

On: 11 June 2014, At: 09:36

Publisher: Taylor & Francis

Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



## Food Additives & Contaminants: Part A

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tfac20>

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Accepted author version posted online: 21 May 2014. Published online: 10 Jun 2014.

To cite this article: Yelko Rodríguez-Carrasco, Guillermina Font, Juan Carlos Moltó & Houda Berrada (2014): Quantitative determination of trichothecenes in breadsticks by gas chromatography-triple quadrupole tandem mass spectrometry, Food Additives & Contaminants: Part A, DOI: [10.1080/19440049.2014.926399](https://doi.org/10.1080/19440049.2014.926399)

To link to this article: <http://dx.doi.org/10.1080/19440049.2014.926399>

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## Quantitative determination of trichothecenes in breadsticks by gas chromatography-triple quadrupole tandem mass spectrometry

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(Received 10 February 2014; accepted 12 May 2014)

Breadsticks are pencil-sized sticks of dry bread widely consumed as a pre-meal appetiser. They are basically wheat-based snacks, which makes them a good matrix to evaluate mycotoxin contamination, since wheat is very susceptible to fungal attack. In this sense, the fast, selective and sensitive gas chromatography-triple quadrupole tandem mass spectrometry (GC-QqQ-MS/MS) method proposed here allows simultaneous determination of deoxynivalenol (DON), 3-acetyldeoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, neosolaniol, HT-2 and T-2 toxin in breadsticks after QuEChERS extraction and clean-up. The performance of the method was assessed with respect to European Commission Regulations by studying the selectivity and specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, matrix effect, accuracy, precision and trueness. Satisfactory results in terms of validation parameters were obtained for all selected mycotoxins (recovery range of 70–110%, RSD < 10%, LOQ < 40  $\mu\text{g kg}^{-1}$ ). The trueness of the method was supported by using certified reference material (DON 1062  $\pm$  110  $\mu\text{g kg}^{-1}$ ). The method was successfully used to evaluate the occurrence of the studied *Fusarium* toxins in 61 breadstick samples. A total of 64% of the samples showed mycotoxin contamination, DON being the most frequently detected toxin. Nonetheless, mean levels obtained were far below the maximum levels permitted by European Union legislation. An additional goal was to carry out a risk-characterisation approach to DON by comparing probable daily intake and provisional maximum tolerable daily intake (PMTDI).

**Keywords:** breadsticks; mycotoxins; risk characterisation; QuEChERS; GC-MS/MS

### Introduction

Bakery products are one of the widest consumed food categories in the world. Among the wide range of this food category, a big portion is dry, shelf-stable foods such as biscuits, crackers and breadsticks. Typical ingredients include wheat flour, water, salt, either oil or fat, and yeast or a chemical raising agent (Ktenioudaki et al. 2012). The basic breadstick 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 may also be combined with ingredients such as peanuts and sunflower seeds, cheese or chocolate. Overwhelmingly, the most popular varieties are made with bleached flour, although whole wheat and mixed grain versions are gaining in popularity. In fact, there is lot of interest in increasing the nutritional value of foods by means of redesigning healthier convenience foods which are consumed 'on the go'. This makes them an alternative source of nourishment to take along (Ktenioudaki et al. 2012). In this line, it has to be highlighted that the European savoury snacks market grew by 3.3% between 2007 and 2010, to reach a total of 2 billion kg in 2011. Moreover, the Spanish savoury market totalled 248.1 million kg in 2011, representing a compound annual growth rate of 1.7% since 2007, and it is forecast to have a volume of 270.9 million kg, an increase of 1.4% by 2016 (Datamonitor 2013).

Nonetheless, agricultural commodities, including wheat, are susceptible to fungal attack in the field, during drying and subsequent storage (Paterson & Lima 2010; Tirado et al. 2010). Fungi may produce as secondary metabolites diverse groups of naturally occurring toxic chemical substances, known as mycotoxins, compromising the safety of food. The *Fusarium* genus is a renowned producer of trichothecenes, considered a highly toxic class of mycotoxins that is subdivided into four groups (A–D). Among them, trichothecenes A, comprising T-2 and HT-2 toxins, and trichothecenes B, including deoxynivalenol, are the most common.

