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Original Research Article

Trichothecenes in breakfast cereals from the Spanish retail market

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ABSTRACT

Deoxynivalenol (DON), nivalenol (NIV), 3-acetyl-DON (3-AcDON), 15-acetyl-DON (15-AcDON) and fusarenone X (Fus-X) were analysed in 148 breakfast cereal samples collected from the Spanish retail market. The samples were taken from the products most commonly consumed by the Spanish population. Major ingredients included corn, wheat and rice, alone or mixed. The trichothecenes were extracted with acetonitrile:water (84:16, v/v). The extracts were cleaned by means of Mycosep 227 columns. For analysis gas chromatography–mass spectrometry (GC–MS) after derivatisation to trimethylsilyl ethers was utilised. Mean recovery values, obtained from different matrices of cereal samples spiked with trichothecenes ranged from 69% to 110% with relative standard deviation lower than 10%. The estimated limits of detection and quantification, calculated at a signal-to-noise ratio of 3:1 and of 6:1, respectively, were between 8.90 and 14.7 μg/kg, and between 15.2 and 23.6 μg/kg, respectively. DON was the most frequently detected mycotoxin and was usually present at the highest concentration. NIV and Fus-X were detected in 4 and 2 samples, respectively; 3- and 15-AcDON were not detected. The calculated dietary intake was compared to tolerable daily intake (TDI) values. The survey demonstrated a regular occurrence of low levels of trichothecenes in breakfast cereals on the Spanish market.

1. Introduction

Trichothecene mycotoxins are secondary metabolites produced by several fungal genera, especially *Fusarium*, which are known to infect agricultural crops throughout the world and in particular cereals, such as wheat, maize, barley, oats, rice and rye. These mycotoxins are chemically stable to heating and survive food processing (Cetin and Bullerman, 2006; Bullerman and Bianchini, 2007). Consequently, a regular contamination can be expected for cereal-based foods, posing a potential risk to human health.

Over 180 trichothecenes are known (Pestka, 2007); deoxynivalenol (DON) is the most commonly found all over the world. Other trichothecenes found in cereals and cereal products are nivalenol (NIV), 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), fusarenon X (Fus-X), T-2 toxin (T-2), HT-2 toxin (HT-2), diacetoxyscirpenol (DAS) and neosolaniol (NEO) (Placinta et al., 1999; JECFA, 2000, 2001). Trichothecenes at acute high doses cause a wide range of toxic effects including vomiting, diarrhoea and haemorrhage (Pestka, 2007), whereas chronic intake of small amounts of trichothecenes leads to an increased susceptibility to infectious diseases as a result of the suppression of the immune system (Schlatter, 2004; Pestka, 2007) and other symptoms as anorexia, anaemia, neuroendocrine changes and immunologic effects (Pestka and Smolinski, 2005; Pestka, 2007). They have been found to inhibit synthesis of protein, DNA and RNA (Visconti et al., 1991; Rotter et al., 1996).

Health risk associated with human exposure to trichothecenes is widely recognised and depends upon the degree they are consumed in a diversified diet. In order to protect health consumers, the European Commission has legislated maximum levels for trichothecenes in cereal grains, flours, and cereal-based products intended for human and animal consumption (Anon., 2006; European Commission, 2006b). Likewise, the Scientific Committee on Food (SCF) of the European Commission established a tolerable daily intake (TDI) for DON (SCF, 1999), NIV (SCF, 2000), and for the sum of T-2 and HT-2 toxins (SCF, 2002) (1, 0.7 and 0.06 μg/kg of bodyweight and day, respectively).

In Spain, different cereal-based foods have been investigated for trichothecenes and zearalenone in recent years (Cerveró et al., 2007; Castillo et al., 2008), with mainly corn-based foods analysed. Cano-Sancho et al. (2011) studied the occurrence of trichothecenes in foodstuffs in the Catalonian market. All of these surveys indicate that *Fusarium* mycotoxins are common contaminants in the Spanish human diet. Generally, the mycotoxins analysed occur at low concentrations, with DON being the most frequent toxin. The hazards associated with chronic exposition make continued

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surveillance necessary concerning the presence of trichothecenes in these types of foods. Moreover, more reliable data on the daily intake of mycotoxins are required in order to contribute in accurate exposure assessment studies. Thus, the present study contributes to increase the awareness of the presence of these toxins in the Spanish diet, expanding the number of trichothecenes analysed and grain spectrum in comparison with Castillo et al. (2008), as well as their trend over a given period and in different regions. To this end, modifications of the analytical methods already reported (Eskola et al., 2001; Castillo et al., 2008) have been carried out and applied to the simultaneous detection of five trichothecenes (DON, NIV, 3-AcDON, 15-AcDON and FUS-X) in different matrices of breakfast cereals in fulfilment with legislation requirements. Method performance characteristics, such as limits of detection and quantification, recoveries, linearity and precision have been evaluated and presented.

