

# Determination of Trichothecenes in Cereal Matrices Using Subcritical Water Extraction Followed by Solid-Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry

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**Abstract** Subcritical water extraction followed by solid-phase extraction and ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection is reported for the first time for the determination of 6 trichothecenes (deoxynivalenol, deoxynivalenol-3-glucoside, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, HT-2 toxin, and T-2 toxin) from different cereals. Water with 1% formic acid was used as the extraction solvent followed by a solid-phase extraction clean-up, achieving good performance with acceptable extraction recoveries, method detection limits between 0.05  $\mu\text{g kg}^{-1}$  and 4.0  $\mu\text{g kg}^{-1}$ , and method quantification limits between 0.4  $\mu\text{g kg}^{-1}$  and 20  $\mu\text{g kg}^{-1}$ . The use of water as the extraction solvent allowed a selective extraction affording low matrix effect levels and the detection and quantification of natural target trichothecenes at very low concentration levels. This extraction method was applied to different cereals, a pseudocereal and an oilseed sample, of which maize, millet, and oat were contaminated by at least one trichothecene.

**Keywords** Trichothecenes · Cereals · Subcritical water extraction · Solid-phase extraction · UHPLC-(ESI)MS/MS

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## Introduction

Cereals are the basis of human nutrition together with the consumption of fruits and vegetables. During recent years, some cereals, pseudocereals, and oilseeds have gained much more relevance than they formerly had, due to an increase in human interest with respect to having healthier nutrition, as well as an increase in food intolerances. Some examples are sorghum, millet, rye, buckwheat, quinoa, sesame seeds, oat, and spelt, among others (Arendt and Dal Bello 2008; Ačanski et al. 2015). The growth in cereal consumption also leads to an increase in the potential ingestion of mycotoxins. Although there are ways to try to reduce mycotoxin concentration, such as milling and cleaning the cereal grains, avoiding their growth is practically impossible (Kostelanska et al. 2011). For this reason, it is necessary to determine their presence in the human diet.

Among all of the reported types of mycotoxins, there is a family of cyclic sesquiterpenoids with low molecular weight (~200–500 Da) called trichothecenes, which appear predominantly in cereals and cereal derivatives, mainly wheat, barley, and corn (Pereira et al. 2014). These mycotoxins are divided into four groups (from type A to D), with types A and B being the most common (Krska et al. 2007). The compounds that generate the greatest interest in view of their toxicity and occurrence classified as type A trichothecenes are HT-2 and T-2 toxins, and those classified as type B are deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3AcDON), and 15-acetyl-deoxynivalenol (15AcDON). Although acetylated forms are DON derivatives produced by fungi, they are considered to be native mycotoxins, which are a classification of free and unmodified mycotoxins (Payros et al. 2016). DON can also be modified biologically by the plant microbiota, producing deoxynivalenol-3-glucoside (DON3G), or animal microbiota, producing de-epoxy DON (DOM-1), 3-epi-DON, and 3-keto-

DON (Payros et al. 2016). Acetylated forms of DON, which display similar or lower toxicity than their precursor (Pestka 2008), commonly appear simultaneously but less frequently than DON (Berthiller et al. 2013; EFSA 2013a). With regard to the glycosylated form, no toxic effects have been demonstrated to date for DON3G in mammals (JECFA 2011), but several authors have reported that colonic microbiota in the large intestine can hydrolyze DON3G, 3AcDON, and 15AcDON, releasing DON, which can be absorbed in the gut (Maresca 2013; Nagl et al. 2014). European regulations have established a maximum permitted level for DON (EC 2007), which varies from 500 to 1750  $\mu\text{g kg}^{-1}$ , depending on the matrices of adult foodstuffs, and recommend a maximum level for HT-2 and T-2 toxins, which varies from 25 to 1000  $\mu\text{g kg}^{-1}$  (EC 2013). Although European regulations are in the process of including DON derivatives within its guidelines (EFSA 2013b), at present, there is no regulation affecting them. With respect to the Joint FAO/WHO Expert Committee on Food Additives (JECFA), a provisional maximum tolerable daily intake (PMTDI) of 1  $\mu\text{g kg}^{-1}$  body weight (bw) for 3AcDON and 15AcDON has been established because the organization considers that toxicity of these derivatives is the same as their precursor's (JECFA 2011). Meanwhile, there is insufficient information on DON3G toxicity to establish a PMTDI (JECFA 2011). Thus, suitable analytical instrumentation and extraction methods can help to establish a clear approach to trichothecene regulation, as it should be able to monitor such low levels.

Previous studies have shown suitable extraction techniques for mycotoxins from different kinds of solid matrices, such as solid-liquid extraction (SLE) (Rubert et al. 2013), QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe extraction) (JiaoJiao et al. 2016; Zhou et al. 2016), pressurized liquid extraction (PLE) (Kokkonen and Jestoi 2009; Campone et al. 2015), and microwave-assisted extraction (MAE) (Pallaroni et al. 2002; Pallaroni and Von Holst 2003). However, SLE and QuEChERS have certain disadvantages in comparison with PLE and MAE, such as they are less automated. The development of extraction methods using water is a sustainable alternative to these classical procedures. PLE and MAE are effective options because they provide effective extractions and they can be used with alternative and less contaminating solvents (Pallaroni and Von Holst 2003; Armenta et al. 2015). Comparing PLE and MAE, PLE might be better as the extraction process can be more automated and it is well-accepted for routine analysis of environmental and food contaminants (Campone et al. 2015). This technique can be also more sustainable if water is used as the extraction solvent, in which case, it is known as subcritical water extraction (SWE) or pressurized hot water extraction (PHWE). Using hot water under pressure, in order to maintain it in liquid state, allows the isolation of valuable components. SWE has largely been used to extract several analytes, such as insecticides and phenolic

compounds, from diverse matrices, such as plants and oils, according to related reviews (Teo et al. 2010; Herrero et al. 2013). However, to the best of our knowledge, SWE has never been used to extract mycotoxins from cereal matrices.