Food contaminated by mycotoxins has been associated with acute and chronic effects, especially hepatic, gastrointestinal and carcinogenic diseases (Juan-García et al. 2013). It has to be highlighted that mycotoxins are relatively stable to cooking and processing, thus food preparation procedures do not guarantee their removal (Kabak 2009). Consequently, the European Union has established maximum mycotoxin levels allowed in several food commodities in Commission Regulation EC No. 1881/2006 and EC No. 1126/2007. Currently, the maximum permitted level set for DON in bread including small bakery wares, pastries, biscuits, cereal snacks and breakfast cereals is 500  $\mu\text{g kg}^{-1}$ . No other

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maximum limit has still been set in foodstuffs for any other trichothecene.

Monitoring of mycotoxin levels in food samples is imperative because of their widespread occurrence. For that reason, the development of sensitive, selective and reliable analytical multi-mycotoxin methods is of major interest (Meneely et al. 2011). Sample preparation should provide clean extracts and be rapid, simple, cheap and environmental friendly as far as possible. Traditionally mycotoxins analyses were performed by the time- and solvent-consuming liquid–liquid extraction, which have been replaced by good alternatives such as pressurised liquid extraction, SPE, matrix-solid-phase dispersion (MSPD) and QuEChERS (Köppen et al. 2010).

MSPD includes sample homogenisation, cellular disruption, exhaustive extraction, fractionation and clean-up in a simple process in which a small amount of sample (0.1–5 g) is blended with the selected solid phase (such as C18, C8, silica, Florisil or alumina) followed by gravitational elution of compounds with a small volume of an appropriate solvent. When necessary, further purification with adsorbents can be performed.

QuEChERS consists in an acetonitrile partitioning and dispersive solid-phase extraction (d-SPE) method well known for its applicability in simultaneous analysis of a large number of compounds in a variety of food matrices. Moreover, the QuEChERS methodology presents some advantages, such as simplicity and effectiveness for complex samples cleaning-up. It generally involves two simple steps. First, the homogenised samples are extracted and partitioned using an organic solvent and salt solution, then a d-SPE technique is used for the supernatant.

As regards the determination of mycotoxins, HPLC and GC are the most widely applied techniques. In the last few years, the number of chromatographic methods coupled with MS/MS detection reported for multiclass analysis of mycotoxins in food has increased considerably, complying with the requirements established in current legislation. More specifically, a high dynamic range and good performance reached in SRM mode make the triple quadrupole (QqQ) one of the most widely employed analyses (Fernandes et al. 2013; Rodríguez-Carrasco et al. 2013).

Considering the above described situation about the determination of mycotoxins in foodstuffs, a method based on GC-QqQ-MS/MS for the simultaneous determination of eight *Fusarium* toxins belonging to the trichothecene mycotoxin group in breadsticks is proposed. To the best of our knowledge, this is the first study to provide detailed information about the occurrence and co-occurrence of *Fusarium* toxins in this widely consumed appetiser.

## 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 C<sub>18</sub>-E (50 µm, 65 Å) was purchased from Phenomenex (Torrance, CA, USA).

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

The standards of the type A and B trichothecenes: deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), diacetoxyscirpenol (DAS), nivalenol (NIV), fusarenon-X (FUS-X), neosolaniol (NEO), T-2 and HT-2 toxin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of all analytes were prepared at the same concentration (1000 mg l<sup>-1</sup>) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (50 mg l<sup>-1</sup>). All standards were stored in darkness and kept at -20°C until the GC-MS/MS analysis.

Certified reference material BRM 003004 (artificially contaminated wheat, DON 1062 ± 110 µg kg<sup>-1</sup>) was purchased from Biopure Referenzsubstanzen GmbH (Tulln, Austria).

### Samples

Sixty-one breadstick snacks were randomly purchased from supermarkets located in different regions of Valencia Metropolitan Area (Spain). All were homogenised (35 g) using a laboratory mill and kept at 4°C in the dark and in dry conditions.

A subdivision of the samples into six groups based on the ingredients was carried out. Samples were categorised as simple breadsticks (*n* = 21), peanuts and sunflower seeds (*n* = 14), whole-grain (*n* = 12), cheese (*n* = 7), chocolate (*n* = 5) and soybean (*n* = 2) breadsticks.

