Food Analytical Methods

DETERMINATION OF TRICHOTHECENES IN CEREAL MATRICES USING SUBCRITICAL WATER EXTRACTION FOLLOWED BY SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

--Manuscript Draft--

Manuscript Number:	FANM-D-17-00713R1
Full Title:	DETERMINATION OF TRICHOTHECENES IN CEREAL MATRICES USING SUBCRITICAL WATER EXTRACTION FOLLOWED BY SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY
Article Type:	Original Research
Keywords:	Trichothecenes; Cereals; Subcritical Water Extraction; Solid-phase extraction; UHPLC-(ESI)MS/MS.
Corresponding Author:	Núria Fontanals Universitat Rovira i Virgili - Campus Sescelades Tarragona, Tarragona SPAIN
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universitat Rovira i Virgili - Campus Sescelades
Corresponding Author's Secondary Institution:	
First Author:	Eugènia Miró-Abella
First Author Secondary Information:	
Order of Authors:	Eugènia Miró-Abella
	Pol Herrero
	Núria Canela
	Lluís Arola
	Rosa Ras
	Núria Fontanals
	Francesc Borrull
Order of Authors Secondary Information:	
Funding Information:	
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 µg kg-1 (for T-2 toxin) and 4.0 µg kg-1 (for deoxynivalenol-3-glucoside), and method quantification limits between 0.4 µg kg-1 (for T-2 toxin) and 20 µg kg-1 (for deoxynivalenol-3-glucoside). The use of water as the extraction solvent allowed a selective extraction which allowed the obtaining of low matrix effect levels, and the detection and quantification of natural target trichothecenes at very low levels. This extraction method was applied to different cereals, a pseudocereal and an oilseed sample, of which millet and oat were contaminated by at least one trichothecene.

COMMENTS FOR THE AUTHOR:

Reviewer #1: General comments :

The authors present a method for « Determination of trichothecenes in cereal matrices using subcritical water extraction followed by solid-phase extraction and liquid chromatography-tandem mass spectrometry.

There are several things in the manuscript that should be addressed :

- I am wondering why authors focused on only trichotecenes B ? Why zearalenone that are produced by the same Fusarium species was not included ? Idem for fumonisins ? Informations about their presence would be more than desirable.

We would like to point out that the aim of this article was to evaluate the extraction of some trichothecene compounds with different polarities and characteristics, and for that we selected several trichothecenes A and B. As the SWE method proposed provided successful results, in line with the reviewer consideration, in further studies we will broaden the list of mycotoxins by including Fumonisins and ZEA.

- I have also a problem with some parameters that are part of method validation but are scattered in the manuscript. Results of ME and recoveries as well as LOQ should be included in Method Validation part, because they are important parts of its evaluation. Results of matrix effect and recoveries are in 2 different places of the manucript. For example, recoveries and matrix effect of maize is in 3.2 ; while the rest of cereals are in 3.4. No information about the linearity and how quantification of samples was made.

I propose to organize the results section in 3 parts : (i) optimisation of MS detection, chromatographic conditions and extraction; (ii) validation method and (iii) application of the developped method.

In agreement with the suggestion, some modifications have been made to the manuscript. First, recoveries and matrix effect of all the cereals detailed in section 3.4 have been moved to the section 3.2. Despite this, these values should be in method optimization part (not in method validation as it is suggested), because these values are used during the optimization of the method; in order to test different method variables, we evaluated and compared recovery values, and for this reason they are detailed in section 3.2.

Regarding quantification of samples, more information has been added to the manuscript. Considering the acceptable repeatability values, quantification was done using external calibration method and applying the recovery for each type of matrix (total recovery, including ME).

Nevertheless, the organization of the four sections has been maintained in order to avoid more complexity of the text.

Specific comments

Abstract :

Why authors do not report results for others mycotoxins

The information provided in the abstract are the intervals of the method detection and quantification limits. The limits corresponding to the rest of mycotoxins are included within this range. In order to avoid confusion, the names of the mycotoxins that appear in parenthesis (the upper and lower limits of the interval) have been removed (line 27-29).

Introduction

L 107, p5 : ..based on SWE followed by SPE clean-up and ….

As suggested, the sentence has been modified (line 108-109).

Mat & Meth :

L183, p8 : What do you mean by « ionization medium » ?

In order to clarify it, it has been changed in the text by « mobile phase » (line 184).