Another advantage of the use of water as the solvent in PLE is that it allows the subsequent selective cleaning of the obtained extracts, using solid-phase extraction (SPE) without any previous solvent exchange, thereby reducing the analysis time. In this respect, the inclusion of a cleaning step reduces or even prevents matrix effects (ME) which can lead to significant overestimation or underestimation of mycotoxin concentration. An effective clean-up prevents or reduces these interferences, enabling sensitive, selective, and robust liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) analysis. Furthermore, the use of water allows milder extraction conditions and at the same time, more selective extraction.

The aim of the present research is to develop a method based on SWE followed by SPE clean-up and ultra-high performance liquid chromatography coupled with tandem mass spectrometry detection (UHPLC-MS/MS), for the simultaneous determination of the six most abundant trichothecenes (DON and its derivatives DON3G, 3AcDON, and 15AcDON; HT-2; and T-2), from different types of cereals, a pseudocereal and an oilseed widely present in the human diet.

## Materials and Methods

### Reagents and Chemicals

The target mycotoxins were six: DON, T-2, HT-2, DON3G, 3AcDON, and 15AcDON (> 99% purity). DON, T-2, and HT-2 were purchased from Trilogy Analytical Laboratory (Washington, MO, USA) and DON3G, 3AcDON, and 15AcDON were purchased from Romer Labs (Union, MO, USA). DON was sold in methanol (MeOH) at 25  $\text{mg L}^{-1}$ , T-2 and HT-2 in acetonitrile (ACN) at 100  $\text{mg L}^{-1}$ , and DON3G in ACN at 50.9  $\text{mg L}^{-1}$ . 3AcDON and 15AcDON were obtained in powder form. A mix solution of all of the mycotoxins at different concentrations was prepared, taking into account their response in (ESI)MS/MS, obtaining similar mycotoxin response values. HT-2 and DON3G were prepared at 1  $\text{mg L}^{-1}$ ; DON, 3AcDON, and 15AcDON at 0.5  $\text{mg L}^{-1}$ ; and T-2 at 0.1  $\text{mg L}^{-1}$ . This mix solution was prepared in water/MeOH (80:20, v/v) and stored at  $-20\text{ }^{\circ}\text{C}$ .

Ultra-pure-grade water was obtained by a Milli-Q water purification system (Millipore, Darmstadt, Germany). MeOH and ACN (both LC-MS grade) were obtained from Panreac (Barcelona, Spain), and acetone was obtained from VWR International (Fontenay-sous-Bois, France). Formic acid (HCOOH) ~ 98% was purchased from Fluka (St. Louis, MO, USA). Ammonium formate ( $\text{NH}_4\text{HCOO}$ ) aqueous solution 10 M was purchased from Sigma-Aldrich (St. Louis, MO,

USA) and diatomaceous earth (DE) was acquired from Thermo Scientific (Sunnyvale, CA, USA). The SPE cartridges were 150-mg OASIS HLB from Waters (Wexford, Ireland) and 200-mg ISOLUTE ENV+ from International Sorbent Technology LTD (Mid Glamorgan, UK).

Working with mycotoxins implies taking various security measures, such as using double gloves (made of latex and nitrile) and cleaning all the materials that have been in contact with mycotoxins with 20% commercial sodium hypochlorite (NaClO).

### Liquid Chromatography-Mass Spectrometry

An Agilent 1290 Infinity LC Series coupled with a 6495 iFunnel Triple Quadrupole MS/MS with electrospray ionization (ESI) interface was used for chromatographic analysis, both from Agilent Technologies (Waldbronn, Germany). Chromatographic separation was achieved using a Cortecs UHPLC C<sub>18</sub> column (100 × 2.1 mm, 1.6 μm) from Waters. A binary mobile phase was used for the chromatographic separation, comprised of water (solvent A) and MeOH (solvent B), both with 5-mM NH<sub>4</sub>HCOO and 0.1% HCOOH. The gradient elution started at 10% B and maintained this percentage for 2 min. Over the next 5.5 min, the gradient increased to 20% and was held again under isocratic conditions for 3.5 min. It was then increased to 95% in 5 min and held under isocratic conditions for 2 min. Finally, it was returned to the initial conditions in 1 min and maintained for 2 min to equilibrate the column. The injection volume was 10 μL, flow rate was fixed at 0.45 mL min<sup>-1</sup>, and the separation was performed at 40 °C. The autosampler was kept at 4 °C.

The optimized source parameters were capillary voltage of 4000 V for DON3G and 3500 V for the rest of compounds; desolvation gas flow and temperature of 18 L min<sup>-1</sup> and 160 °C, respectively; nebulizer pressure of 35 psi; nozzle voltage of 2000 V for DON3G and 500 V for the rest; fragmentor voltage of 380 V; cell acceleration voltage of 5 V; and sheath gas flow and temperature of 11 min<sup>-1</sup> and 350 °C, respectively. The high- and low-pressure funnel parameters were, respectively, 90 and 60 V for DON3G and 150 and 60 V for the rest of mycotoxins. Multiple reaction monitoring (MRM) experiments were carried out in positive polarity for all of the studied compounds with three representative MRM transitions for each mycotoxin, in accordance with the European Commission guidelines (SANTE 2015). The collision energy was optimized for each product ion and they are detailed in Table 1, together with all MRM parameters obtained.