### Dispersive-based sample preparation

A preliminary study was performed in order to determine the extraction procedure that achieves the best results. In this sense, MSPD and QuEChERS methods were compared for the extraction of mycotoxins from breadsticks followed by determination by GC-MS/MS. As a starting

point, a triplicate set of samples spiked at  $100 \mu\text{g kg}^{-1}$  was extracted following the above-mentioned extraction methods. Recovery and precision data of both methods were compared.

#### QuEChERS

In brief, 5 g of homogenised sample were added to 25 ml of distilled water and sonicated for 15 min. The main extraction involved the addition of 8 ml of acetonitrile, 4 g of  $\text{MgSO}_4$  and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the supernatant was submitted to d-SPE with a mixture of 900 mg of  $\text{MgSO}_4$  and 300 mg of  $\text{C}_{18}$  and centrifuged for 1 min at 1500 rpm. Finally the extract was evaporated to dryness under nitrogen flow.

#### Matrix solid-phase dispersion

The MSPD procedure described by Blesa et al. (2004) was taken as a starting point and optimised for the selected mycotoxins. Briefly, 1 g of sample was placed into a glass mortar (50 ml capacity) and gently blended with 1 g of  $\text{C}_{18}$  for 5 min using a pestle to obtain a homogeneous mixture. The homogeneous mixture was introduced into a 100 mm  $\times$  9 mm i.d. glass column with a coarse frit (No. 2) and covered with a plug of silanised glass wool at the top of the column, and eluted drop wise with 15 ml of acetonitrile/methanol (50/50, v/v) by applying a slight vacuum. Then the extract was transferred to a 25 ml conical tube and evaporated to dryness at  $35^\circ\text{C}$  with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA).

#### Derivatisation

The dry extract was added with 50  $\mu\text{l}$  of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatised sample was diluted to 250  $\mu\text{l}$  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.

#### GC-QqQ-MS/MS conditions

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

Separation was achieved on a HP-5MS 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$  capillary column. A total of 1  $\mu\text{l}$  of the final clean extract of mycotoxins was injected in splitless mode at  $250^\circ\text{C}$  in a programmable temperature vaporisation (PTV) inlet employing helium as the carrier gas at a fixed pressure of 20.3 psi. The oven temperature programme was initially  $80^\circ\text{C}$ , and the temperature was increased to  $245^\circ\text{C}$  at  $60^\circ\text{C min}^{-1}$ . After a 3 min hold time, the temperature was increased to  $260^\circ\text{C}$  at  $3^\circ\text{C min}^{-1}$  and finally to  $270^\circ\text{C}$  at  $10^\circ\text{C min}^{-1}$  and then held for 10 min. Chromatographic analysis time was performed in 15 min. The GC-MS/MS parameters of the studied mycotoxins are shown in Table 1.

#### Method performance

The developed method was validated following SANCO 12495/2011. Linearity was first evaluated. Eight concentration levels for independent determination for three parallel sets between LOQ and  $100 \times \text{LOQ}$  were employed for linearity evaluation. Matrix-matched calibration curves were built by spiking blank sample

Table 1. GC-MS/MS parameters for mycotoxin determination.

MS/MS parameters	DON	3-ADON	FUS-X	DAS	NIV	NEO	HT-2	T-2
Quantitation transition (Q)	392 > 259	392 > 287	450 > 260	350 > 229	289 > 73	252 > 195	347 > 157	350 > 259
Collision energy (eV)	10	5	10	15	15	10	10	10
Dwell time (ms)	25	35	35	35	35	25	25	25
Confirmation transition (q)	407 > 197	467 > 147	450 > 245	378 > 124	379 > 73	252 > 167	347 > 185	350 > 229
Collision energy (eV)	10	10	20	10	15	15	10	15
Dwell time (ms)	25	25	35	25	35	35	25	35
Ion ratio (Q/q $\pm$ RSD, %)	42 $\pm$ 3	48 $\pm$ 12	12 $\pm$ 7	57 $\pm$ 10	30 $\pm$ 3	41 $\pm$ 4	87 $\pm$ 8	82 $\pm$ 6
Retention time (min)	8.28	9.42	9.48	9.53	9.89	11.24	14.66	14.71

extracts with selected mycotoxins at the same concentration levels than standard calibration curves.

