Rev. Toxicol. (2013) 30: 198-202

Determination of deoxynivalenol in wheat-based snacks by gas chromatography-triple quadrupole tandem mass spectrometry

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Recibido 10 de septiembre de 2013 / Aceptado 20 de diciembre de 2013

Abstract: Deoxynivalenol (DON) is the most frequently detected mycotoxin in cereal and cereal-based products, and a continuous monitoring of this toxin in foodstuffs is highly desirable. In this sense, a QuEChERS based extraction and gas chromatography-tandem mass spectrometry detection is proposed to determine DON in an appetizer largely consumed, the wheat-based snacks. In this study, a total of 40 samples were analyzed. The samples were divided into two groups based on the composition. Extraction was carried out with acetonitrile followed by a dispersive solid phase extraction and analyzed for DON content by gas chromatography-tandem mass spectrometry (GC-QqQ-MS/MS) method. The overall occurrence of samples with DON was 67.5%, with maximum content of 61µg/kg. In spite of its high incidence, DON concentrations found in samples were much lower than the maximum limit established in the current European legislation (500 µg/kg) for the foodstuff evaluated. Data obtained indicated a low exposure to DON through the consumption of this food commodity.

Keywords: wheat-based snacks, mycotoxins, occurrence, GC-MS/MS, exposure.

Resumen: Determinación de deoxinivalenol en rosquilletas mediante cromatografía de gases acoplada a espectrometría de masas en tándem. El deoxinivalenol (DON) es la micotoxina producida por hongos del género *Fusarium* que con más frecuencia se detecta en cereales y productos a base de cereales. Por ello, es recomendable realizar una continua monitorización de su incidencia en los alimentos. Este trabajo propone un procedimiento analítico basado en una extracción tipo QuEChERS seguido de una cromatografía de gases acoplada a un detector de triple cuadrupolo para la determinación de DON en rosquilletas. Se analizaron un total de 40 muestras las cuales se dividieron según su composición en dos grupos. El DON fue identificado en el 67,5% de las muestras analizadas con un contenido máximo de 61 µg/kg. A pesar de su incidencia elevada, los niveles de DON hallados fueron muy inferiores a los límites máximos legislados en la actual legislación europea (500 µg/kg). Los resultados obtenidos muestran una baja exposición a DON a través del consumo de esta matriz alimentaria.

Palabras clave: rosquilletas, micotoxinas, GC-MS/MS, exposición.

Introduction

Wheat-based snacks are a traditional ready to eat appetizer made by an heterogeneous recipe that incorporates wheat flour and different amount of other ingredients depending on the nutrition objectives for which have been designed [1]. For instance, whole-grain could be incorporated into the product to increase the fiber intake. The fiber fraction has also functional properties used to improve textural and organoleptic attributes such as water and oil retention, swelling and viscosity. Inclusion of fatty acids also decreases the hardness of snack products and increases the crispiness of the final product as well as its potential nutritional benefit, especially unsaturated fatty acids, as oleic acid [2].

Nonetheless, agricultural commodities, including wheat, are susceptible to fungal attack in the field, during drying and subsequent storage [3]. Fungi may produce as secondary metabolites diverse groups of naturally occurring toxic chemical substances, known as mycotoxins. Mycotoxins are frequent contaminants of grains and cereal products thus they suppose an important threat to food safety. In fact, it is reported that 25-50% of harvested crops in the world are contaminated by mycotoxins annually [4]. Hence, dietary exposure through the consumption of contaminated food is frequent in many populations.

Thrichothecenes is one of the major mycotoxins groups. They are chemically designated as 12,13-epoxy trichothecenes because they are esters of sesquiterpenoid alcohols containing the trichothecene tricyclic ring, which possesses a chemical double bond at C9-C10 and an epoxide at C12-C13. Over 200 thrichothecenes have been reported and categorized into four groups. Group A trichothecene is mainly represented by T-2 and HT-2 mycotoxins; group B by DON and nivalenol; group C by crotocin and baccharin; and group D by satratoxin and roridin [5].