### Sampling

Prior to the extraction and analysis, studied matrices were ground with the mill Taurus Aromatic (Taurus Group, Oliana, Spain), sifted twice in 500- and 100-μm sieves and

homogenized. For spiked samples, 2 mL of acetone was added to 1 g of each sample in a 100-mL beaker, in order to spike the matrix homogeneously. Subsequently, 100 μL of the mix solution (see “Reagents and Chemicals” for concentrations) was added to the suspension and left overnight in a stirrer to let the mycotoxins come into contact with the sample and until the acetone was completely evaporated. Matrices were spiked at three different mycotoxin concentrations according to their sensitivity in UHPLC-(ESI)MS/MS, in order to obtain similar analytes response. The matrix used for method development and validation was maize, and the other matrices studied were three different cereals (spelt, millet, and oat), one pseudocereal (quinoa), and one oilseed (sesame seed), all obtained from local markets.

### Sample Extraction

For the SWE, a homogeneous mix of 1 g of sample and 1 g of DE was poured into an 11-mL stainless steel extraction cell, which was packed by inserting a layer of DE at the bottom and at the top (approximately 0.3 g for each layer) and a cellulose filter at the bottom, following the manufacturer’s recommendations. Extractions were achieved on a Dionex ASE 350 accelerated solvent extractor (Dionex Corp., Sunnyvale, CA, USA). The SWE conditions were as follows: water with 1% HCOOH as the extraction solvent, 80 °C with 5 min of cell preheating, 1500-psi extraction pressure, flush volume of 50%, purge time of 60 s, and a single extraction cycle of 5 min. The obtained extracts of volumes around 15 mL were cleaned up in OASIS HLB cartridges, previously conditioned with 10 mL of MeOH and 10 mL of water with 1% HCOOH (pH 2.0). The mycotoxins were eluted with 5 mL of MeOH and evaporated to dryness with a miVac vacuum concentrator (Genevac LTD, Ipswich, UK). The mycotoxins were re-suspended with 2 mL of water/MeOH (80:20, v/v) and filtered with a 0.45-μm nylon filter (Phenomenex, Torrance, CA, USA) just prior to analysis.

## Results and Discussion

### Instrumental Optimization

Precursor ions were selected testing positive and negative modes with the mobile phase based on previous studies developed for similar mycotoxin groups (Zachariasova et al. 2010; Rubert et al. 2014; Veprikova et al. 2015; Miró-Abella et al. 2017). That is, the solvents tested were water/MeOH (50:50, v/v) with two acids (formic and acetic acid) at 0.1% (v/v) and two salts (ammonium formate and acetate) at 5 mM being added to both solvents, either alone or in combination, resulting in 6 different solutions. The mycotoxins were injected individually in order to select the ions from the target

**Table 1** LC-MS/MS parameters for trichothecenes determination

Start time (min)	Mycotoxin	Retention time (min)	Molecular weight (g mol <sup>-1</sup> )	Precursor ion (m/z)	Dwell time (ms)	Product ion (q/Q ratio)	CE (eV)
0	DON	2.9	296.3	297.1	[DON + H] <sup>+</sup>	248.9	8
						231.1 (66)	10
						203.1 (59)	8
4.4	DON3G	3.6	458.4	297.1	[DON3G - 3G + H] <sup>+</sup>	248.9	8
						231.1 (66)	10
						203.1 (59)	8
4.4	3AcDON	9.8	338.3	339.2	[3AcDON + H] <sup>+</sup>	231.0	8
						203.0 (39)	24
						175.0 (23)	18
5.8	15AcDON	10.1	338.3	356.2	[15AcDON + NH <sub>4</sub> ] <sup>+</sup>	339.1	16
						321.0 (42)	12
						136.9 (36)	4
5.8	HT-2	14.7	424.4	442.2	[HT-2 + NH <sub>4</sub> ] <sup>+</sup>	215.1	8
						262.9 (90)	8
						196.9 (31)	20
5.8	T-2	15.1	466.5	484.2	[T-2 + NH <sub>4</sub> ] <sup>+</sup>	214.9	16
						304.9 (80)	12
						185.0 (66)	10

compounds by flow injection analysis (FIA) at a flow rate of 0.45 mL min<sup>-1</sup>, at the following concentrations: 1 mg L<sup>-1</sup> for HT-2 and DON3G; 0.5 mg L<sup>-1</sup> for DON, 3AcDON, and 15AcDON; and 0.1 mg L<sup>-1</sup> for T-2. Taking into account adducts with the greater response in each mobile phase combination, the solution with ammonium formate and formic acid was the one that provided the highest response. In consequence, this was chosen as the mobile phase for the chromatographic separation. With this mobile phase, precursor ions appeared in greater abundance in positive mode. DON was ionized as [DON + H]<sup>+</sup> in the more abundant form, and DON3G gave the same transition than DON by losing the glucoside fragment. Therefore, DON and DON3G had the same precursor ion. With respect to both acetylated DON derivatives, their most abundant ion was the protonated form [M + H]<sup>+</sup>. However, [15AcDON + NH<sub>4</sub>]<sup>+</sup> was selected as the ion for 15AcDON, whereas the protonated adduct [3AcDON + H]<sup>+</sup> was selected for 3AcDON, not only to avoid possible interferences, but also to enhance analyte selectivity and sensitivity. Finally, the ammonium adducts [M + NH<sub>4</sub>]<sup>+</sup> of HT-2 and T-2 toxins were selected, as they are the most abundant forms.