Accuracy was verified by measuring the recoveries from spiked blank samples at  $100 \mu\text{g kg}^{-1}$ . Precision (expressed as %RSD) of the method was determined by repeatability (intra-day precision) and reproducibility (inter-day precision). Intra-day variation was evaluated in six determinations in a single day, while inter-day variation was tested on three different working days within 20 days. Sensitivity was evaluated by LOD and LOQ values. LOD was determined as the analyte concentration that produced a peak signal of three times the background noise from the chromatogram regarding SRM2, confirmation transition. LOQ was determined as the analyte concentration that produced a peak signal of 10 times the background noise from the chromatogram regarding SRM1, quantification transition.

### Exposure assessment

For the exposure assessment based on a deterministic approach, the probable daily intake (PDI,  $\mu\text{g kg}^{-1}$  bw day) was determined by multiplying the mean concentration by the consumption data. Exposure assessment was calculated for children and adults assuming an average body weight (bw) of 25 and 70 kg, respectively. The ratio PDI/PMTDI expressed as a percentage (% of relevant PMTDI) was used for health risk characterisation.

### Results and discussion

The effectiveness of the clean-up of the breadstick extract was compared for both dispersive-based sample preparation methods. The most time-consuming step of the single sample preparation procedure is the evaporation under nitrogen flow. In this sense, the most time-consuming and most laborious method was MSPD. Despite both methods requiring a dryness step to derivatise, the MSPD volume to evaporate was significantly higher than those obtained by QuEChERS. Therefore, QuEChERS extraction is the fastest and less laborious method. In addition, it is possible to assemble up to 12 samples in the QuEChERS methodology, whereas in MSPD only a simultaneous operation up to six assemblies can be carried out.

The vast majority of recovery results exceeded 80% in QuEChERS, whereas a range from 46% to 89% was obtained by the MSPD method. In terms of repeatability (expressed as %RSD), all analytes gave RSDs < 15% in QuEChERS, while up to 37% was obtained in MSPD. Taking into account the above results and considering the advantages offered by QuEChERS compared with MSPD, it was chosen to complete the validation process. The differences between both methods as well as the demands on chemicals, material and time are illustrated in Table 2.

Chromatographic analysis time was performed in 15 min, which meets the requirement for a high throughout determination. Moreover, the developed analytical procedure offers reliable results in terms of sensibility and sensitivity (Table 3).

Table 2. MSPD and QuEChERS preliminary data for the studied mycotoxins.

Extraction procedure	Sample weight (g)	Extraction time (h)	Recovery range (at $100 \mu\text{g kg}^{-1}$ ) (%)	Solvent volume (ml)	Equipment cost	Simultaneous operation
MSPD	1	3	46–89	15 ml (AcN:MeOH)	Low	Up to 6 assemblies
QuEChERS	5	1.5	70–110	8 ml (AcN)	Low	Up to 12 assemblies

Table 3. Performance characteristics of the proposed method in breadsticks.

Mycotoxin	LOD ( $\mu\text{g kg}^{-1}$ )	LOQ ( $\mu\text{g kg}^{-1}$ )	REC (%)	Intra-day precision (% RSD) ( $n = 6$ )	Inter-day precision (% RSD) ( $n = 3$ )	ME (%)
DON	0.6	1.25	116	3	9	18
3-ADON	5	10	92	1	5	50
FUS-X	20	40	100	4	10	38
DAS	5	10	80	3	12	54
NIV	2.5	5	93	4	9	9
NEO	5	10	86	3	10	47
HT-2	1.25	2.5	80	1	7	50
T-2	20	40	73	5	11	67

Notes: ME, matrix effect; REC, Apparent recovery. The ratio  $(A/B \times 100)$  is defined as the absolute matrix effect expressed as percentage where 'A' is the matrix-matched calibration slope and 'B' is the slope of the standard calibration in solvent.

**QuEChERS validation**

The performance of the method was assessed to meet European Commission regulations by studying the selectivity and specificity, LOD, LOQ, linearity, matrix effect, accuracy, precision and trueness.