Among all group B trichothecenes, DON (also known as vomitoxin) is the most commonly detected trichothecene in cereal grains. It is primarily produced by *Fusarium graminearum* and *Fusarium culmorum* in different geographical regions. *F. graminearum* grows at 25ºC and at water activity above 0.88, whereas *F. culmorum* grows optimally at 21ºC and at water activity above 0.87 [6].

Similarly to other trichothecenes, acute exposure of mice to DON doses causes histopathologic effects ranging from hemorrhage/necrosis of the intestinal tract, necrosis in bone marrow and lymphoid tissues, to kidney and heart lesions. The most common effects of prolonged dietary exposure of experimental animals to DON are decreased weight gain and anorexia [7].

Due to their toxic potential, regulatory limits were introduced in several foodstuffs for DON in many countries, including the European Union (EU). Currently, maximum permitted level set for DON in bread including small bakery wares, pastries, biscuits, cereal snacks and breakfast cereals is 500 µg/kg [8]. None other maximum limit has still been set in foodstuffs for any other trichothecene. It should be noted that DON is reported to be a very stable compound, both during storage/milling and the processing/cooking of food [9].

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In addition, detailed risk assessment for DON was carried out by the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) resulting in a provisional maximum tolerable intake (PTMDI) of 1.0 µg per kg bodyweight (bw). In 2010, the JEFCA updated its evaluation for DON and concluded to include its acetylated forms 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol to define the proposed value as a group PMTDI [10].

The goal of risk characterization is to assess hazard/toxicity levels by exposure doses to determine if risk may occur under the specific scenarios.

Selection of an appropriate extraction and clean-up methods play important roles in DON determination. Recovery value is a key parameter in assessment of extraction and clean-up efficiency. Recovery values between 70 and 120% are considered acceptable by Document SANCO/12495/2011 [11]. Extraction of DON is usually carried out throughout polar solvents because of its physicochemical properties.

Conventional liquid-liquid extraction techniques have been replaced by sample preparation procedures such as matrix solid phase dispersion (MSPD) [12], pressurized liquid extraction (PLE) [13], solid-phase microextraction (SPME) [14], molecular imprinted polymers (MIPs) [15], dispersive liquid–liquid microextraction (DLLME) [16] and QuEChERS [17]. The QuEChERS method has several advantages over most traditional methods of analysis such as the fastness and the high simple throughput, the smaller volumes of no chlorinated organic solvents used and the good recoveries obtained . As regards analytical method for mycotoxins determination, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the most extensively technique used. However, gas chromatography methods coupled to mass spectrometry detector offer a narrower analytical scope allowing simultaneously a very useful and relatively inexpensive analytical performance with some clear advantages as lower detection limits and greater selectivity [19].

Considering the above described situation, the aims of this survey were to develop a method based on GC-MS/MS for the determination of deoxynivalenol in wheat-based snacks, to evaluate the occurrence of this mycotoxin in a total of forty samples and to estimate deoxynivalenol exposure in children and adults through wheat-based snacks intake.

Material and methods

Chemical and reagents

Solvents (acetonitrile, hexane and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate (thin powder) was obtained from Alfa Aesar GmbH & Co (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, USA).

The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was purchased from Supelco (Bellefonte, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

The standard of deoxynivalenol (DON) was obtained from Sigma-Aldrich (St. Louis, USA). A stock solution of was prepared at 1000 mg/L in methanol. The stock solution was diluted with acetonitrile in order to obtain the appropriate working standard solution (50 mg/L). Standards was stored in darkness and kept at -20ºC until the GC-MS/MS analysis.

Certified reference material BRM 003004 (artificially contaminated wheat, DON 1062 ± 110 µg/kg) was purchased from Biopure Referenzsubstanzen GmBH (Tulln, Austria).