After the selection of the correspondent precursor ions and the mobile phase, different product ions were selected for each mycotoxin by applying different collision energies, in order to obtain the three most abundant MRM transitions that will facilitate the correct mycotoxin identification, as recommended by the EU Directive (SANTE 2015), and these are detailed in Table 1. Further source parameters were also optimized and are detailed in “Liquid Chromatography-Mass Spectrometry.”

With regard to the chromatographic gradient, it was mainly focused on the separation of DON and DON3G which were well-resolved and it was possible to select the same precursor ion for both.

Once MS values were optimized and chromatographic separation was achieved, instrumental linearity and limits of detection (LOD) and quantification (LOQ) were established. LODs and LOQs were calculated as the lowest mycotoxin concentration that the quantifier and qualifier transitions displayed a signal-to-noise ratio (S/N) ≥ to 3 and 10, respectively. The LODs obtained were from 0.01 to 0.2 µg L<sup>-1</sup> for all compounds, except for DON3G, for which was 0.7 µg L<sup>-1</sup>. The LOQs ranged from 0.2 to 0.5 µg L<sup>-1</sup> for all compounds, except for DON3G, for which was 2.5 µg L<sup>-1</sup>. The linearity was suitable (with  $r^2 \geq 0.994$ ) and it ranged from LOQs used as the lowest concentration to 20 µg L<sup>-1</sup> for T-2, to 100 µg L<sup>-1</sup> for DON, to 500 µg L<sup>-1</sup> for acetylated forms, and to 1000 µg L<sup>-1</sup> for DON3G and HT-2.

### Optimization of Extraction

Taking into consideration that in previous studies (Sánchez Maldonado et al. 2014; Plaza and Turner 2015), the SWE of several compounds in a wide range of matrices was achieved successfully, a SWE was tested to extract the target mycotoxins from the cereal matrices. Water was acidified with 1% of HCOOH (pH 2.0) in order to improve the extraction, as in the aforementioned studies. Using acidified water as the



extraction solvent, it is not necessary to do any change of the solvent for a clean-up process using a SPE cartridge.

Prior to SWE, the SPE process was optimized. Two different cartridges were tested: an OASIS HLB and an ISOLUTE ENV+. A total volume of 25 mL of water solution with target mycotoxins at 25  $\mu\text{g L}^{-1}$  for T-2; 125  $\mu\text{g L}^{-1}$  for DON, 3AcDON, and 15AcDON; and 500  $\mu\text{g L}^{-1}$  for HT-2 and DON3G, was loaded into the previously conditioned cartridge. The mycotoxins were then eluted with three sequential fractions of MeOH: a first fraction of 3 mL, a second fraction of 2 mL, and a third of 2 mL. Most of the mycotoxins eluted at the first 3 mL. The second fraction also contained some mycotoxins, with a recovery up to 10%. But in the third fraction, the mycotoxins' presence was insignificant. Consequently, a single elution of 5 mL of MeOH was selected. Table 2 details all the recovery results. Obtained recovery values (%Rec SPE std) were slightly higher for OASIS HLB, especially for the more polar compounds. However, both cartridges obtained good recovery values, all higher than 76%. Further tests were performed in order to discard interactions between the cartridge and the matrix. For that, instead of water solution, extracts from SWEs of non-spiked maize samples were used, which were spiked at the same concentration as above after SWE extraction. The obtained recoveries (%Rec SPE matrix) were lower than in water solution, decreasing equally in both cartridges. However, recoveries were slightly higher for OASIS HLB (detailed in Table 2).

Then, SWE optimization was performed taking into consideration the parameters with the greatest influence, namely temperature and number of cycles, as well as the extraction solvent, and maintaining the other parameters as described in "Sample Extraction." To do so, 1 g of homogenized maize sample was poured into a stainless steel extraction cell with DE, as explained in "Sample Extraction," and two different SWE temperatures were examined: 80 °C and 100 °C. Both temperatures provided suitable results in a similar order of

magnitude, so a temperature of 80 °C was selected. Moreover, the number of SWE cycles was tested. The second cycle obtained an insignificant signal response, and a single extraction cycle was finally selected.

Once SWE parameters were optimized, the SWE extract was loaded into both SPE cartridges, and the mycotoxins were eluted; the extract was evaporated and re-suspended with the same solvent conditions as the initial mobile phase: 1 mL of water/MeOH (80:20, v/v), in order to obtain their recovery of the whole extraction. Two different groups of concentrations were tested to calculate the recoveries of the entire method. These two groups were chosen in order to obtain similar response values of all compounds and taking into account their linear ranges. One group was at 1  $\mu\text{g kg}^{-1}$  (for T-2), at 5  $\mu\text{g kg}^{-1}$  (for DON, 3AcDON, and 15AcDON), and at 20  $\mu\text{g kg}^{-1}$  (for HT-2 and DON3G). The other concentration group was at 15  $\mu\text{g kg}^{-1}$  (for T-2), at 75  $\mu\text{g kg}^{-1}$  (for DON, 3AcDON, and 15AcDON), and at 200  $\mu\text{g kg}^{-1}$  (for HT-2 and DON3G). The %Rec SWE + SPE was calculated by comparing the concentration obtained from samples spiked before the extraction process with the concentration obtained from samples spiked after the extraction process. The obtained recovery values were similar at both tested groups, and just values when the sample was spiked at the lower concentration are shown in Table 2. As can be seen, the recovery values (%Rec SWE + SPE) obtained when OASIS HLB was used in the SPE are slightly higher than those achieved with ISOLUTE ENV+. Thus, OASIS HLB was selected for further experiments. In addition, from the %Rec SWE + SPE values, we can confirm that the SWE parameters as well as the use of water as solvent are a suitable option to extract these mycotoxins from cereals.