L187, p8 : I suggest to replace « medium compositions » by « solution »

The expression has been modified (lines 188 and 192).

L195, p8 : Authors tested negative mode but they did not give any results.

According with the suggestion, more information about negative results has been added (line 194-195).

L196, p8 : I suggest : « DON-3G give the same transition than DON by loosing the glucoside fragment ».

The sentence has been modified (line 196).

L198-201, p8 : « If, …whole analysis » I suggest to delete this sentence because we're not in this situation.

This information would have caused confusion, and we have deleted all the detailed information.

L203, p8 : What do you mean by « their main ion » ?

In order to avoid confusion, it has been changed from the text by «their most abundant ion» (line 198).

L203-216, p9 : In this section, authors do not give any important additional information to table 1, and can thus be deleted

According to the comment, this section has been widely simplified.

Please replace « toxin » by the term of « mycotoxin » throughout the text.

The term has been replaced throughout the manuscript.

L226, p9 : Both LOD and LOQ determination are part of method validation (see general comments). Authors did not indicate how they evaluated them.

There was some confusion with the abbreviations of LOD and LOQ (resolved six questions later). During the manuscript, we detailed two kinds of limits: the instrumental limits and the limits of the method. In section 3.1 (Instrumental optimization) we detail the limits of the instrument LC-MS/MS (with the standard solution). Then, in section 3.3 (Method validation) we detail the limits of the whole method, when the samples are analyzed by SWE/SPE/LC-MS/MS.

Regarding the indications of how we calculated them, they are in section 3.1: "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." (line 247-250). The same approach was used in section 3.3 to calculate the method detection and quantification limits (line 351-352).

L231, p9 : I suggest to replace « PLE and SPE optimization » by « Optimization of extraction »

As suggested, the title of this section has been modified (line 255).

L232-250, p10 : The authors should review the English for this section.

The English of the whole manuscript has been revised.

L269-270, p11 : The sentence « It should be noted that, in these experiments, ME was not considered just to evaluate the extraction recovery values » is confusing. Could authors give more details on calculated recovery and ME.

The reviewer is right since the sentence was confusing and for this reason it has been rewritten (line 297-300). "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." For obtaining the ME value, the concentration obtained from samples spiked after the extraction was compared with the standard solution concentration. In order to clarify it, it has been also modified from the manuscript (lines 309-313).

L281, p11 : The authors explain the absence of ME of acetylated DON by the fact that they are not eluted at the beginning of the chromatogram (rt 9.8 and 10.1). Why this is not the case for HT-2 and T2 which are eluted further (rt 14.7 and 15.1, respectively).

Thank you for the suggestion, we were mistaken. The corresponding modifications have been done (line 313-315).

L282, p11 : Even after dilution, there is still a ME (>20%) for these mycotoxins. There are only 2 ways to remove ME : the use of labelled internal standard and matrix-matched calibration curve. The authors did not use labelled mycotoxins in this study. How about the second solution ? If it is the case, could authors explain how they built the calibration curve.

Regarding these two approaches to compensate ME, the use of isotopically-labeled standards for each compound could not be afforded because of their elevated costs. Moreover, first of all we did a dilution in order to check if this procedure could be sufficient. Thanks to the dilution, ME were reduced and they were between the acceptable range of 20 and -20%, in most of the samples (in maize, and in the rest of cereals) (lines 317-323).

After addressing ME by dilution, matrix-matched calibration was used as quantification tool in the case of maize. On the other hand, for the mycotoxins quantification in the rest of

samples, matrix-matched calibration and instrumental curve were the two possible options, and the use of the instrumental curve applying the total recovery was selected. These explanations have been added to the manuscript (lines 395-400).

L294, p12 : Use the same abbreviation for limit of detection (LOD) and quantification (LOQ) throughout the manuscript.

In order to simplify it and taking into account your comment, the same abbreviation has been established throughout the manuscript.

L294, p12 : I suggest to replace « analytes » by « mycotoxins » throughout the manuscript.

This term has been replaced along the manuscript.

L311, p12 : I recommend presenting the performances of the method (repeatability, reproducibility, recovery, ..) in a table.

We had already considered this option, but in order to avoid to include another table, an interval of the repeatability and reproducibility parameters has been added in the text (line 375-376).

L323, p12 : What do you mean by « results were unsuitable » ?