Selectivity and specificity were assessed by recognising the quantitation (Q) and confirmation (q) transitions of each mycotoxin studied at values close to the LOQ in the presence of the other evaluated mycotoxins. There were neither interferences resulted from the presence of the other mycotoxins nor from the presence of the matrix. Additionally, the ratio between both transitions (Q/q) demonstrated the good correlation between peak areas and concentration of trichothecenes (Table 3).

Correlation coefficients higher than 0.990 were obtained for all mycotoxins in the concentration range studied (from LOQ to  $100 \times \text{LOQ}$ ). The LODs and

LOQs obtained (from 0.6 to  $20 \mu\text{g kg}^{-1}$  and from 1.25 to  $40 \mu\text{g kg}^{-1}$ , respectively) were lower than the maximum limits established by Commission Regulation (EC) No. 401/2006) showing the suitability of the developed method for the determination of trace amounts of *Fusarium* toxins in the food matrix studied. The method sensitivity is similar to other surveys, as shown in Table 4.

The matrix effect (ME), defined as the ratio between the slopes of the matrix matched extract and the slopes of external calibration, was calculated. A value of 100% indicated that there was no matrix effect. There was signal enhancement with a value higher than 100% and signal suppression with value lower than 100%. ME values from 9% to 67% were obtained for the studied mycotoxins (Table 3) and matrix-matched calibrations were used for quantitation purpose.

Table 4. Comparison of the sensitivity obtained by several authors after MSPD and QuEChERS extraction and chromatography techniques coupled to mass spectrometry determination.

Mycotoxin	Commodity	Extraction procedure	Confirmatory technique	LOQ ( $\mu\text{g kg}^{-1}$ )	Reference
DON	Wheat flour		GC-MS	25	Cunha & Fernandes (2010)
	Wheat semolina	QuEChERS	GC-MS/MS	1	Rodríguez-Carrasco et al. (2012)
	Wheat flour		LC-MS	10	Sospedra et al. (2010)
	Wheat		UHPLC	50	Zachariasova et al. (2010)
3-ADON	Wheat flour	MSPD	LC-MS/MS	4	Zhang et al. (2013)
	Wheat	QuEChERS	UHPLC	45	Rubert et al. (2012)
	Wheat semolina		GC-MS/MS	50	Zachariasova et al. (2010)
	Wheat flour	MSPD	LC-MS/MS	1	Rodríguez-Carrasco et al. (2012)
FUS-X	Wheat flour	QuEChERS	GC-MS	18	Rubert et al. (2012)
	Wheat semolina		GC-MS/MS	30	Cunha & Fernandes (2010)
DAS	Wheat flour	MSPD	LC-MS/MS	5	Rodríguez-Carrasco et al. (2012)
	Wheat semolina	QuEChERS	GC-MS/MS	30	Rubert et al. (2012)
	Wheat flour		LC/MS	5	Sospedra et al. (2010)
NIV	Wheat flour	MSPD	LC-MS/MS	6	Rubert et al. (2012)
	Wheat semolina	QuEChERS	GC-MS	40	Cunha & Fernandes (2010)
	Wheat flour		GC-MS/MS	3	Rodríguez-Carrasco et al. (2012)
	Wheat		LC/MS	100	Sospedra et al. (2010)
NEO	Wheat flour		UHPLC	100	Zachariasova et al. (2010)
	Wheat flour	MSPD	LC-MS/MS	110	Rubert et al. (2012)
HT-2	Wheat			75	Rubert et al. (2011)
	Wheat semolina	QuEChERS	GC-MS/MS	5	Rodríguez-Carrasco et al. (2012)
HT-2	Wheat flour	MSPD	LC-MS/MS	45	Rubert et al. (2012)
	Wheat semolina	QuEChERS	GC-MS/MS	3	Rodríguez-Carrasco et al. (2012)
	Wheat		UHPLC-MS/MS	3	Romero-González et al. (2011)
	Wheat flour		LC/MS	18	Sospedra et al. (2010)
T-2	Wheat		UHPLC	30	Zachariasova et al. (2010)
	Wheat flour	MSPD	LC-MS/MS	4	Zhang et al. (2013)
	Wheat		LC-MS/MS	10	Rubert et al. (2012)
	Wheat				Rubert et al. (2011)
T-2	Wheat semolina	QuEChERS	GC-MS/MS	5	Rodríguez-Carrasco et al. (2012)
	Wheat		UHPLC-MS/MS	5	Romero-González et al. (2011)
	Wheat flour		LC/MS	4	Sospedra et al. (2010)
	Wheat		UHPLC	10	Zachariasova et al. (2010)
T-2	Wheat		LC-MS/MS	2.5	Zhang et al. (2013)
	Wheat	MSPD	LC-MS/MS	6	Rubert et al. (2012)
	Wheat			5	Rubert et al. (2011)