In addition, ME were evaluated and the values were obtained by comparing the concentration obtained when the samples were spiked after the whole extraction process with the concentration obtained with the pure standard, and considering ME = 0 (no matrix effect), ME > 0 (ion enhancement),

**Table 2** Trichothecene recoveries from SPE (%Rec SPE) and extraction recoveries from SWE and SPE (%Rec SWE + SPE) of both tested cartridges. Matrix effects from OASIS HLB (%ME) are also

detailed, from a dilution of 1 and 2 mL. Matrix was the maize sample spiked with the mycotoxins mixture, which concentrations are specified in the text

	ISOLUTE ENV+			OASIS HLB				
	%Rec SPE std	%Rec SPE matrix	%Rec SWE + SPE	%Rec SPE std	%Rec SPE matrix	%Rec SWE + SPE	%ME 1 mL	%ME 2 mL
DON	76	70	69	92	73	77	-48	-24
DON3G	84	69	49	101	76	63	-35	-21
3AcDON	105	87	51	94	93	69	13	10
15AcDON	106	98	46	110	103	59	-8	3
HT-2	94	76	45	94	78	56	-39	-25
T-2	88	37	41	82	41	53	-39	-24

$n = 3$ , RSD lower than 3% in both %Rec SPE, lower than 12% in both %Rec SWE + SPE. Results obtained from a final resuspension of 2 mL

and  $ME < 0$  (ion suppression). The obtained ME values are shown in Table 2, and it can be observed that all of the mycotoxins, except the acetylated forms of DON, are highly affected by ion suppression due to the complexity and composition of the maize samples. In order to reduce these ME values, an option could be the use of isotopically labeled standards for each compound, but it could not be afforded because of their elevated cost. For that, the mycotoxins were diluted in a re-suspension of 2 mL of water/MeOH (80:20, *v/v*) solution instead of 1 mL. The results improved slightly as can also be observed in Table 2, with the percentage of ME reduced in all cases. Even in the case of some mycotoxins, such as DON, HT-2 and T-2, the ME reduced by nearly half.

Once the recovery and ME results for maize were obtained, and in order to evaluate the applicability of the developed method to other samples, three different cereals (spelt, millet, and oat), one pseudocereal (quinoa), and one oilseed (sesame seed) were spiked with the target mycotoxins, in the same way and concentrations as the maize samples. Different extraction recoveries and ME were obtained from each matrix after a dilution of 2 mL, as detailed in Table 3. The obtained results were similar to those obtained in maize samples, especially in the case of spelt and quinoa samples. Oat, millet, and sesame displayed slightly lower recoveries. In the case of 3AcDON, in sesame matrices, the recovery was not calculated since there was an interference which masked the mycotoxin and it was not possible to quantify it; thereby, they are not collected in Table 3. With regard to ME for all matrices, they were considerably low. A previous extraction research was based on the use of PLE with organic solvents (Kokkonen and Jestoi 2009), and the ME obtained were higher for the same analytes due to the use of a less selective extraction solvent. Thus, using water as extraction solvent could be a suitable alternative because it extracts the mycotoxins and at the same time, does not extract many interferences as can be observed with the lower percentage of ME obtained from the extracts diluted with 2 mL. The reported method is adequate to quantify

trichothecenes which appear naturally in complex matrices and at low concentrations. In addition, the present procedure allows a more effective and selective extraction, with lower ME, and it is more sustainable than classical PLE.

### Method Validation

Method validation parameters, such as linear range, LOD, LOQ, repeatability, and reproducibility, were evaluated using 1 g of maize samples spiked with the target trichothecenes. First of all, the presence of natural contamination was evaluated and taken into account by substrating the signal from contaminated samples. Then, the linear range was assessed from LOQs to 40  $\mu\text{g kg}^{-1}$  for T-2, to 200  $\mu\text{g kg}^{-1}$  for DON and its acetylated forms, and to 400  $\mu\text{g kg}^{-1}$  for DON3G and HT-2. The linearity was acceptable with  $r^2$  higher than 0.990. LODs and LOQs were obtained in the same way as in the case of instrumental limits described above in “Instrumental Optimization.” The LODs obtained were 0.05  $\mu\text{g kg}^{-1}$  for T-2; between 0.5 and 1.0  $\mu\text{g kg}^{-1}$  for DON, 3AcDON, 15AcDON, and HT-2; and 4.0  $\mu\text{g kg}^{-1}$  for DON3G. With respect to LOQs, they ranged between 0.4 and 1.0  $\mu\text{g kg}^{-1}$  for DON, 3AcDON, 15AcDON, and T-2; 4.0  $\mu\text{g kg}^{-1}$  for HT-2; and 20  $\mu\text{g kg}^{-1}$  for DON3G. The regulation for maize samples permits a maximum level for DON of 1750  $\mu\text{g kg}^{-1}$  (EC 2007), recommends a maximum level for the sum of T-2 and HT-2 of 100  $\mu\text{g kg}^{-1}$  (EC 2013), and recommends a maximum level for 3AcDON and 15AcDON of 1  $\mu\text{g kg}^{-1}$  (JECFA 2011). Taking into account these regulated levels and using them as reference values, the obtained LOQs are acceptable because they are below them. In some mycotoxins such as DON, HT-2, and T-2, LOQ values are more than 100 times lower than the regulation values, denoting that it could be a good method to detect possible food and feed trichothecene natural contamination. There is in the literature previous researches which analyze diverse mycotoxins, by PLE with organic solvents and LC-MS/MS (Kokkonen and Jestoi 2009;

