Original research article

Trichothecenes in breakfast cereals from the Spanish retail market

Rosa Montes, Ramón Segarra and María-Ángeles Castillo*

Departamento de Biotecnología, Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural, Universidad Politécnica de Valencia, Camino de Vera, 14, 46022 Valencia, Spain.

* Corresponding author: María-Ángeles Castillo, Departamento de Biotecnología, Escuela Técnica Superior de Ingeniería Agronómica y del Medio Natural. Universidad Politécnica de Valencia, Camino de Vera 14, 46022- Valencia, Spain

Fax: 34-963877429. Tel.: + 34-963877423.

E-mail address: mcastill@btc.upv.es

Abstract

Deoxynivalenol (DON), nivalenol (NIV), 3-acetyl-DON (3-AcDON), 15-acetyl-DON (15-AcDON) and fusarenone X (Fus-X) were analyzed 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%–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.

Keywords: Mycotoxin contamination in food; Deoxynivalenol; Nivalenol; Fusarenon-X; Trichothecenes; GC-MS; Breakfast cereals; Spanish foods; Public health risk; Food safety; Food analysis; Food composition

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; JEFCA,

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 per 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 analyzed. 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 analyzed occur at low concentrations, with DON being the most frequent toxin. The hazards associated with chronic exposition make continued surveillance necessary concerning the presence of trichothecenes in these type of foods. Moreover, more reliable data on daily intake of mycotoxins are required in order to contribute in

accurate exposure assessment studies. Thus, the present study contributes to increasing the awareness of the presence of these toxins in the Spanish diet, expanding the number of trichothecenes analyzed 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 Material 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 ºC 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^{TM} 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 ºC 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, twelve 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 1,832.6 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 x 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, 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 ºC.

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 ºC. 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 shaking. After 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 a Agilent 7683 B autosampler injector and coupled to a Agilent 5975 quadrupole mass selective detector. Separation was achieved on the HP-5 capillary column (30 m x 0.25 mm I.D., 0.25 µm film thickness) from Agilent Technologies (Waldbrom, Germany).

The temperature of injection port was 270 ºC 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 ℃, held for 1 min. It was increased from 80–240 ℃ at a rate of 30 ℃/min. At a 240 ℃, the heating rate was changed to 5 ℃/min. Final temperature was 280 ℃. The mass spectrometer worked at electron impact mode at 70 eV. Interface, ion source and quadrupole temperatures were 300, 230 and 150 ℃, respectively. Mass scan range covered from 35–500 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 analyzed 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 Figure 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. Recovery studies were carried out by spiking in triplicate mycotoxin-free samples at two levels of each mycotoxin (50 and 500 µg/kg), according to the method described previously (Castillo et al., 2008).

2.7 Statistical analyses

The statistical analyses were performed with STATGRAPHICS PLUS software (Statistical Graphics Corp. version 5.1) and SPSS (version 14.0.1 for Windows, SPSS Inc., Chicago).

Data of toxin contents were checked for normal distribution (Shapiro-Wilks test) and variance homogeneity (Cochran test). Kruskal-Wallis test was used when data not fulfil to normal distribution. Fisher's Test was employed to establish significant association between presence of mycotoxin and certain characteristics of the samples.

3 Results and discussion

The method described has allowed for the simultaneous detection and quantification of five trichothecenes in a short time (Figure 1) in the 148 breakfast cereal samples collected in the province of Valencia (Spain).

3.1 Analytical quality control

Mycotoxins were calibrated by an internal calibration procedure. Calibration curves were linear over the range studied, showing correlation coefficients of > 0.99. The estimated limits of detection and quantification were between 8.90 and 14.7 µg/kg, and between 15.2 and 23.6 µg/kg, respectively (Table 2). These results are in agreement with those reported in the literature (Castillo et al., 2008; Edwards, 2009; González-Osnaya et al., 2011).

The recoveries and relative standard deviation (RSDr) of mycotoxins in the different matrices at two different spiking levels are summarised in Table 3. Recovery rates at the lower spiking level were between 90% for DON and 110% for 3-AcDON. At the higher spiking level, the recoveries varied between 69% for NIV and 108% for DON. The lowest recoveries corresponded to NIV at the higher spiking level (500 µg/kg) for all matrices. Low NIV recoveries from cereals have been previously reported (Langseth and Rundberget, 1998; Berthiller et al., 2005). As reported by Jestoi et al. (2004), the recoveries of NIV were increased significantly by rinsing the Mycosep column with eluent, but it remained below 80% at the higher spiking level (data not shown). Weingaertner et al.

(1997) and Krska et al. (2001) reported the adsorption of NIV on polar sites in the packing material of Mycosep columns. Recently, higher NIV recoveries have been reported, by using clean-up methods other than Mycosep (Lattanzio et al., 2007).

The RSDr (%) of the mean recoveries for all trichothecenes ranged from 0.6–9.7% at the lower spiking level and from 1.0–9.2% at the higher spiking level. The recovery and RSDr values for each mycotoxin highlight the good repeatability of the method, specifically for DON. They are in accordance with Commission Directive No. 401/2006 for methods of analysis of mycotoxins in foodstuffs (European Commission, 2006a).