Recovery studies for the whole procedure were carried out by spiking blank samples with the working standard solutions at  $100 \mu\text{g kg}^{-1}$  to correct the matrix signal suppression. Satisfactory results in terms of recoveries were found (70–110%) (Table 3). The results obtained are according to recoveries accepted by the Commission Regulation (EC) No. 401/2006. Precision studies showed that the method was repeatable ( $n = 6$ ) (RSD < 5%) and reproducible ( $n = 3$ ) (RSD < 12%).

The trueness of the method was supported by certified reference material (BRM003004). The analysis of the certified reference material showed a mean value of  $1025 \pm 23 \mu\text{g kg}^{-1}$  ( $n = 6$ ) when matrix-matched calibration was used. These calculated concentrations were satisfactory according to the certificated values  $1062 \pm 110 \mu\text{g kg}^{-1}$ . Considering the results of the

proposed method, this could be applied for regular monitoring of mycotoxins in wheat-based food by routine laboratories.

SRM chromatograms of a blank breadstick sample spiked at  $100 \mu\text{g kg}^{-1}$  of the multi-mycotoxin working solution (A), a certificated reference material (wheat artificially contaminated by DON at  $1032 \pm 110 \mu\text{g kg}^{-1}$ ) (B), and a naturally contaminated breadstick sample with DON and HT-2 at 42 and  $18 \mu\text{g kg}^{-1}$  (C), respectively, are shown in Figure 1.

#### Quantitation of mycotoxins in snack samples

The proposed QuEChERS-based procedure followed by GC-MS/MS determination was used to evaluate the mycotoxins occurrence in 61 breadstick samples (Figure 2). Results showed that 64% of samples were contaminated