**Table 3** Trichothecene extraction recoveries (%Rec SWE + SPE) and matrix effects (%ME) of the studied samples. Spiking concentrations are specified in the text

	Spelt		Millet		Oat		Quinoa		Sesame	
	%Rec SWE + SPE	%ME	%Rec SWE + SPE	%ME	%Rec SWE + SPE	%ME	%Rec SWE + SPE	%ME	%Rec SWE + SPE	%ME
DON	60	-18	48	-17	53	-34	45	-8	72	-45
DON3G	49	-3	39	-6	35	-15	42	-6	48	-5
3AcDON	40	13	28	15	41	-18	35	28	-	-
15AcDON	46	8	34	9	27	2	33	9	50	14
HT-2	52	-7	34	4	25	1	39	7	44	6
T-2	47	-17	39	-14	30	-15	37	-12	33	-9

*n* = 3, RSD lower than 20%

**Table 4** Maximum trichothecene concentration ( $\mu\text{g kg}^{-1}$ ) extracted from the studied samples

	Maize	Spelt	Millet	Oat	Quinoa	Sesame
DON	15.6 to 17.8	< LOD to < LOQ	< LOQ to 8.1	< LOQ to 64.5	< LOD to < LOQ	–
DON3G	< LOQ to 2.9	–	–	< LOD to 8.7	–	–
3AcDON	< LOQ	–	–	–	< LOD to < LOQ	–
15AcDON	15.8 to 16.7	< LOD to < LOQ	< LOQ to 3.3	< LOQ to 10.6	–	–
HT-2	< LOD to 7.4	–	–	< LOQ to 35.2	< LOD to < LOQ	–
T-2	< LOD to 2.1	–	< LOD to < LOQ	< LOQ to 4.5	–	–

$n = 3$ ,

(–) not detected

Desmarchelier et al. 2010). In these researches, target mycotoxins also were extracted, among others, obtaining LOD and LOQ values higher than those obtained in the present research, denoting that SWE could be a good tool to extract type A and type B trichothecenes.

Method repeatability (intra-day,  $n = 5$ ) and reproducibility (inter-day,  $n = 5$ ) were obtained from different trichothecene concentration tests: T-2 at  $1 \mu\text{g kg}^{-1}$ ; DON, 3AcDON, and 15AcDON at  $5 \mu\text{g kg}^{-1}$ ; and HT-2 and DON3G at  $10 \mu\text{g kg}^{-1}$ . Repeatability and reproducibility were expressed as relative standard deviation percentage (%RSD), and they were acceptable in accordance with current guidelines (SANTE 2015). The obtained results were between 6 and 9% for the repeatability and between 16 and 18% for the reproducibility.

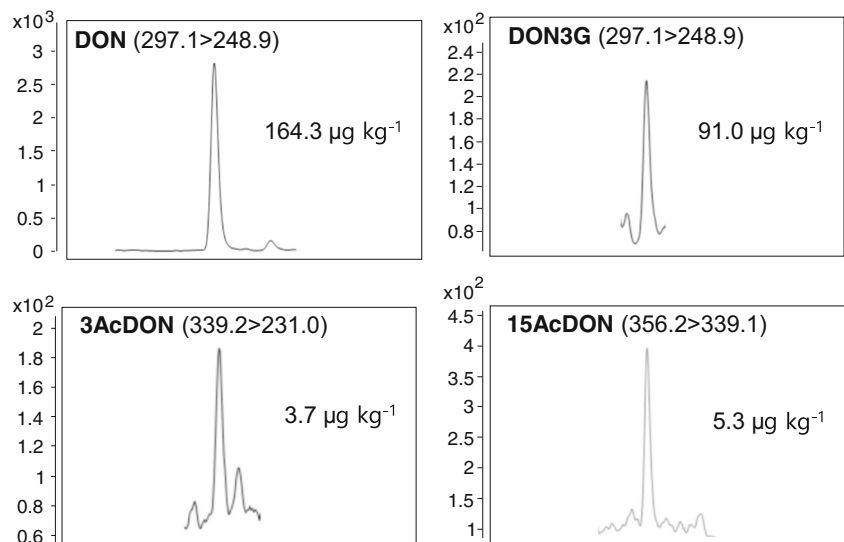
### Application to Different Samples

Once the method was successfully applied to maize samples, the natural presence of trichothecenes was studied using three different commercial brands of each cereal, pseudocereal, and oilseed ( $n = 18$ ). Considering that the extraction recoveries were satisfactory and the repeatability of the method too,

quantification of mycotoxins in the cereal samples was proposed using external calibration curve and applying the total recovery values (recovery explained in “Optimization of Extraction”). This was further proved by quantifying the mycotoxins present in maize sample by using the two approaches: matrix-matched calibration curve and external calibration curve plus total recovery percentage. The accuracy of both approaches was from 76 to 112%.