In comparison with prior analytical method used in our laboratory (Castillo et al., 2008), a method optimisation has been achieved. The total analysis time was reduced, first in sample preparation and extraction; secondly, the applied GC conditions yielded a successfully simultaneous determination in a shorter time. Moreover, the use of mass spectrometer allowed the unambiguous identification of mycotoxins and its presence in the samples.

3.2   Occurrence of trichothecenes in breakfast cereal samples

In this study, 148 breakfast cereal samples were examined for five trichothecenes using a proven reliable method. Toxins contents between the detection and quantification limit were calculated as the average of both limits. DON was the most commonly detected mycotoxin (Table 4); it was found above the detection limit in 25.7% of all samples with a median content of 74.4 µg/kg in positive samples. According to cereal-base, the incidence of DON varied between 0%, 18.5%, 30.4% and 30.6% for rice-, wheat-, multigrain- and corn-based samples, respectively. No significant association was detected between the incidence of DON and type of cereal base ($P = 0.087$). The DON contamination levels fluctuated between 31.5 and 468 µg/kg in positive samples analyzed. The greatest levels of

DON were found among the wheat-based samples with quantities (429 and 468 µg/kg) very close to the established limit in Europe (500 µg/kg) (EC, 2006b). Out of the 38 samples that showed presence of DON, nine samples contained below 50.0 µg/kg and other nine samples were above of 100 µg/kg, while the more numerous group (20 samples) showed contents in the range of 51.0-100 µg/kg. No significant differences were found among the median DON contents for all commodities ($P > 0.05$). In Figure 2 a total ion chromatogram of a naturally contaminated sample with DON is shown.

Twenty-four DON positive samples were products with chocolate, honey, vanilla or sugar. The statistical analysis was not significant ($P > 0.05$) for DON median levels and incidence between this sweetened and no sweetened samples.

Corn, rice and wheat were typically listed as ingredients in multigrain-based samples. Of the 46 multigrain samples, twelve contained also oats; DON was detected in eight of them. Fisher exact test showed a strong association between DON occurrence and presence/absence of oats ($P = 0.0030$).

NIV was detected in 2.7% of all samples analyzed, corresponding to three corn- and one wheat-based cereal samples, with a median content of 15.1 µg/kg of positive samples. The detected levels in corn-based samples were below quantification limit. The highest level was 56.7 µg/kg detected in the wheat-made sample.

FUS-X was only detected in two multigrain-based cereal samples, one of them with content below quantification limit. No samples analyzed were positive for acetyl derivatives of DON.

Results showed the co-occurrence of DON and FUS-X toxins at concentrations of 51 and 42 µg/kg, respectively in one multigrain sample with bran; and 49.1 and 15.1 µg/kg, of

DON and NIV respectively, in one corn-made cereal sample, representing an occurrence of 0.7%. Previous studies have also reported low percentages of trichothecene co-occurrence in maize samples (Cavaliere et al., 2005) and wheat-based samples (Schollenberger et al., 2005; Lattanzio et al., 2007).

The levels of trichothecenes detected in breakfast cereals in the present study are generally in accordance with previous reports from Spanish market in which the incidence and levels of trichothecenes are generally low. In the first study carried out in Spain, a range of 28.3-195 µg/kg of DON was reported for corn made foods (Cerveró et al., 2007). Castillo et a. (2008) reported concentrations of 30-121 µg/kg of DON and 51-107 µg/kg of NIV in corn-made breakfast cereals, with occurrences of 40% and 11%, respectively. More recently, in a wider study, Cano-Sancho et al. (2011) found higher occurrences of DON (74-79%) in similar samples, especially breakfast cereals and corn snacks; median contents fluctuated between 93–157 µg/kg. However, higher contents of DON were reported in wheat based samples (pasta and bread) and in one sample of corn flakes, achieving levels above 500 µg/kg. These findings are similar as those found in a recent Spanish study carried out by González-Osnaya et al. (2011) in Valencia City on bread and pasta samples, where DON was detected in 28% and 62% of the samples, respectively. None of the samples exceeded the maximum permitted limit, 500 and 750 µg/kg for bread and pasta, respectively (European Commission, 2006b).

In our study, the incidence of DON was relatively low compared to certain reports from European countries, like 64% reported in Italy (Cirillo et al., 2003a,b), 67% in Germany (Schollenberger et al., 2005), 56% in France (SCOOP, 2003), even in United Kingdom (35%) (FSA, 2005). More recently, breakfast cereals have been surveyed for *Fusarium*

toxins in Canada (Roscoe et al., 2008): DON was also the predominant mycotoxin with a total incidence of 46%, ranging from 3% in rice-based to 72% in wheat-based cereals.

