant cereals purchased in Canada from 2002 to 2005 contained detectable (> 0.002 ng/g) amounts of AFB₁. Although the incidence of aflatoxin contamination in these foods was high, the concentrations found in most of the products were < 0.1 ng/g; two products contained > 0.1 ng/g AFB₁, and none of the cereals contained greater than 1 ng/g AFB₁. Other surveys of products obtained in the U.S. (Zhang, et al., 2014), Italy (Juan, Raiola, Mañes, & Ritieni, 2014), Spain (Beltrán, et al., 2011) and Portugal (Alvito, et al., 2010) found low incidence and concentrations of aflatoxins in infant cereal products and other baby foods. Dissimilarities in the results from these studies may in part be due to differences in the analytical methods used to detect and quantify aflatoxins in the products. The diversity in concentrations of aflatoxins may also be explained by the fact that the cereal ingredients were likely obtained from different geographical locations and at different times, both factors of which are known to affect fungal and mycotoxin contamination of food (Magan, Medina & Aldred, 2011).

OTA and DON have been reported to be the most commonly occurring mycotoxin contaminants in cereal and other grain-based foods (Duarte, et al., 2010; Lombaert, et al., 2003). Out of the 64 surveyed infant cereal samples, 10% of rice-based, 13% of barley-based, 55% of oat-based and 31% of mixed-grain based infant cereals were contaminated with OTA at concentrations ranging from 1.0 – 14.4 ng/g (Table 4). Similar incidences of contamination of baby cereals from Spain (Araguás, González-Peñas, & De Cerain, 2005), Italy (Beretta, et al., 2002; Juan, et al., 2014) and Canada (Lombaert, et al., 2003) were reported. In survey of breakfast cereals (not specifically described as infant foods) obtained in Malaysia, Soleimany et al. (2012) found that 70% of the samples were contaminated with OTA (0.1 – 5.76 ng/g). Soleimany et al. (2012) found OTA mostly in rice-based cereal products while Lombaert et al. (2003) noticed an even distribution of OTA among the oat, barley, and multi-grain cereals.

According to Rubert et al. (2012), OTA was detected in oat-based powdered baby food and in mixed-cereal liquid “ready-to-eat” baby food. In contrast, no OTA was found in a recent survey (10 total) of infant cereal products purchased in the U.S. (Zhang, et al., 2014).

DON (42 positive samples out of 64 total samples; 66%) was the most commonly detected mycotoxin in infant cereals analyzed in this limited survey, and concentrations ranged from 1.4 – 147 ng/g (Table 4). However, the concentrations of DON found in the cereal products did not exceed the FDA advisory level for DON in wheat products for human consumption (1000 ng/g) or the regulatory limits in the EU for DON in infant foods (200 ng/g). Nevertheless, it is important to recognize that DON represents the mycotoxin with the highest percentage of positive samples (35 % of rice-based, 75% of barley-based, 94% of oat-based and 88% of mixed-grain cereals) and highest concentrations in all types of matrices. Our results are similar to those reported in cereal products sampled from West Germany (Schollenberger, Suchy, Jara, Drochner, & Muller, 1999), Malaysia (Soleimany, et al., 2012) and Canada (Lombaert, et al., 2003). Schollenberger, et al. (1999) detected DON in 60% of surveyed baby foods (25 total) which included cereals, cookies and rusks (hard dry biscuits), and 56% of breakfast cereals (not infant foods) with positive samples in the range of 15.0 – 314 ng/g and 15 – 238 ng/g, respectively. Similarly, Soleimany, et al. (2012) also detected DON in 40 out of 100 all infant cereals at concentrations ranging from 12.0 ng/g to 113 ng/g (rice, wheat, barley, oat, maize). According to Lombaert, et al. (2003) who evaluated 367 cereal-based infant foods in Canada, DON was the most frequently detected mycotoxin (63% of all samples). Barley- and soy-based cereal products were the most frequently contaminated with DON, while no DON was detected in the few rice-based cereals that were analyzed. Jestoi, et al. (2004) reported that DON

was detected in 92% of the baby-food samples obtained in Finland and Italy; however, concentrations of DON in these products were typically below 100 ng/g.