Sampling and sample preparation

The basic wheat-based snack is a pencil shaped stick of bread that has been rolled and baked to a crispy texture and seasoned lightly, usually with a little salt. They are widely consumed as an appetizer in Valencia. In this work, forty wheat-based snack samples (35 g) were randomly purchased from April to June 2013, from different retailers, including supermarkets and smaller shops located in different regions of Valencia Metropolitan Area (Spain). Samples were divided in two groups: (A) simple wheat-based snacks (n = 20) and (B) wheat-based snacks containing other main ingredients such as fiber or dried fruits (n = 20). All samples were homogenized using a laboratory mill and kept at 4 ºC under dark and dry conditions.

A previously reported QuEChERS based sample preparation [19] was taken as a starting point and was modified and optimized for wheat-based snack mycotoxin extraction. In brief, 5 g of homogenized sample were added to 25 mL of distilled water and were sonicated for 15 min. The main extraction involved the addition of 8 mL of acetonitrile, 4 g of MgSO4 and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the supernatant was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of MgSO4 and 300 mg of C18 and centrifuged for 1 min at 1500 rpm. Finally the extract was evaporated to dryness under nitrogen flow.

The dry extract was added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 250 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7). Finally, the hexane layer was transferred to an autosampler vial for the chromatographic analysis.

Gas chromatography triple quadrupole mass spectrometry

A GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, USA) were used for MS/MS analysis. The mass spectrometer operated in electron impact ionization (EI, 70 eV). The transfer line and source temperatures were 280 and 230 ºC, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent Masshunter version B.04.00 software.

The separation was achieved on a HP-5MS 30 m x 0.25 mm x 0.25 µm capillary column. One microliter of the final clean extract was injected in splitless mode at 250ºC in programmable temperature vaporization (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially 80ºC, and the temperature was increased to 245ºC at 60ºC/min. After a 3 min hold time, the temperature was increased to 260ºC at 3ºC/min and finally to 270ºC at 10ºC/min and then held for 10 min. GC-MS/MS parameters are shown in table 1.

Table 1. *MS/MS data and validation parameters of DON*

Mycotoxin	DON
Retention time (min)	8.31
Quantitation transition (Q)	392>259
Confirmation transition (q)	407>197
Ratio Q/q (± % RSD)	41.6 (3.2)
Limit of detection (μg kg^{-1})	0.6
Limit of quantitation (μg kg^{-1})	1.25
Recovery (%)	116
Intra-day precision (% RSD) (n=5)	3
Inter-day precision (% RSD) (n=3)	9
Matrix effect (%)	18
External standard regression equation	y=20.244x-13040.790
External standard correlation coefficient	r^2=0.994
Matrix-matched regression equation	y=3.645x-2236.382
Matrix-matched correlation coefficient	r^2=0.993

Data analysis and validation parameters

For identification purposes, retention time of DON in the standard and in the sample was compared at tolerance of ± 0.5%. Moreover, in accordance with the 2002/657/EC Decision [20], the relative ion intensity of DON standard solution and the spiked samples at the concentration levels used for the calibration curve were compared. Spiking samples were left to stand overnight to allow mycotoxin absorption onto the sample prior to extraction. The linearity of the MS/MS method was established with eight calibration points, using external standards over a concentration range of LOQ to 160 μg/kg. Peak area of target analyte was calculated using Mass Hunter software (Agilent). Each calibration point was obtained as the mean of three injections (n =3).

The sensitivity of the method was estimated by establishing the limit of detection (LOD) and quantitation (LOQ). The LOD was determined as the lowest mycotoxin concentration whose qualifier transition presented a signal-to-noise ratio (S/N) ≥ 3. The LOQ was determined as the minimum detectable amount of analyte with S/N ≥ 10 for the quantifier transition.

For the assessment of matrix effect, a comparison of the slopes of the regression lines for DON, in pure solvent and in matrix-matched standards was performed. Matrix-matched calibration solutions were prepared by spiking sample extracts at equal concentrations in the final extracts as the solvent standard. Eight concentration levels of mycotoxins were applied for calibration: 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μg/kg.