The median DON levels detected in the present study were similar to those found in the studies previously mentioned but lower than those registered in samples surveyed in the United Kingdom (FSA, 2005) or in France (SCOOP, 2003) in which the highest DON concentration (27,500 µg/kg) was found in breakfast cereals. With regard to more recent studies, only one sample of corn flakes reported by Cano-Sancho et al. (2011) in Spain, and 3 breakfast cereal samples in the Canadian study (Roscoe et al., 2008) exceeded the EU legally limit. Conversely DON contamination detected in the present study is greater than that reported by Martins et al. (2008) where DON was not detected in any of the 105 corn-based samples. This lower occurrence could be explained among other things by the relatively high detection limits estimated in this study (100 µg/kg). The relative low levels and percentages found in the present data may be attributed, among others, to a different origin of basing cereals and environmental conditions; it is well known that *Fusarium* contamination and toxin production is more likely under wet and cold weather conditions of Northern and Central European countries.

Most of all, these surveys have demonstrated that DON is the predominant trichothecene found in cereal-based foods, mainly in corn and wheat-based. This is consistent with results described here. Moreover, the higher levels of DON have been detected in wheat-based samples, similar to research by Martins and Martins (2001) in Portugal, Roscoe et al. (2008) in Canada, and Fan et al. (2009) in China. By contrast, none of the mycotoxins tested were detected in rice-based samples. Similar results to these were reported by Lombaert et al. (2003) and Roscoe et al. (2008). Mycotoxin contamination is less common for rice than for many other cereals (Tanaka et al., 2007). During cultivation the *Fusarium*

infection is less likely; however, rice storage conditions can be an ideal substrate for other mycotoxigenic fungi (Reiter et al., 2010), i.e. *Aspergillus* or *Penicillium*, aflatoxins, ochratoxin, citrinin producers.

With regard to NIV, incidence (ca. 3%) was lower than that found previously in Spain (4%) (Castillo et al., 2008) but greater than registered on Canadian breakfast cereals (0.6%) (Roscoe et al., 2008). It is interesting to point out that in an earlier Spanish study all samples were corn-made. Considering only the corn-samples studied herein, the incidence of NIV was slightly superior (4.8%).

Few studies have explored the FUS-X contamination of cereal-based foods. Cavaliere et al. (2005) detected this mycotoxin in 13% of maize samples collected from different farms located in Italy. No sample was found to contain FUS-X in cereal-based foods or wheat flour samples analyzed in Germany (Schollenberger et al., 1999, 2002, 2005). This is the first report of detection FUS-X in Spanish foods, which was present in two multigrain cereal samples containing wheat, rice and bran.

Despite the fact that DON and its acetylated derivatives – 3-and 15-AcDON – are frequently found together in cereal-based products (Eriksen and Petterson, 2004; González et al., 2008; Edwards, 2009), none of the samples analysed here contained acetyl derivatives. These acetylated forms of DON co-occur with DON at much lower levels (Pestka, 2010) and when DON is present at a high concentration (Placinta et al., 1999).

Different revised works have shown that mycotoxin levels vary between different survey areas even at different times in the same area. This may to be attributed to different harvest, origin of cereals and climate conditions (Langseth and Elen, 1997; Müller et al., 1997a,b, 2001; Scott, 1997) in addition to the related aspects to storage conditions (Viquez et al., 1996). FUS-X occurs more frequently in the warmer and subtropical climates in

corn, oats and wheat (Weidenbörner, 2000) and NIV more often in years with dry and warm conditions (Yazar and Omurtag, 2008).

3.3   Exposure assessment of trichothecenes

The exposure to analysed toxins has been estimated and compared to Tolerable Daily Intake (TDI) proposed by Scientific Committee on Food (SCF) of the European Commission, in order to assess the risk to public health from consumption of breakfast cereals by Spanish people. According to the Spanish Ministry of Agriculture, Fisheries and Food (MAPA), the Spanish population consumes 18.6 g of breakfast cereals per day, which correspond to 3.58 kg/person/year. Table 5 shows the calculated human DON, NIV and FUS-X dietary intake, according to our results and MAPA. DON and NIV dietary intake were compared with the TDI levels (1µg/kg bw/day for DON and 0.7 µg/kg bw/day – temporary TDI for NIV), and expressed as a percentage. The intake of DON ranged from 0.01–0.15 µg/kg bw/day, and 0.02–0.35 µg/kg bw/day for adults and children, respectively. The intake of NIV fluctuated from 0.005-0.018 µg/kg bw/day for adults, and 0.011-0.042 µg/kg bw/day for children population. Finally, the range of intake of FUS-X was 0.004-0.013 µg/kg bw/day, and 0.009-0.032 µg/kg bw/day for adult and children consumers, respectively. Compared with media values, the highest detected DON levels, corresponding to wheat-based samples, represent an increment of both intake and percentages of TDI (13–15 % of TDI for adults and 32–35% for children). These values, although they comply with the current European legislation, must be taken into account, particularly for vulnerable populations such as children. Breakfast cereals represent only a part of the diet, so a higher consumption of this cereal is expected, and consequently these intake values could be higher.