Only one cereal sample (a barley infant cereal), was contaminated with FB₁ while five cereals (one barley, three oat, and one mixed grain) contained FB₂ (Table 4). Concentrations of FB₁ and FB₂ detected were below 10 ng/g, which is considerably lower than the advisory levels established by FDA (2000 ng/g total fumonisins for corn-based human foods) and the EU (200 ng/g total fumonisins for baby foods and infants). Our results are consistent with Rubert, et al. (2012) and Zhang, et al. (2014), both of whom reported low incidence and concentrations of fumonisins in infant cereals. However, Lombaert, et al. (2003) found a high incidence of fumonisin contamination in soy-based and multi-grain cereals.

In our study, six cereals (four oat-based and two mixed-grain cereals) contained detectable amounts of HT-2 toxin (concentrations from 2.4 – 9.6 ng/g), while eighteen out of 64 samples (28%) were contaminated with T-2 at concentration levels ranging from 0.4 to 3.6 ng/g. Overall, the results from our survey were consistent with those reported by Rubert, et al. (2012) for these toxins in commercial baby foods purchased in Spain.

ZEN was found primarily in oat and mixed-grain infant cereals in our study (Table 4). ZEN was detected in 21% of the infant cereals ranging from 0.5 – 32.0 ng/g. Among the 13 ZEN-contaminated cereals, one sample had ZEN levels above EU limit of 20 ng/g. At present, there are no regulatory limits in the U.S. for ZEN in foods. Similar to our findings, Lombaert, et al. (2003) reported that ZEN was detected in 33% of all samples. In that study, ZEN was present in soy and multi-grain cereals with mean values of 1.4 and 4.5 ng/g, respectively and not detected in oat cereals (Lombaert, et al., 2003).

4. Conclusions

A triple quadrupole mass spectrometer method operating in the ESI+ mode was developed and validated for the simultaneous determination of aflatoxins B₁, B₂, G₁, G₂, OTA, ZEN, DON, FB₁ and FB₂, T2 and HT2-toxins using a single-step extraction and a stable isotope dilution assay. The 11 mycotoxins were separated in less than 10 min on the HPLC, and the sensitivity of the method enable detection of the toxins at concentrations far lower than the regulatory limits in the U.S. and Europe. Application of this method on commercial infant cereals collected from the U.S. markets showed that 78% of infant cereals were contaminated with at least one of these mycotoxins. In particular, co-occurrence of multiple mycotoxins was found most frequently for oat-based infant cereals followed by mixed grain cereals. Although none of the cereals contain mycotoxin levels that exceeded the U.S. regulatory limits, 21 samples exceeded EU limits for mycotoxins including OTA, aflatoxin B₁, and ZEN. This survey is the first ever done specifically of infant cereals for the U.S. market. This study suggests that cereals intended for infants should be routinely monitored for mycotoxin content.

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538

539 **Figure Legends**

540

541 **Figure 1.** Overlay of the MRM transitions for unlabeled mycotoxins spiked into an oatmeal
542 cereal at 25 ng/g before extraction. The isotopically labeled internal standards are also shown.

543

544 **Figure 2.** Extracted ion chromatogram of DON and OTA spiked into infant rice cereal at a
545 concentration of 10 ng/g and the corresponding signal-to-noise value for the mass transitions.

546

547 **Figure 3.** Overlay of the MRM transitions for a naturally incurred barley infant cereal
548 containing 5.03 ng/g DON and 1.05 ng/g AFB₂. The isotopically labeled internal standards are
549 also shown.

550

551 **Table 1. MS/MS acquisition parameters for mycotoxins**

Analyte	<i>m/z</i> precursor ion	<i>m/z</i> product ion	Fragmentor (V)	Collision Energy (V)
Aflatoxin B ₁	313.1	285, 241	190	20
[¹³ C ₁₇]-Aflatoxin B ₁	330.1	301.1	180	20
Aflatoxin B ₂	315.1	287.1, 259	190	24
[¹³ C ₁₇]-Aflatoxin B ₂	332.2	303.1	190	24
Aflatoxin G ₁	329.1	311, 243	190	20
[¹³ C ₁₇]-Aflatoxin G ₁	346.1	212	180	44
Aflatoxin G ₂	331.1	313, 245.1	190	24
[¹³ C ₁₇]-Aflatoxin G ₂	348.1	259	175	28
Deoxynivalenol	297.3	248.9, 203	90	4, 12
[¹³ C ₁₅]-Deoxynivalenol	312.1	262.9	90	4
Fumonisin B ₁	722.4	352.3, 334.2	190	40
[¹³ C ₃₄]-Fumonisin B ₁	756.5	374.2	190	40
Fumonisin B ₂	706.4	336.3, 318.3	260	40
[¹³ C ₃₄]-Fumonisin B ₂	740.5	358.4	210	36
HT-2 toxin	442.2	263.1, 215	110	8
[¹³ C ₂₂]-HT-2 toxin	464.5	278.4	110	8
T-2 toxin	484.3	305, 215.2	110	8
[¹³ C ₂₄]-T-2 toxin	508.3	322	110	8
Ochratoxin A	404.1	358, 239, 193	140	8, 25, 45
[¹³ C ₂₀]-Ochratoxin A	424.2	250.2	120	24
Zearalenone	319.2	301.1, 283.1	80	4