Recovery and precision, expressed as percentage relative standard deviation (% RSD), were determined by analyzing wheat-based snack samples at 100 μg/kg. Quantitation to validate the method was carried out using matrix-matched standards to avoid overlap between concepts such as recovery and matrix effects. Intra-day precision data were obtained from five analyses performed on one day; inter-day data were tested on three different working days within 20 days. The recovery was determined as the average of a five analyses of a spiked sample.

The trueness of the method was also supported by certified reference material (BRM003004).

Dietary intakes estimates

The probable daily intake (PDI) was determined as indicated in equation 1 [21]:

$$PDI = {(C \times K)}/{bw} \qquad \text{(eq. 1)}$$

where *PDI* is the probable daily intake (μg kg^{-1} *bw* day^{-1}) of DON; *C* is the average concentration of a DON found in the analyzed samples (μg kg^{-1}); *K* is the mean data consumption of the food commodity evaluated (g day^{-1}) and *bw* is the body weight (kg) used for different population groups. As this food commodity is consumed by general population including children, two different body weights were considered (25 and 70 kg for adults and adults, respectively).

Results and discussion

QuEChERS validation

Selectivity and specificity were assessed by recognizing the quantitation (Q) and confirmation (q) transitions of DON at same concentration levels as used for the construction of the calibration curve. There were no interferences that resulted from the presence of the matrix. Additionally, the ratio between both transitions (Q/q) demonstrated the correlation between DON peak areas and concentration of DON (Table 1).

Correlation between the response and the amount of analyte was verified by plotting signal intensity against analyte concentration. A correlation coefficient higher than 0.990 was obtained for the concentration range studied (from LOQ to 160 μg/kg). Both external standard and matrix-matched calibration curves are shown in Table 1.

LOD and LOQ obtained (0.6 and 1.25 μg/kg, respectively) in wheat-based snacks were far below the concentrations of DON established by the Commission Regulation (EC) No. 401/2006 [22], showing the suitability of the developed method for the determination of trace amounts of DON in the food matrix studied.

Considerable signal suppression was observed (18%) (Table 1). This fact justifies the use of matrix-matched calibration to compensate the matrix effect. Matrix-matched calibrations were prepared fresh daily and injected at the end of each injection sequence.

Precision and accuracy are summarized in Table 1. Precision studies showed that the method was repeatable (n=5) (RSD<3%) and reproducible (n=3) (RSD<9%). Satisfactory results in terms of recoveries were found (116%). The results obtained are in agreement with the recoveries admitted by the Commission Regulation (EC) No. 401/2006 [22].

Additionally, trueness of the method was evaluated through the analysis of the certified reference material. Results showed a mean value of 1025 μg/kg with a standard deviation of 23 μg/kg (n=6). These calculated concentrations were satisfactory according to the certificated values 1062 ± 110 μg/kg.

Quantitation of mycotoxins in snacks samples

Table 2 shows the results of the forty wheat-based snack analyzed samples. An overall DON incidence of 67.5% was found. According to the occurrence of DON based on the samples classification specified in 2.2 section, 60% of simple wheat-based snacks and 75% of wheat-based containing other main ingredients showed DON contamination. This slightly difference could be attributed to the type of ingredients presents in the sample such as whole wheat and dried fruits. Nonetheless, mean values found and the ranges of concentrations of both groups of samples were similar. A certificated reference material (wheat artificially DON contaminated at 1062 ± 110μg/kg) MRM chromatogram (A), as well as a naturally

contaminated wheat-based snack sample with DON at 42 µg/kg (B) is shown in Figure 1.