4   Conclusions

Our results indicate that mycotoxin contamination in breakfast cereal samples of the Spanish market is present at levels far below the guidelines. DON was the most frequent, with the highest mean content detected in wheat-based samples, two of them showing levels close to the established limits. The NIV levels were low; FUS-X was detected in only two samples, and acetyl derivatives of DON were not detected in any of the samples. This is the first report of detection FUS-X in Spanish foods. The results obtained in this study added to previous ones (as mentioned above) contribute to increased knowledge on mycotoxin occurrence, particularly trichothecenes, in cereal products on the Spanish market. On the other hand, data on the estimated daily intake testify a possibility of chronic intake of mycotoxins, specifically DON. Thus, detection of mycotoxins confirms the importance of continued surveillance in Spain, both for raw materials and foods.

Acknowledgment

References

Anon. (2006). Commission recommendation on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. *Official Journal of the European Union L, 229,* 7-9.

Berthiller, F., Schuhmacher, R., Buttinger, G, Krska R.. (2005). Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize

by high performance liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A, 1062,* 209-216.

Bullerman, L. B., Bianchini, A. (2007). Stability of mycotoxins during food processing. *International Journal of Food Microbiology, 119,* 140-146.

Cano-Sancho, G., Valle-Algarra, F. M., Jiménez, M., Burdaspal, P., Legarda, T. M., Ramos, A. J., Sanchis, V., Marín, S. (2011). Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). *Food Control, 22,* 490-495.

Castillo, M. A., Montes, R., Navarro, A., Segarra, R., Cuesta, G., Hernández, E. (2008). Occurrence of deoxynivalenol and nivalenol in Spanish corn-based food products. *Journal of Food Composition and Analysis, 21,* 423-427.

Cavaliere, C., D'Ascenzo, G., Foglia, P., Pastorini, E., Samperi, R., Laganà, A. (2005). Determination of type b trichothecenes and macrocyclic lactone mycotoxins in field contaminated maize. *Food Chemistry, 92,* 559-568.

Cerveró, M. C., Castillo, M. A., Montes, R., Hernández, E. (2007). Determination of trichothecenes, zearalenone and zearalenols in commercially available corn based foods in Spain. *Revista Iberoamericana de Micología, 24,* 52-55.

Cetin, Y., Bullerman, L. B. (2006). Confirmation of reduced toxicity of deoxynivalenol in extrusion-processed corn grits by the MTT bioassay. *Journal of Agricultural and Food Chemistry, 54,* 1949-1955.

Cirillo, T., Ritieni, A., Galvano, F., Amodio-Cocchieri, R. (2003a). Natural co-occurrence of deoxynivalenol and fumonisins B_1 and B_2 in Italian marketed foodstuffs. *Food Additives and Contaminants, 20,* 566-571.

Cirillo, T., Ritieni, A., Visone, M., Amodio-Cocchieri, R. (2003b). Evaluation of

conventional and organic Italian foodstuffs for deoxynivalenol and fumonisins B_1

and B_2. *Journal of Agricultural and Food Chemistry, 51,* 8128-8131.

Edwards, S. G. (2009). *Fusarium* mycotoxin content of UK organic and conventional

wheat. *Food Additives and Contaminants: Part A, 26,*496-506.

Eriksen, G. S., Pettersson, H. (2004). Toxicological evaluation of trichothecenes in animal

feed. *Animal Feed Science Technology, 114,* 205-239.

Eskola, M., Boonzaaijer, G., van Osenbruggen, W. A., Rizzo, A., Tijmensen, G. (2001). A

study of the suitability of gas chromatography-electron capture detection for the

analysis of deoxynivalenol in cereals. *Mycotoxin Research, 16,* 73-90.

European Commission. (2006a). Commission Regulation (EC) No. 401/2006 of 23

February 2006. Laying down the methods of sampling and analysis for the official

control of the levels of mycotoxins in foodstuffs. *Official Journal of the European*

Union L, 70, 12-34.

European Commission (2006b). Commission Regulation (EC) No. 1881/2006 of 19

December 2006. Setting maximum levels for certain contaminants in foodstuffs.

Official Journal of the European Union L, 364, 5-24.

Fan, P., Zhang, Y., Zhou, M., Chen, C., Wang, J. (2009). Incidence of trichothecenes in

wheat-based foods from China. *International Journal of Environmental Analytical*

Chemistry, 89, 269-276.

FSA. (2005). Survey of maize-based retail products for mycotoxins. *Food Survey*

Information Sheet, 72/05.

González, H. H., Moltó, G. A., Pacin, A., Resnik, S. L., Zelaya, M. J., Masana, M., Martínez, E. J. (2008). Trichothecenes and mycoflora in wheat harvested in nine locations in Buenos Aires province, Argentina. *Mycopathologia, 165,* 105-114.

González-Osnaya, L., Cortés, C., Soriano, J. M, Moltó, J. C., Mañes, J. (2011). Occurrence of deoxynivalenol and T-2 toxin in bread and pasta commercialised in Spain. *Food Chemistry, 124,* 156-161..