In the results obtained for the mycotoxin 3-acetyl-deoxynivalenol in sesame samples there was an interference which masked the mycotoxin and it was not possible to quantify it, and for this reason we did not include them in the table. This has been also explained in the text (line 330-332).

L324-330, p12 : I completely disagree with this comparison. The method developed in this study targeted trichothecenes B that are more polar namely DON and their derivatives ; while the method developed by Kokkonen and Jestoi (2009) analyse more than 30 mycotoxins including trichothecenes B and some apolar mycotoxins as aflatoxins, ochratoxins, zearalenone and thus they use a different solvent mixture.

We agree with the suggestion and for this reason, the statement has been modified (line 363-368).

L336, p13 : replace « as expected, DON … » by « DON was found in …. but at low level ….

The sentence has been replaced as suggested by the reviewer (line 404-405).

L346-354, p14 : I have doubt about this statement. Based on the literature and my own experience, Fusarium species called "field fungi" are not competitive enough with "storage fungi" to grow and produce mycotoxins under any storage conditions. I strongly suggest to omit this statement from the text as it is not the purpose of this manuscript.

According to your suggestion, this statement has been modified. The affirmations about storage conditions have been eliminated, leaving the detected concentrations because of their interest (line 415-421).

Table 1 : I suggest to add column with molecular weight

The column with the molecular weight has been added to the Table 1 (page 21-22).

Table 2 is not a good presentation of the results. The results are from 2 experiments (optimisation of SPE extraction vs determination of recovery) with different levels of

contamination. I am wondering why the authors do not use the same level of contamination in order to compare recovery of SPE alone and PLE+SPE.

Thank you for your comment and we apologize because there was a mistake. The mycotoxin concentrations used for the optimization of the SPE extraction was wrong. They were at 25 µg L^{-1} for T-2, 125 µg L^{-1} for DON, 3AcDON and 15AcDON, and 500 µg L^{-1} for HT-2 and DON3G instead of at 10 µg L^{-1} for T-2, 50 µg L^{-1} for DON, 3AcDON and 15AcDON, and 100 µg L^{-1} for HT-2 and DON3G. Thus, levels of contamination used for %Rec SPE and %Rec SWE+SPE, were at 1 µg kg^{-1} for T-2, 5 µg kg^{-1} for DON, 3AcDON and 15AcDON, and 20 µg kg^{-1} for HT-2 and DON3G.

We thought that presenting the results in only one table, the readers can evaluate and compare different parameters of the extraction: the recovery of the standard solutions with the SPE, the recovery of the SPE with a matrix solution, the recovery of the whole extraction, etc.

The spiked concentrations have been modified in the text (lines 265-267 and 372-373).

Figure : Authors should show a chromatogram with transitions and retention times

We have not considered the addition of a chromatogram since all the information (transitions and t_R) are already detailed in Table 1. Instead, we displayed a MRM chromatogram for a contaminated maize sample.

DETERMINATION OF TRICHOTHECENES IN CEREAL MATRICES USING SUBCRITICAL WATER EXTRACTION FOLLOWED BY SOLID-PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Eugènia Miró-Abella[1,2], Pol Herrero[2], Núria Canela[2], Lluís Arola[3], Rosa Ras[2], Núria Fontanals[1*] and Francesc Borrull[1]

[1]Department of Analytical Chemistry and Organic Chemistry, Universitat Rovira i Virgili, Sescelades Campus, Marcel·lí Domingo, s/n, 43007 Tarragona, Spain

[2]Group of Research on Omic Methodologies (GROM), Centre for Omic Sciences (COS), Universitat Rovira i Virgili, Av. Universitat 1, 43204 Reus, Spain

[3]Unitat de biotecnologia - EURECAT, Av. Universitat 1, 43204 Reus, Spain

*Corresponding author.

Phone: (+34) 977 55 86 29

Fax: (+34) 977 55 84 46

E-mail: nuria.fontanals@urv.cat

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 µg kg^{-1} ~~(for T-2 toxin)~~ and 4.0 µg kg^{-1} ~~(for deoxynivalenol-3-glucoside)~~, and method quantification limits between 0.4 µg kg^{-1} ~~(for T-2 toxin)~~ and 20 µg kg^{-1} ~~(for deoxynivalenol-3-glucoside)~~. The use of water as the extraction solvent allowed a selective extraction ~~which allowed the obtaining of~~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.*

1. 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 that 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 type 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 is 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 hydrolyse 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 μg kg^{-1} to 1,750 μg 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 μg kg^{-1} to 1,000 μg 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 μg 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 ~~or the unpredictable ionization suppression of the studied mycotoxins~~. 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, ~~while~~and~~,~~ at the same time, ~~enabling~~ 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-~~(ESI)~~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.