Table 2. *Occurrence of DON in wheat-based snacks analyzed*

Snacks		DON
Containing wheat only (n = 20)	Incidence	12/20
	Contaminated samples (%)	60
	Average contamination (µg kg^{-1})	40
	Contamination range (µg kg^{-1})	31 - 61
Containing wheat + more ingredients (n = 20)	Incidence	15/20
	Contaminated samples (%)	75
	Average contamination (µg kg^{-1})	35
	Contamination range (µg kg^{-1})	30 - 48
Total samples (n = 40)	**Overall incidence (%)**	**67.5**

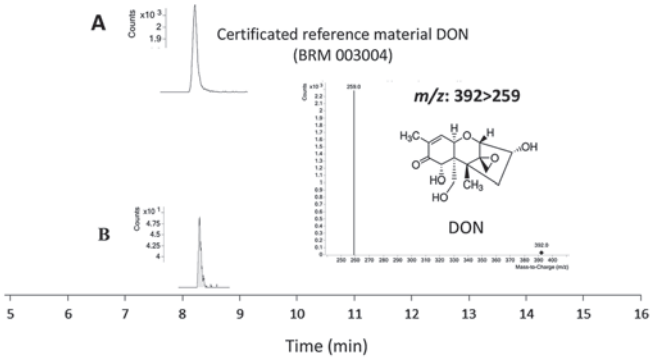

Figure 1. MRM chromatograms of the certificated reference material (wheat artificially DON contaminated at 1062 ± 110µg/kg) (A) and a naturally DON contaminated wheat-based snack sample at 42 µg/kg (B)

Up to now, no previous studies have been conducted as regards determination of DON in wheat-based snack samples. Thus, this fact justifies the novelty of this research work. Authors have compared published DON occurrence data at similar food commodities. Similar DON occurrence were found by Lindbland et al. [23], in winter wheat from 2009 (n=31) to 2011 (n=33) being 81 and 64% and median concentrations at 38 and 31 µg/kg, respectively. Vidal et al. [24], reported an occurrence of DON of 62% in the 37 wheat fiber samples. Differences between organically and conventionally produced samples were also detected being the occurrence 72 and 58%, respectively. The occurrence of DON in wheat during 2010 and 2011 was reported by Slikova et al. [25]. Of the 299 grain wheat samples analyzed, 76.6% were contaminated with DON. In 2010, 82.2% of the samples were DON contaminated whereas 70.7% was the DON incidence reported in 2011. The average DON content found was 930 and 300 µg/kg in 2010 and 2011, respectively. These high contents are rarely reported in literature and could be related to the nature of analyzed samples (grain samples).

Calculation of DON intake

A deterministic analysis was performed, in a first attempt to assess the dietary exposure of DON. Official consumption data about the food matrix selected was not available and authors have estimated that the individual pack (35 g) is consumed for typical Valencian brunch. As specified in 2.7 Section, the PDI was calculated. The PDI obtained was 0.045 and 0.016 µg/kg bw day for children and adults, respectively. A risk characterization, expressed as percentage of PMTDI was calculated by comparing PDI to PMTDI. Results shown in Table 3 indicated a low exposure to DON through wheat-based snacks consumption.

Table 3. *Deoxynivalenol exposure calculated for children and adults through wheat-based snacks consumption*

Group population	PDI (µg/kg bw)	PMTDI (µg/kg bw)	%PMTDI
Children	0.045	1	4.5
Adults	0.016		1.6

Conclusions

Validation parameters as well as the unambiguous identification allowed by mass spectrometry shows the suitability of the proposed methodology for the analysis of the most frequently detected *Fusarium* toxin (DON) in wheat-based snack samples. 27 out of 40 samples analyzed were DON contaminated. In spite of that, mean values found were much lower than the maximum limit (500 µg/kg) established by the current European legislation (EC) 1881/2006 for this food commodity. Different DON occurrence was found between simple wheat-based snacks and those samples containing other main ingredients with incidences of 60 and 75%, respectively. It could be because of the type of ingredient such as fiber and dried fruits. Nonetheless, differences as regards average contents were not observed. The widely consumed wheat-based snacks by Valencian population do not constitute an important source of DON based on obtained results.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Innovation (AGL2010-17024/ALI). Y. Rodríguez-Carrasco thanks the F.P.U. Grant (No. AP2010-2940) provided by the Ministry of Education.

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