Jestoi, M., Ritieni, A., Rizzo, A. (2004). Analysis of the *Fusarium* mycotoxins fusaproliferin and trichothecenes in grains using gas chromatography-mass spectrometry. *Journal of Agricultural and Food Chemistry, 52,* 1464-1469.

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (2000). Safety evaluation of certain food additives and contaminants. Zearalenone. *WHO Food Additives Series, 44.* Retrieved March 23, 2012 from:

http://www.inchem.org/documents/jecfa/jecmono/v44jec14.htm

Joint FAO/WHO Expert Committee on Food Additives (JECFA). (2001). Safety evaluation of certain mycotoxins in food. Deoxynivalenol, HT-2 and T-2 toxin. *FAO Food and Nutrition Paper 74.* Retrieved March 23, 2012 from:

http://www.inchem.org/documents/jecfa/jecmono/v47je01.htm

Krska, R., Baumgartner, S., Josephs, R. (2001). The state-of-the-art in the analysis of type-A and -B trichothecene mycotoxins in cereals. *Fresenius Journal of Analytical Chemistry, 371,* 285-299.

Langseth, W., Elen, O. (1997). The occurrence of deoxynivalenol in Norwegian cereals—differences between years and districts, 1998–1996. *Acta Agriculturae Scandinavica, B 47,* 176-184.

Langseth, W., Rundberget, T. (1998). Instrumental methods for determination of nonmacrocyclic trichothecenes in cereals, foodstuffs and cultures. *Journal of Chromatography, A 815,* 103-121

Lattanzio, V. M. T., Solfrizzo, M., Visconti, A. (2007). Determination of trichothecenes in cereals and cereal-based products by liquid chromatography-tandem mass spectrometry. *Food Additives and Contaminants A, 25,* 320-330.

Lombaert, G. A., Pellaers, P., Roscoe, V., Mankotia, M., Neil, R., Scott, P. M. (2003). Mycotoxins in infant cereal foods from the Canadian retail market. *Food Additives and Contaminants, 20,* 494–504.

Martins, H. M., Almeida, I., Marques, M. F., Guerra, M. M. (2008). Fumonisins and deoxynivalenol in corn-based food products in Portugal. *Food and Chemical Toxicology, 46,* 2585-2587.

Martins, M. L., Martins, H. M. (2001). Determination of deoxynivalenol in wheat-based breakfast cereals marketed in Portugal. *Journal of Food Protection, 64,* 1848-1850.

Müller, H. M., Reimann, J., Schumacher, U., Schwadorf, K. (1997a). *Fusarium* toxins in wheat harvested during six years in an area of southwest Germany. *Natural Toxins, 5,* 24-30.

Müller, H. M., Reimann, J., Schumacher, U., Schwadorf, K. (1997b). Natural occurrence of *Fusarium* toxins in barley harvested during five years in an area of southwest Germany. *Mycopathologia, 137,* 185-192.

Müller, H. M., Reimann, J., Schumacher, U., Schwadorf, K. (2001). Further survey of the occurrence of *Fusarium* toxins in wheat grown in southwest Germany. *Archives of Animal Nutrition, 54,* 173-182.

Pestka, J. J. (2007). Deoxynivalenol: toxicity, mechanisms and animal health risks. *Animal Feed Science and Technology, 137,* 283-298.

Pestka, J. J. (2010). Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Archives of Toxicology, 84,* 663-679.

Pestka, J. J., Smolinski, A. T. (2005). Deoxynivalenol: toxicology and potential effects on humans. *Journal of Toxicology and Environmental Healt, B, 8,* 39-69.

Placinta, C. M., D'Mello, J. P. F., Macdonald, A. M. C. (1999). A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Animal Feed Science and Technology, 78,* 21-37.

Reiter, E. V., Vouk, F., Böhm, J., Razzai-Fazeli, E. (2010). Aflatoxins in rice- A limited survey of products marketed in Austria. *Food Control, 21,* 988-991.

Roscoe, V., Lombaert, G. A., Huzel, V., Neumann, G., Melietio, J., Kitchen, D., Kotello, S., Krakalovich, T., Trelka, R., Scott, P. M. (2008). Mycotoxins in breakfast cereals from the Canadian retail market: a 3-year survey. *Food Additives and Contaminants A, 25,* 347-355.

Rotter, B. A., Prelusky, D. B., Pestka, J. J. (1996). Toxicology of deoxynivalenol (vomitoxin). *Journal of Toxicology and Environmental Health, 48,* 1-34.

Scientific Committee on Food (SCF). (1999). Opinion on *Fusarium* Toxins - Part 1: Deoxynivalenol (DON) (expressed on 2 December 1999).Retrieved March 23, 2012 from: http://ec.europa.eu/food/fs/sc/scf/out44_en.pdf

Scientific Committee on Food (SCF) (2000). Opinion on *Fusarium* Toxins - Part 4: Nivalenol (NIV) (expressed on 19 October 2000). Retrieved March 23, 2012 from: http://ec.europa.eu/food/fs/sc/scf/out74_en.pdf

Scientific Committee on Food (SCF) (2002). Opinion on *Fusarium* Toxins - Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol (expressed on 26 February 2002). Retrieved March 23, 2012 from: http://ec.europa.eu/food/fs/sc/scf/out123_en.pdf

Schlatter, J. (2004). Toxicity data relevant for hazard characterization. *Toxicology Letters, 153,* 83-89.