At least one mycotoxin was detected in all of the six samples studied, and they could be quantified in three cases: maize, millet, and oat. Different interval concentrations were found in the three different brands, and they are detailed in Table 4. DON was found in all the samples at low level, except in sesame samples. DON was detected in spelt and quinoa samples and quantified in maize in values up to  $17.8 \mu\text{g kg}^{-1}$ , in oat up to  $64.5 \mu\text{g kg}^{-1}$ , and in millet up to  $8.1 \mu\text{g kg}^{-1}$ . This mycotoxin displayed the greatest trichothecene incidence ratio. Previous studies have also reported the presence of this trichothecene in the samples indicated (Jestoi et al. 2004; Schollenberger et al. 2005; Krysińska-Traczyk et al. 2007; Juan et al. 2013). Furthermore, 15AcDON also was quantified in maize up to  $16.7 \mu\text{g kg}^{-1}$  and in oat up to  $10.6 \mu\text{g kg}^{-1}$ . With regard to the oat matrix, mycotoxin co-exposure is

**Fig. 1** Quantitative transition MRM chromatograms of detected trichothecenes in highly contaminated maize sample



common, as identified in the previous studies (Schollenberger et al. 2005). As such, three more mycotoxins were quantified in oat: DON3G up to  $8.7 \mu\text{g kg}^{-1}$ , HT-2 up to  $35.2 \mu\text{g kg}^{-1}$ , and T-2 up to  $4.5 \mu\text{g kg}^{-1}$ . The concentration found in these samples is similar to those described in a previous study (Gottschalk et al. 2007).

From all the studied samples, there were some maize samples which were visually contaminated by fungi. The results obtained showed the presence of DON at  $164.3 \mu\text{g kg}^{-1}$ , DON3G at  $91.0 \mu\text{g kg}^{-1}$ , 3AcDON at  $3.7 \mu\text{g kg}^{-1}$ , and 15AcDON at  $5.3 \mu\text{g kg}^{-1}$ , the quantitative transition MRM chromatograms of which are shown in Fig. 1. These values are not detailed in Table 4, since this sample was singular. If these concentrations are compared with those quantified in the maize samples without visual contamination, it can be observed that, for example, DON concentration was more than 5-fold. Therefore, it has been shown how visual contamination can anticipate the presence of mycotoxins.

From all these obtained results, one of the most important facts is that it was possible to extract these six different trichothecenes without using organic solvents at very low concentrations and with low ME.

## Conclusions

For the first time, a method has been developed for the determination of six trichothecenes using SWE followed by an SPE clean-up and UHPLC-(ESI)MS/MS. The improved alternative extraction used acidified water as solvent followed by a straight-forward clean-up step. Although better recoveries would be obtained using an organic extraction solvent, water allowed better selectivity by obtaining lower ME levels. This decrease in ME levels involved the quantification of the target mycotoxins at very low concentrations and a selective detection of the natural presence of trichothecenes in the studied samples. The performance of the method may indicate a benefit of using alternative solvents, such as water, able to obtain results as sensitive and reliable as those provided by organic solvents.

Further research should be focused on the improvement of the purification step, by using less organic solvents and becoming more alternative, apart from broadening the applicability of the method by including more mycotoxins in different type of samples.

## Compliance with Ethical Standards

**Conflict of Interest** Eugènia Miró-Abella declares that she has no conflict of interest. Pol Herrero declares that he has no conflict of interest. Núria Canela declares that she has no conflict of interest. Lluís Arola declares that he has no conflict of interest. Rosa Ras declares that she has no conflict of interest. Núria Fontanals declares that she has no conflict of interest. Francesc Borrull declares that he has no conflict of interest.

## Research Involving Human Participants and/or Animals

**Ethical Approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed Consent** Not applicable.

## References

- Ačanski MM, Vujić DN, Psodorov DB (2015) Practical method for the confirmation of authentic flours of different types of cereals and pseudocereals. *Food Chem* 172:314–317
- Arendt E, Dal Bello F (2008) Gluten-free cereal products and beverages. *Food Sci Technol* 4-8:81–190
- Armenta S, Garrigues S, de la Guardia M (2015) The role of green extraction techniques in Green Analytical Chemistry. *Trends Anal Chem* 71:2–8
- Berthiller F, Crews C, Dall'Asta C et al (2013) Masked mycotoxins: a review. *Mol Nutr Food Res* 57:165–186
- Campone L, Piccinelli AL, Celano R et al (2015) A fully automated method for simultaneous determination of aflatoxins and ochratoxin A in dried fruits by pressurized liquid extraction and online solid-phase extraction cleanup coupled to ultra-high-pressure liquid chromatography-tandem mass spectrometry. *Anal Bioanal Chem* 407:2899–2911
- Commission Recommendation EC (2013) No 2013/165/EU of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. *Off J Eur Union* L91:12–15
- Commission Regulation EC (2007) No 1126/2007 of 28 September 2007 amending regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. *Off J Eur Union* L255:14–17
- Desmarchelier A, Oberson J-M, Tella P et al (2010) Development and comparison of two multiresidue methods for the analysis of 17 mycotoxins in cereals by liquid chromatography electrospray ionization tandem mass spectrometry. *J Agric Food Chem* 58:7510–7519
- European Food Safety Authority, EFSA-Q-2013-00721 (2013a) Request for a scientific opinion on the risks for animals and public health related to the presence of deoxynivalenol, metabolites of deoxynivalenol and masked deoxynivalenol in food and feed. Available at [www.efsa.europa.eu](http://www.efsa.europa.eu). Accessed 12 Mar 2017
- European Food Safety Authority, EFSA (2013b) Deoxynivalenol in food and feed: occurrence and exposure. *EFSA J* 11:3379–3434
- Gottschalk C, Barthel J, Engelhardt G et al (2007) Occurrence of type A trichothecenes in conventionally and organically produced oats and oat products. *Mol Nutr Food Res* 51:1547–1553
- Herrero M, Castro-Puyana M, Mendiola JA, Ibañez E (2013) Compressed fluids for the extraction of bioactive compounds. *Trends Anal Chem* 43:67–83
- Jestoi M, Somma MC, Kouva M et al (2004) Levels of mycotoxins and sample cytotoxicity of selected organic and conventional grain-based products purchased from Finnish and Italian markets. *Mol Nutr Food Res* 48:299–307
- JiaoJiao X, Jian Z, BaiFen H et al (2016) Simultaneous and rapid determination of deoxynivalenol and its acetylate derivatives in wheat flour and rice by ultra high performance liquid chromatography with photo diode array detection. *J Sep Sci* 39:2028–2035
- Joint FAO/WHO Expert Committee on Food Additives, JECFA (2011) Evaluation of certain contaminants in food. *World Heal Organ Tech Rep Ser* 959:1–105
- Juan C, Ritieni A, Mañes J (2013) Occurrence of Fusarium mycotoxins in Italian cereal and cereal products from organic farming. *Food Chem* 141:1747–1755