2. Materials and methods

2.1 Reagents and chemicals

The target mycotoxins were six ~~Fusarium toxins~~: 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 mg L^{-1}; T-2 and HT-2 in acetonitrile (ACN) at 100 mg L^{-1} and DON3G in ACN at 50.9 mg L^{-1}. 3AcDON and 15AcDON were obtained in powder form. A mix solution of all of the ~~analytes~~ 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 mg L^{-1}, DON, 3AcDON and 15AcDON at 0.5 mg L^{-1} and T-2 at 0.1 mg L^{-1}. This mix solution was prepared in water/MeOH (80:20, v/v) and stored at -20ºC.

Ultrapure-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). 10 M ammonium formate

(NH_4HCOO) aqueous solution 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).

2.2 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_{18} column (100 mm x 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_4HCOO and 0.1% HCOOH. The gradient elution started at 10% B and maintained this percentage for 2 minutes. Over the next 5.5 minutes, the gradient increased to 20% and was held again under isocratic conditions for 3.5 minutes. It was then increased to 95% in 5 minutes and held under isocratic conditions for 2 minutes. Finally, it was returned to the initial conditions in 1 minute and maintained for 2 minutes to equilibrate the column. The injection volume was 10 µL, flow rate was fixed at 0.45 mL min^{-1} and the separation was performed at 40ºC. The autosampler was kept at 4ºC.

The optimized source parameters were: ~~a~~ capillary voltage of 4,000 V for DON3G and 3,500 V for the rest of compounds; desolvation gas flow and temperature of 18 L min^{-1} and 160ºC, respectively; nebulizer pressure of 35 psi; nozzle voltage of 2,000 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 L min^{-1} and 350ºC, 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 ~~analytes~~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.

2.3 Sampling

Prior to the extraction and analysis, stud~~y~~ied matrices were ground with the mill Taurus Aromatic (Taurus Group, Oliana, Spain), sifted twice in 500 µm and 100 µm sieves and homogenized. For spiked samples, 2 mL of acetone were added to 1 g of each sample in a 100 mL beaker, in order to spike the matrix homogenously. Subsequently, 100 µL of the mix solution (see Section 2.1 for concentrations) was added to the suspension and left overnight in a stirrer to let the ~~analytes~~ mycotoxins come into contact with the sample and until the acetone was completely evaporated. Matrices were spiked at three different ~~analytes~~ mycotoxin concentrations according to their sensitivity in UHPLC-(ESI)MS/MS, in order to obtain similar analytes response. ~~Spiking concentrations were the following: 10 µg kg^{-1} for T-2, 50 µg kg^{-1} for DON, 3AcDON and 15AcDON, and 100 µg kg^{-1} for HT-2 and DON3G.~~ 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.

2.4 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% of HCOOH as the extraction solvent, 80ºC with 5 minutes of cell preheating, 1,500 psi extraction pressure, flush volume of 50%, purge time of 60 s and a single extraction cycle of 5 minutes. 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 ~~analytes~~ mycotoxins were eluted with 5 mL of MeOH and evaporated to dryness with a miVac vacuum concentrator (Genevac LTD, Ipswich, UK). The ~~analytes~~

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.

3. Results and discussion

3.1 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 5mM 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 compounds by flow injection analysis (FIA) at a flow rate of 0.45 mL min^{-1}, at the following concentrations: 1 mg L^{-1} for HT-2 and DON3G, 0.5 mg L^{-1} for DON, 3AcDON and 15AcDON, and 0.1 mg L^{-1} for T-2. Taking into account adducts with the greater response in each mobile phase combination, the ~~composition~~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 $[DON+H]^+$ 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 $[M+H]^+$. However, $[15AcDON+NH_4]^+$ was selected as the ion for 15AcDON, whereas the protonated adduct $[3AcDON+H]^+$ was selected for 3AcDON, to avoid possible interferences, but also to enhance analyte selectivity and sensitivity. Finally, the ammonium adducts $[M+NH_4]^+$ of HT-2 and T-2 toxins were selected, as they are the most abundant forms. ~~Then, the ionization medium was optimized based on previous studies in the literature developed for similar mycotoxin groups (Zachariasova et al. 2010; Rubert et al. 2014; Veprikova et al. 2015; Miró-Abella et al. 2017). For the present study, 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 5mM being added to both solvents, either alone or in combination, resulting in 6 different medium~~