Schollenberger, M., Jara, H. T., Suchy, S., Drochner, W., Müller, H. M. (2002). *Fusarium* toxins in wheat flour collected in an area in southwest Germany. *International Journal of Food Microbiology, 72,* 85-89.

Schollenberger, M., Müller, H. M., Rüfle, M., Suchy, S., Planck, S., Drochner, W. (2005). Survey of *Fusarium* toxins in foodstuffs of plant origin marketed in Germany. *International Journal of Food Microbiology, 97,* 317-326.

Schollenberger, M., Suchy, S., Jara, H. T., Drochner, W., Müller, H. M. (1999). A survey of *Fusarium* toxins in cereal-based foods marketed in an area of southwest Germany. *Mycopathologia, 147,* 49-57.

SCOOP (Reports on Tasks for Scientific Cooperation). (2003). Task 3.2.10. Collection of occurrence data of *Fusarium* toxins in food and assessment of dietary intake by the population of EU Member States. Brussels: Directorate-General Health and Consumer Protection. Retrieved March 23, 2012 from: http://ec.europa.eu/food/fs/scoop/task3210.pdf

Scott, P. M., 1997. Multi-year monitoring of Canadian grains and grain-based foods for trichothecenes and zearalenone. *Food Additives and Contaminants, 14,* 333-339.

Tanaka, K., Sago, Y., Zheng, Y., Nakagawa, H., Hushiro, M. (2007). Mycotoxins in rice. *International Journal of Food Microbiology, 119,* 59-66.

Viquez, O. M., Castell-Castell Pérez, M. E., Shelby, R. A. (1996). Occurrence of fumonisin B1 in maize grown in Costa Rica. *Journal of Agricultural and Food Chemistry, 44,* 2789-2791.

Visconti, A., Minervini, F., Lucivero, G., Gambatesa, V. (1991). Cytotoxic and immunotoxic effects of *Fusarium* mycotoxins using a rapid colorimetric bioassay. *Mycopathologia, 113,* 181-186.

Weidenbörner, M. (2000). *Encyclopedia of Food Mycotoxins.* Berlin, Germany: Springer.

Weingaertner, J., Krska, R., Praznik, W., Grasserbauer, M., Lew, H. (1997). Use of Mycosep multifunctional clean-up columns for the determination of trichothecenes

in wheat by electron-capture gas chromatography. *Fresenius Journal of Analytical Chemistry, 357,* 1206-1210.

Yazar, S., Omurtag, G. Z. (2008). Fumonisins, trichothecenes and zearalenone in cereals. *International Journal of Molecular Sciences, 9,* 2062-2090.

Figure captions

Fig. 1. Total ion chromatogram for five trichothecenes standards (4μg/mL). DON, deoxynivalenol; 3-ADON, 3-acetyldeoxynivalenol; FUS-X, fusarenon-X; 15-ADON, 15-acetyldeoxynivalenol; NIV, nivalenol; NEO, neosolaniol, internal standard.

Fig. 2. Total ion chromatogram of a naturally contaminated cornflakes sample with 91.35 μg/kg of deoxynivalenol (DON); NEO, neosolaniol, internal standard; (a): ion cromatograms (m/z 235, 259, 295) and mass spectra of DON.

Fig. 1. Total ion chromatogram for five trichothecenes standards (4µg/ml). DON, deoxynivalenol; 3-ADON, 3-acetyldeoxynivalenol; FUS-X, fusarenon-X; 15-ADON, 15-acetyldeoxynivalenol; NIV, nivalenol; NEO, neosolaniol, internal standard