- Kokkonen MK, Jestoi MN (2009) A multi-compound LC-MS/MS method for the screening of mycotoxins in grains. *Food Anal Methods* 2: 128–140
- Kostelanska M, Dzuman Z, Malachova A et al (2011) Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *J Agric Food Chem* 59:9303–9312
- Krska R, Welzig E, Boudra H (2007) Analysis of Fusarium toxins in feed. *Anim Feed Sci Technol* 137:241–264
- Krysińska-Traczyk E, Perkowski J, Dutkiewicz J (2007) Levels of fungi and mycotoxins in the samples of grain and grain dust collected from five various cereal crops in eastern Poland. *Ann Agric Environ Med* 14:159–167
- Maresca M (2013) From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. *Toxins (Basel)* 5:784–820
- Miró-Abella E, Herrero P, Canela N et al (2017) Determination of mycotoxins in plant-based beverages using QuEChERS and liquid chromatography-tandem mass spectrometry. *Food Chem* 229:366–372
- Nagl V, Woechtl B, Schwartz-Zimmermann HE et al (2014) Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in pigs. *Toxicol Lett* 229:190–197
- Pallaroni L, Von Holst C (2003) Comparison of alternative and conventional extraction techniques for the determination of zearalenone in corn. *Anal Bioanal Chem* 376:908–912
- Pallaroni L, Von Holst C, Eskilsson CS, Björklund E (2002) Microwave-assisted extraction of zearalenone from wheat and corn. *Anal Bioanal Chem* 374:161–166
- Payros D, Alassane-Kpembi I, Pierron A et al (2016) Toxicology of deoxynivalenol and its acetylated and modified forms. *Arch Toxicol* 90:2931–2957
- Pereira VL, Fernandes JO, Cunha SC (2014) Mycotoxins in cereals and related foodstuffs: a review on occurrence and recent methods of analysis. *Trends Food Sci Technol* 36:96–136
- Pestka JJ (2008) Mechanisms of deoxynivalenol-induced gene expression and apoptosis. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 25:1128–1140
- Plaza M, Turner C (2015) Pressurized hot water extraction of bioactives. *Trends Anal Chem* 71:39–54
- Rubert J, Soriano JM, Mañes J, Soler C (2013) Occurrence of fumonisins in organic and conventional cereal-based products commercialized in France, Germany and Spain. *Food Chem Toxicol* 56:387–391
- Rubert J, León N, Sáez C et al (2014) Evaluation of mycotoxins and their metabolites in human breast milk using liquid chromatography coupled to high resolution mass spectrometry. *Anal Chim Acta* 820:39–46
- Sánchez Maldonado AF, Mudge E, Gänzle MG, Schieber A (2014) Extraction and fractionation of phenolic acids and glycoalkaloids from potato peels using acidified water/ethanol-based solvents. *Food Res Int* 65:27–34
- SANTE (2015) European Commission Document No SANTE/11945/2015. Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed
- Schollenberger M, Müller HM, Rühle M et al (2005) Survey of Fusarium toxins in foodstuffs of plant origin marketed in Germany. *Int J Food Microbiol* 97:317–326
- Teo CC, Tan SN, Yong JWH et al (2010) Pressurized hot water extraction (PHWE). *J Chromatogr A* 1217:2484–2494
- Veprikova Z, Zachariasova M, Dzuman Z et al (2015) Mycotoxins in plant-based dietary supplements: hidden health risk for consumers. *J Agric Food Chem* 63:6633–6643
- Zachariasova M, Lacina O, Malachova A et al (2010) Novel approaches in analysis of fusarium mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. *Anal Chim Acta* 662:51–61
- Zhou Q, Li F, Chen L, Jiang D (2016) Quantitative analysis of 10 mycotoxins in wheat flour by ultrahigh performance liquid chromatography-tandem mass spectrometry with a modified QuEChERS strategy. *J Food Sci* 81:T2886–T2890