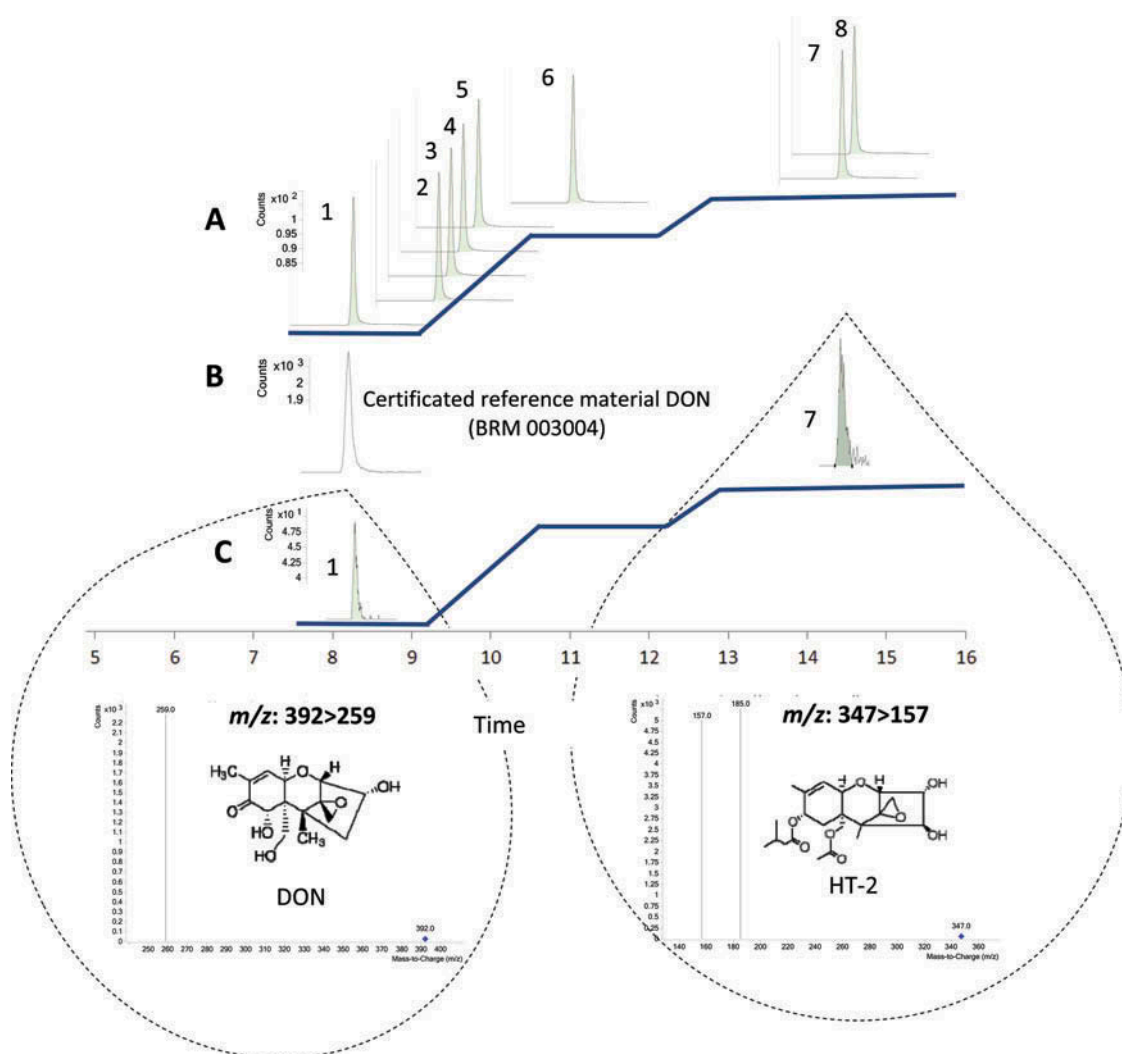


Figure 1. (colour online) SRM chromatogram of a blank breadstick with a chocolate sample spiked at  $100 \mu\text{g kg}^{-1}$  of the multi-mycotoxin working solution (A); certificated reference material (wheat artificially DON contaminated at  $1032 \pm 110 \mu\text{g kg}^{-1}$ ) (B); and breadstick with chocolate naturally contaminated with DON (1) and HT-2 toxin (7) at 42 and  $18 \mu\text{g kg}^{-1}$ , respectively (C).

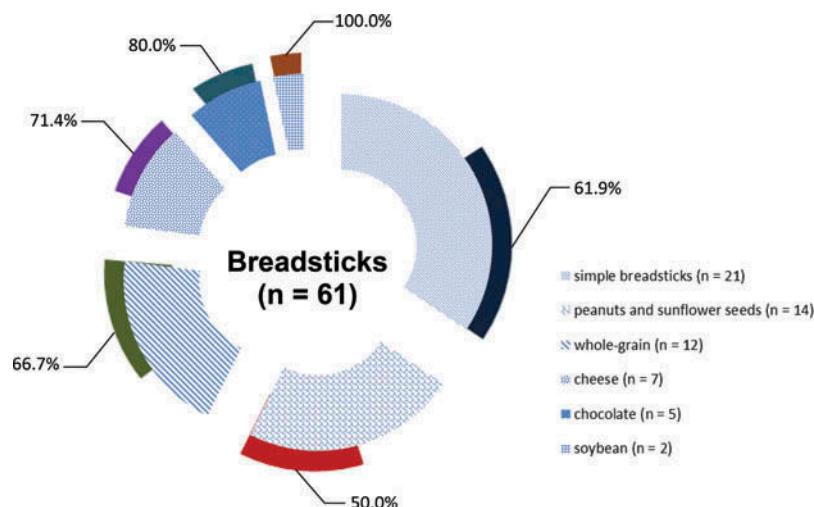


Figure 2. (colour online) Incidence of mycotoxins contamination (%) in breadstick samples analysed.