Fig. 2. Total ion chromatogram of a naturally contaminated cornflakes sample with 91.35 µg/kg of deoxynivalenol (DON); NEO, neosolaniol, internal standard; (a): ion cromatograms (m/z 235, 259, 295) and mass spectra of DON
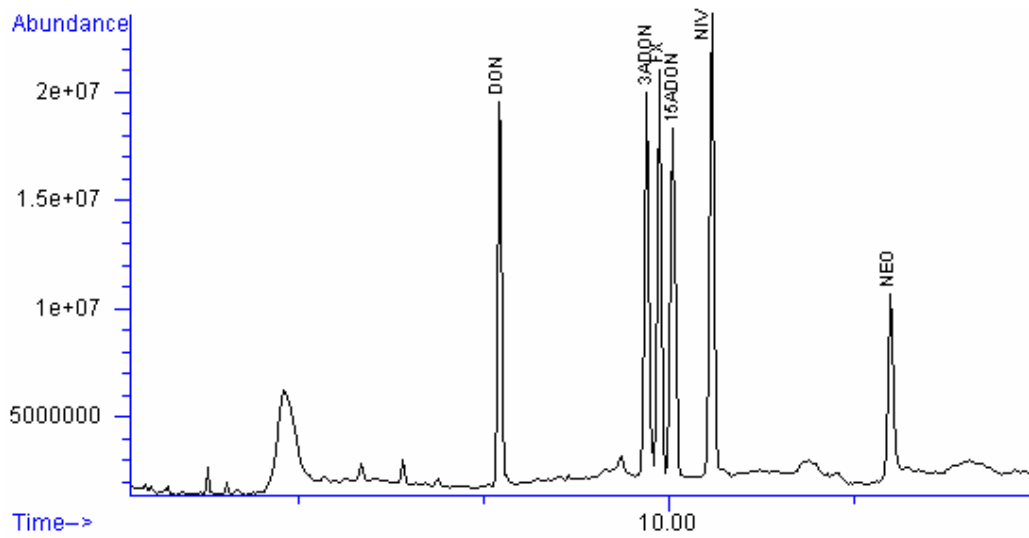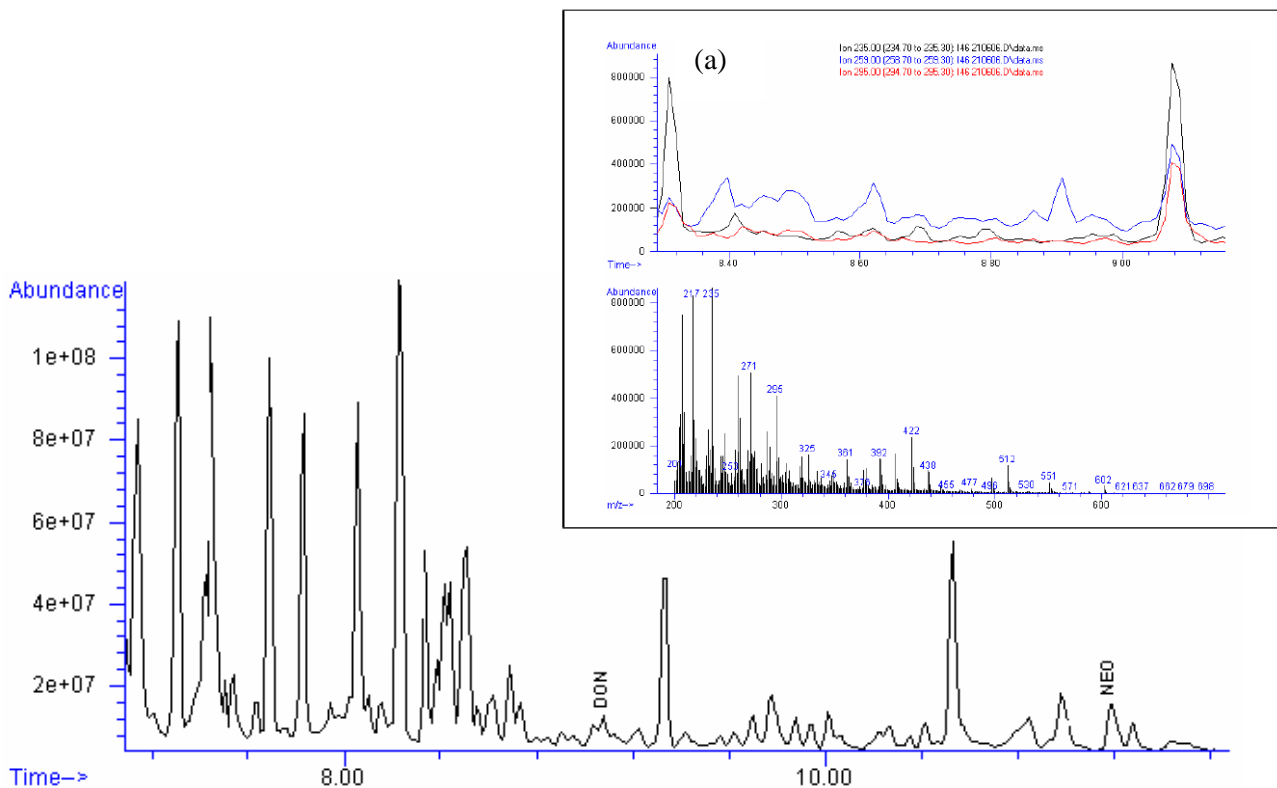
Table 1

Retention times and monitored fragment ions of the studied trichothecenes and the internal standard

Mycotoxin	Retention time (min)	Monitored fragment ions *(m/z)*
DON	9.031	235, 259, 295
3-AcDON	9.819	295, 377, 467
FUS-X	9.886	450, 480
15-AcDON	9.953	350, 392, 407
NIV	10.164	289, 349, 379
NEO neosolaniol (ISTD)	11.119	252, 290