552	$[^{13}\text{C}_{18}]$ -Zearalenone	337.3	301.4	110	8
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Table 2. Method performance and matrix effects

Mycotoxin	Spike level (ng/g)	Rice Cereal (% Recovery Internal Std ± RSD)	Barley Cereal (% Recovery Internal Std ± RSD)	Oat Cereal (% Recovery Internal Std ± RSD)	Mixed-Grain Cereal (% Recovery Internal Std ± RSD)
DON	12.5	83.0 ± 10.4	43.2 ± 3.4	116.4 ± 6.1	87.0 ± 10.6
	25.0	109.5 ± 14.2	92.2 ± 6.9	100.7 ± 3.0	124.7 ± 7.6
	50.0	109.8 ± 4.2	73.8 ± 2.3	98.2 ± 1.5	113.6 ± 3.8
AFB ₁	12.5	112.0 ± 6.0	100.1 ± 2.7	116.4 ± 4.2	116.8 ± 13.0
	25.0	119.3 ± 6.5	108.6 ± 7.7	115.8 ± 1.9	117.4 ± 2.3
	50.0	124.9 ± 5.7	115.2 ± 4.3	111.2 ± 3.7	122.2 ± 5.5
AFB ₂	12.5	100.3 ± 6.7	94.0 ± 4.1	113.5 ± 5.3	114.3 ± 5.6
	25.0	115.2 ± 3.7	103.5 ± 8.9	109.5 ± 1.1	118.6 ± 1.2
	50.0	117.7 ± 5.8	110.7 ± 4.2	108.4 ± 2.3	123.4 ± 7.9
AFG ₁	12.5	132.3 ± 19.3	79.9 ± 7.5	112.0 ± 2.3	125.1 ± 2.1
	25.0	122.4 ± 9.7	85.3 ± 5.1	112.6 ± 3.2	125.1 ± 3.7
	50.0	119.1 ± 2.7	102.9 ± 7.1	107.1 ± 1.6	120.3 ± 4.3
AFG ₂	12.5	120.6 ± 6.1	95.9 ± 1.5	118.5 ± 2.2	117.1 ± 4.9
	25.0	123.9 ± 3.0	103.9 ± 8.8	111.8 ± 3.2	122.7 ± 3.7
	50.0	133.9 ± 10.0	116.9 ± 6.2	109.7 ± 2.0	121.6 ± 2.8
OTA	12.5	100.1 ± 31.0	90.6 ± 3.1	88.3 ± 4.6	79.3 ± 10.1
	25.0	93.3 ± 18.0	102.0 ± 9.2	86.0 ± 4.7	83.7 ± 4.7
	50.0	91.0 ± 3.7	123.4 ± 5.0	86.2 ± 2.3	84.3 ± 5.4
ZEN	12.5	102.5 ± 6.0	110.2 ± 10.9	102.8 ± 6.1	81.6 ± 25.6
	25.0	86.1 ± 2.6	108.3 ± 7.6	116.2 ± 11.2	97.2 ± 23.8
	50.0	95.8 ± 10.0	138.0 ± 4.7	105.6 ± 6.5	85.2 ± 24.8
FB ₁	12.5	32.6 ± 36.8	43.7 ± 27.9	55.1 ± 6.0	132.9 ± 18.0
	25.0	60.6 ± 16.4	29.0 ± 19.0	60.2 ± 17.5	128.8 ± 6.6
	50.0	59.8 ± 11.0	27.9 ± 24.5	45.3 ± 19.6	119.2 ± 8.9
FB ₂	12.5	65.9 ± 24.8	21.8 ± 56.6	77.8 ± 15.8	72.7 ± 10.2
	25.0	96.4 ± 21.5	26.3 ± 25.8	69.5 ± 12.4	68.4 ± 2.4
	50.0	81.3 ± 15.5	41.0 ± 11.9	60.4 ± 2.4	60.5 ± 13.7
HT-2 toxin	12.5	95.7 ± 15.8	117.8 ± 16.5	119.2 ± 14.3	132.9 ± 18.0
	25.0	125.5 ± 15.2	118.3 ± 13.7	129.5 ± 6.9	128.8 ± 6.6
	50.0	132.2 ± 7.7	122.2 ± 3.3	119.9 ± 3.5	119.2 ± 8.7
T-2 toxin	12.5	129.7 ± 8.8	77.7 ± 6.7	109.8 ± 3.4	113.2 ± 4.8
	25.0	131.6 ± 8.0	77.3 ± 7.2	102.4 ± 6.9	120.2 ± 6.9
	50.0	127.9 ± 8.7	97.0 ± 9.7	103.8 ± 4.1	118.3 ± 6.6