with at least one mycotoxin. DON was the mycotoxin detected in higher amount. Despite its considerable incidence, the concentration range found (from 30 to 60  $\mu\text{g kg}^{-1}$ , mean =  $32 \pm 8 \mu\text{g kg}^{-1}$ ) was much lower than the maximum limit established in the current legislation for this type of matrix (500  $\mu\text{g kg}^{-1}$ ). Taking into account the subdivision of the samples, breadstick samples with cheese, chocolate and soybean were the samples with the highest occurrence of DON, with incidences of 71.4%, 80% and 100%, respectively. Furthermore, two out of five samples containing chocolate showed co-occurrence of mycotoxins. The *Fusarium* toxins found were DON and HT-2 toxin, which were quantified at 32 and 35  $\mu\text{g kg}^{-1}$  of DON and at 17 and 22  $\mu\text{g kg}^{-1}$  of HT-2, respectively. As regards the other types of breadstick samples, incidences of DON of 50.0%, 61.9% and 66.7% were found in peanuts and sunflower seeds, simple and whole grain, respectively. NEO was also found in one whole-grain sample at 19  $\mu\text{g kg}^{-1}$ . No other studied *Fusarium* toxins were found in any of the samples analysed. Recently, Tolosa et al. (2013) reported the occurrence of *Fusarium* toxins in nuts, dried fruits and dates from Valencia, and thus the results obtained in the breadsticks combined with other ingredients could be justified. In this way, Sirot et al. (2013) reported DON contamination in bread and dried bread products at mean value of 132.1  $\mu\text{g kg}^{-1}$ . Similar values were obtained by González-Osnaya et al. (2011) in Spanish bread samples with a maximum concentration of 146.6  $\mu\text{g DON kg}^{-1}$  and in a recent study carried out in 34 fibre-enriched bread samples by De Boevre et al. (2013) where an average DON content of 34  $\mu\text{g kg}^{-1}$  was found.

A comparison of all whole-grain and simple breadstick samples was carried out in order to investigate the amount of mycotoxins in fibre-rich samples. Eight out of 12 whole-grain breadsticks were DON contaminated

(66.7%), whereas 13 out of 21 simple breadsticks contained DON (61.9%). In spite of the low DON contamination, the mean levels were similar being  $36 \pm 5$  and  $40 \pm 10 \mu\text{g kg}^{-1}$  for whole-grain and simple breadsticks, respectively. Statistical analysis (Student's *t*-test) of repeated measures was applied to analyse the results obtained. No significant statistical differences for a confidence interval of 95% were found between whole-grain and simple breadsticks. These results are in line with the study conducted by Vrček et al. (2014) who compared the nutritional value and food safety of organically and conventionally produced wheat flours. Although average concentrations of detected mycotoxins were higher in conventional than in organic flours, this difference was not significant. Similarly, Edwards (2009) and Ok et al. (2011) did not find statistically significant differences in the trichothecene content in the analysed cereals obtained from different agricultural practices.

#### DON intake

A deterministic analysis was performed in a first attempt to assess the dietary exposure of DON by breadsticks. Official consumption data were not available and the authors have estimated that the complete pack acquired from a supermarket is consumed on a daily basis (35 g). As specified in the exposure assessment section, the PDI was calculated. The PDIs obtained were 0.045 and 0.016  $\mu\text{g kg}^{-1}$  bw day for children and adults, respectively. The PDI was compared with PMTDI (1  $\mu\text{g kg}^{-1}$  bw day) as an approach to DON risk characterisation (SCF 2002). Results showed that the intake of the breadsticks amounted to 1.6% DON PMTDI in adults and 4.5% DON PMTDI in children.



## Conclusions

The GC-QqQ-MS/MS method proposed in this paper allowed the unambiguous identification of all mycotoxins studied below  $\mu\text{g kg}^{-1}$  levels, fulfilling the requirements established by the European Union and satisfactory results in terms of recovery were reached by QuEChERS-based sample preparation. Considering the advantages of the proposed method, this could be applied for regular monitoring of mycotoxins in wheat-based food by routine laboratories.

As regards occurrence of mycotoxins, a considerable number of contaminated breadstick samples (64%) contained at least one mycotoxin, but at concentrations lower than the European Union maximum level. Nonetheless, it has to be highlighted that several cereal-based commodities eaten daily should be considered for exposure assessment studies.

## Funding

This work was supported by the Spanish Ministry of Science and Innovation [grant number AGL2010-17024/ALI]. Y. Rodríguez-Carrasco thanks the FPU Grant [number AP2010-2940] provided by the Ministry of Education.

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