DON, deoxynivalenol; 3-AcDON, 3-acetyldeoxynivalenol; FUS-X, fusarenon-X; 15-AcDON, 15-acetyldeoxynivalenol; NIV, nivalenol; NEO, neosolaniol; ISTD: internal standard

Table 2

Linearity data and limits of detection (LOD) and quantification (LOQ) of trichothecenes[a]

Mycotoxin	Curve equation[b]	r^2	LOD (µg/kg)	LOQ (µg/kg)
DON	$y = 8.30 \times 10^6 x - 1.36 \times 10^6$	0.997	11.4	20.6
3-AcDON	$y = 5.50 \times 10^6 x - 9.76 \times 10^5$	0.998	14.7	23.6
FUS-X	$y = 4.34 \times 10^6 x - 5.26 \times 10^5$	0.999	8.90	15.2
15-AcDON	$y = 1.00 \times 10^7 x - 1.71 \times 10^6$	0.997	12.6	21.4
NIV	$y = 8.19 \times 10^6 x - 1.27 \times 10^6$	0.998	10.7	19.4

DON, deoxynivalenol; 3-AcDON, 3-acetyldeoxynivalenol; FUS-X, fusarenon-X; 15-AcDON,

15-acetyldeoxynivalenol; NIV, nivalenol.

[a]: number of independent determinations = 3

[b]: linear range: 0.1 - 5 µg/ml

Table 3

Recoveries and relative standard deviations (%) of trichothecenes in spiked samples[a]

Mycotoxin	Spiking levels (µg/kg)	Corn-based	Wheat-based	Rice-based
DON	500	94.3 ± 9.1	96.1 ± 4.9	98.0 ± 1.0
	50	102 ± 0.6	90.0± 9.1	106 ± 1.7
3-AcDON	500	97.0 ± 2.7	106 ± 2.1	98.9 ± 3.8
	50	98.7 ± 3.4	110 ± 1.5	104 ± 2.8
FUS-X	500	90.5 ± 9.2	92.7 ± 4.5	103 ± 3.9
	50	96.3 ± 4.6	103 ± 7.3	103 ± 3.2
15-AcDON	500	108 ± 4.6	102 ± 5.8	105 ± 2.8
	50	96.6 ± 4.1	101± 3.0	96.3 ± 3.9
NIV	500	71.8 ± 2.9	75.2 ± 4.3	69.2 ± 7.7
	50	96.3 ± 3.1	100 ± 9.7	101 ± 1.5

DON, deoxynivalenol; 3-AcDON, 3-acetyldeoxynivalenol; FUS-X, fusarenon-X; 15-AcDON, 15-acetyldeoxynivalenol;

NIV, nivalenol.

[a]: number of independent determinations= 3

Table 4

Incidence and levels of trichothecene toxins in different groups of breakfast cereal samples

Grain (No. samples)	Toxin detected	No. (%) of positive samples	Toxin in positive samples (µg/kg)		
			Range	Mean	Median
Corn (62)	DON	19 (30.7)	32.8 – 191	86.3	83.1
	NIV	3 (4.8)	< LOQ[a]	15.1	15.1
Wheat (27)	DON	5 (18.5)	58.7 – 468	223	93.8
	NIV	1 (3.7)	56.7	56.7	56.7
Multigrain (46)	DON	14(30.4)	31.6 – 127	65.8	55.3
	FUS-X	2 (4.3)	< LOQ[b] – 42.4	27.2	27.2
Total samples	DON	38 (25.7)	31.5 – 468	96.7	74.4
	3-AcDON	0	-	-	-
	FUS-X	2 (1.3)	< LOQ[b] – 42.4	27.2	27.2
	15-AcDON	0	-	-	-
	NIV	4 (2.7)	< LOQ[a] – 56.7	25.5	15.1

DON, deoxynivalenol; 3-AcDON, 3-acetyldeoxynivalenol; FUS-X, fusarenon-X; 15-AcDON, 15-acetyldeoxynivalenol;

NIV, nivalenol; LOQ, limit of quantification.

[a]: LOQ (NIV) = 19.4 µg/kg

[b]: LOQ (FUS-X) = 15.2 µg/kg

Table 5

Estimated trichothecene daily intake[a] (µg/kg bw/day) and the percentage that it represents of the tolerable daily intake (TDI) proposed by the Scientific Committee on Food of the European Union (SCF)

	DON			NIV			FUS-X	
	Intake (µg/kg bw/day)	SCF (%)		Intake (µg/kg bw/day)	SCF (%)		Intake (µg/kg bw/day)	SCF (%)[d]
Adults [b]	0.030	3.00		0.008	1.14		0.009	-
Children [c]	0.072	7.20		0.019	2.71		0.020	-

DON, deoxynivalenol; NIV, nivalenol; FUS-X, fusarenon-X

[a] Intake data obtained from Spanish Ministry of Agriculture, Fisheries and Food (MAPA) (18.6 g/day) is assumed.

[b] A body weight (bw) of 60 kg is assumed for adults.

[c] A body weight (bw) of 25 kg is assumed for children.

[d] Limit not established