2. Materials and methods

2.1. Reagents

Trichothecene standards were purchased from Sigma–Aldrich (Madrid, Spain); according to the manufacturer, purity was 96% or higher. Stock and working trichothecene standard solution mixture of DON, 3-AcDON, 15-AcDON, NIV and Fus-X were prepared by appropriate dilution in acetonitrile to assess the linearity, accuracy and precision of method. An internal standard solution of 10 μg/mL in hexane was prepared from the NEO standard. These solutions were kept at −20 °C when not in use. The derivatisation reagent N-trimethylsilylimidazole–N,O-bis(trimethysilyl)acetamide–trimethylchlorosilane (TMSI–BSA–TMCS) (3:3:2) (Tri-Sil TBT) was purchased from Supelco (Madrid, Spain). All solvents (acetonitrile, and hexane) were of HPLC grade and purchased from J.T. Baker (Deventer, Holland). The water used was purified with a Millipore Milli-Q Plus system (Millipore, Billerica, MA, USA). Potassium dihydrogen phosphate (KH_2PO_4) and sodium hydroxide (NaOH) used to prepare phosphate buffer, were purchased from Panreac (Barcelona, Spain). The Mycosep™ 227 columns were purchased from Romer Labs, Inc., USA.

2.2. Samples

A total of 148 packaged samples of commercial breakfast cereals were randomly collected during 8 months (February–September, 2009) from supermarkets and retail outlets located in the province of Valencia (Spain). A wide range of brands were covered to ensure that the survey was representative of the products available to Spanish consumers. For each commercial sample, at least 0.50 kg (0.50–0.75 kg, 2–3 commercial packages, respectively) was collected and finely ground at particle size about 1 mm, using an Osterizer mill (Oster Co., USA) for 3 min. Each commercial ground sample was thoroughly mixed before taking subsample for analysis and then kept at −20 °C until analysis.

Samples included the following major ingredients, alone or mixed: corn, wheat and rice. The samples were categorised as corn (n = 62), wheat (n = 27), rice (n = 13) and multigrain-based (n = 46). Corn, rice and wheat were typically listed as ingredients in multigrain-based samples. Of the 46 multigrain samples, 12 contained also oats. In general, a total of 88 samples contained additional ingredients, such as chocolate, honey, vanilla and sugar.

2.3. Extraction and clean-up

The analytical method used for trichothecenes was a modification of the method presented by Eskola et al. (2001) and Castillo et al. (2008). A 25 g sample of finely ground cereal was homogenised with 100 mL of acetonitrile:water (84:16, v/v) for 3 min, using an UltraTurrax T 25 (Jankle & Kunkel IKA-Labortechnik, Staufen, Germany) at 1832.6 rad/s. The extracted sample was then filtered through a filter paper (Whatman grade 2V) from Whatman (Maidstone, UK). The filtrate was defatted with n-hexane (2× 20 mL). A total of 8 mL defatted extract was purified by MycoSep 227 column, following the instructions of the manufacturer. Four to five millilitres of purified extract were collected, and the procedure was repeated with 8 mL of acetonitrile:water (84:16, v/v) in order to increase the recovery of the more polar compounds (Jestoi et al., 2004). Both fractions were combined and evaporated to dryness under a gentle stream of nitrogen at 50 °C.

2.4. Derivatisation

The derivatisation procedure is a modification of previously used in our laboratory (Castillo et al., 2008). A 50 μL volume of the derivatisation reagent (Tri-Sil TBT) was placed into a vial containing the dry residue. The mixture was allowed to react for 30 min at 80 °C. After cooling, the derivatised sample was diluted to 175 μL with hexane and mixed thoroughly. The hexane was then washed with 1 mL of phosphate buffer (0.1 M, pH 7.2) and, finally, 75 μL of previously derivatised internal standard (NEO) (final concentration 3 μg/mL) was added and shaken. After the separation of two layers, the upper hexane layer (250 μL) with the trimethylsilyl derivatives was transferred to an autosampler vial for GC–MS.

2.5. GC–MS analysis of trichothecenes

Separation and quantification of five trichothecenes were performed using a GC–MS with electron impact ionisation. The GC was an Agilent model 6890N equipped with an Agilent 7683 B autosampler injector and coupled to an Agilent 5975 quadrupole mass selective detector. Separation was achieved on the HP-5 capillary column (30 m × 0.25 mm I.D., 0.25 μm film thickness) from Agilent Technologies (Waldbrom, Germany).

The temperature of injection port was 270 °C and the mode of injection was splitless. The carrier gas was helium at 1.8 mL/min flow rat and injection volume was of 2 μL in splitless mode. The initial GC oven temperature was 80 °C, and held for 1 min. It was increased from 80 to 240 °C at a rate of 30 °C/min. At a 240 °C, the heating rate was changed to 5 °C/min. Final temperature was 280 °C. The mass spectrometer worked at electron impact mode at 70 eV. Interface, ion source and quadrupole temperatures were 300, 230 and 150 °C, respectively. Mass scan range covered from 35 to 500 m/z. All spectra were monitored with a total ion current (TIC) and selected ion monitoring (SIM) modes. In the SIM mode, the spectrum of each mycotoxin was analysed at least one specific molecular ion selected for the target. The retention time and the monitored fragment ions are given in Table 1. Signals were processed by Chemstation software (Agilent).

A total ion chromatogram of a silylated standard solution of trichothecenes is presented in Fig. 1.

2.6. Quality control of procedure

Validation experiments established the performance characteristics of the method. The following parameters were investigated: linearity, limits of detection and quantification, recovery and repeatability. Linearity was established by injecting increasing concentrations of the mixed standard solutions (0.1, 0.5, 1.0, 2.0, 3.0 and 5 μg/mL). Standard curves were generated by linear regression of ion abundance of each toxin versus concentration. The detection and quantification limits for all trichothecenes were calculated at a signal-to-noise ratio of 3:1 and of 6:1, respectively.