Table 3. Linearity, limits of detection and limits of quantitation

Mycotoxin	Linear Range (ng/ml)	Standard solution		Rice		Barley		Oat		Mixed	
		LOD (ng/mL)	LOQ (ng/mL)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)	LOD (ng/g)	LOQ (ng/g)
AFB ₁	0.1 - 100	0.025	0.1	0.1	0.5	0.1	0.5	0.5	1.0	0.1	0.5
AFB ₂	0.1 - 100	0.01	0.05	0.5	1.0	0.5	1.0	0.5	1.0	0.1	0.5
AFG ₁	0.1 - 100	0.01	0.05	0.1	0.5	0.1	0.5	0.5	1.0	0.1	0.5
AFG ₂	0.1 - 100	0.01	0.05	0.5	1.0	0.1	0.5	0.5	1.0	0.1	0.5
DON	0.1 - 100	0.1	0.5	10.0	50.0	1.0	5.0	10.0	50.0	0.5	1.0
HT-2 toxin	0.1 - 100	0.1	1.0	1.0	5.0	1.0	5.0	0.5	1.0	1.0	5.0
T-2 toxin	0.1 - 100	0.01	0.05	0.05	0.1	0.05	0.1	0.05	0.1	0.1	0.5
FB ₁	0.1 - 100	0.5	1.5	5.0	10.0	5.0	10.0	1.0	5.0	5.0	10.0
FB ₂	0.1 - 100	0.125	0.25	1.0	5.0	1.0	5.0	1.0	5.0	1.0	5.0
OTA	0.1 - 100	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.5	0.1	0.5
ZEN	0.1 - 100	0.025	0.1	*	*	5.0	10.0	5.0	10.0	*	*

* Unable to determine LOD and LOQ for ZEN because of interference peaks

Table 4. Mycotoxin contamination of infant cereals in the U.S. market

Mycotoxin	Type of cereal			
	Rice	Barley	Oat	Mixed-grain
DON	7/20 (1.4 – 55.0)*	6/8 (3.6 – 125.6)	15/20 (2.5 – 146.5)	14/16 (4.4 – 48.2)
AFB ₁	1/20 (5.9)	0/8 (ND)	0/20 (ND)	2/16 (2.4 – 5.6)
AFB ₂	4/20 (1.1 – 5.0)	1/8 (1.1)	5/20 (1.1 – 2.6)	4/16 (1.4 – 1.7)
AFG ₁	0/20 (ND)	0/8 (ND)	0/20 (ND)	0/16 (ND)
AFG ₂	0/20 (ND)	0/8 (ND)	8/20 (0.4 – 1.7)	1/16 (1.0)
OTA	2/20 (1.3 – 1.4)	1/8 (14.4)	11/20 (0.6- 11.8)	5/16 (1.0 – 2.7)
ZEN	1/20 (9.0)	0/8 (ND)	9/20 (1.2 – 13.6)	3/16 (0.5 – 32.1)
FB ₁	0/20 (ND)	1/8 (6.2)	0/20 (ND)	0/16 (ND)
FB ₂	0/20 (ND)	1/8 (7.5)	3/20 (1.5 – 15.8)	1/16 (2.3)
HT-2 toxin	0/20 (ND)	0/8 (ND)	4/20 (2.4 – 9.6)	2/16 (5.6 – 6.4)
T-2 toxin	3/20 (1.1 – 3.6)	1/8 (1.5)	8/20 (0.6 – 2.3)	6/16 (0.4 – 1.2)

*Number of cereal samples containing >LOQ for each mycotoxin/total number of cereals surveyed (range of contamination, ng/g)

ND = not detected

Figure 1.

404.1 → 239.0 S/N 11.26

297.3 → 248.9 S/N 2.18

Figure 2.

Figure 3.