compositions. The analytes mycotoxins were injected individually in order to select the ions from the target compounds, by flow injection analysis (FIA) at a flow rate of 0.45 mL min^{-1}, at the following concentrations: 1 mg L^{-1} for HT-2 and DON3G, 0.5 mg L^{-1} for DON, 3AcDON and 15AcDON, and 0.1 mg L^{-1} for T-2. For each medium composition solution, different adducts were studied. Taking into account adducts with the greater response in each mobile phase combination, the composition 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.

Once the mobile phase was selected, precursor ions were selected, testing positive and negative modes. From all the obtained results, precursor ions appeared in greater abundance in positive mode. DON was ionized as $[DON+H]^+$ in the more abundant form, and DON3G was ionized as $[DON3G-3G+H]^+$, which resulted from the loss of the glucoside fragment gave the same transition than DON by losing the glucoside fragment. Therefore, DON and DON3G had the same precursor ion. If, in the subsequent chromatographic separation, these analytes present different retention times, this precursor ion would be used for both analytes. However, if their peaks appear overlaid, the formate adduct $[DON3G+HCOO]^-$ would be used, which also provided a high response but in negative mode that might restrict the sensitivity of the whole analysis. With respect to both acetylated DON derivatives, their main ion most abundant ion was the protonated form $[M+H]^+$. However, $[15AcDON+NH_4]^+$ was selected as the ion for 15AcDON, whereas the protonated adduct $[3AcDON+H]^+$ was selected for 3AcDON, to avoid possible interferences, but also to enhance analyte selectivity and sensitivity. In any case, the final ion selection would be subjected to the following chromatographic separation. Finally, the ammonium adducts $[M+NH_4]^+$ of HT-2 and T-2 toxins were selected, as they are present in more abundant forms.

With regard to the chromatographic gradient, it was mainly focused on the separation of DON and DON3G, and on the separation of both acetylated forms of DON. As such, the initial conditions included a very low percentage of organic solvent, which was gently increased over the chromatographic time. As a result, DON and DON3G were well-resolved and it was possible to select the most sensitive precursor ion for DON3G (i.e. $[DON3G-3G+H]^+$), because it will not interfere with DON. With respect to acetylated forms of DON, retention times were still close and the election of both

protonated adducts and their transitions would not have suitable selectivity, due to the fact that acetylated derivatives are isomeric compounds. These are the reasons that their precursor ions were chosen from the protonate and ammonium adducts form for 3AcDON and 15AcDON, respectively, as explained above. After the selection of the correspondent precursor ions and the mobile phase, different product ions were obtained selected for each analyte 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 Section 2.2.

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 determinedcalculated as the lowest trichothecene mycotoxin concentration with athat the quantifier and qualifier transitions (Q in Table 1) that displayed a signal-to-noise ratio (S/N) equivalent ≥ to 3 and 10, respectively. The LODs obtained were from 0.01 µg L^{-1} to 0.2 µg L^{-1} for all compounds, except for DON3G, for which was 0.7 µg L^{-1}. The LOQs ranged from 0.2 µg L^{-1} to 0.5 µg L^{-1} for all compounds, except for DON3G, for which was 2.5 µg L^{-1}. The linearity was suitable (with $r^2 \geq 0.994$) and it ranged from LOQs used as the lowest concentration to 20 µg L^{-1} for T-2, to 100 µg L^{-1} for DON, to 500 µg L^{-1} for acetylated forms and to 1,000 µg L^{-1} for DON3G and HT-2.

3.2 PLE and SPE oOptimization of extraction

Taking into consideration that in previous studies (Sánchez Maldonado et al. 2014; Plaza and Turner 2015), the successful the SWE of different several compounds in various kindsa wide range of matrices was achieved successfully, (Sánchez Maldonado et al. 2014; Plaza and Turner 2015), this extractiona SWE was tested to extract the target trichothecenes mycotoxins from the studycereal matrices. Water was acidified with 1% of HCOOH (pH 2.0) in order to improve the extraction, as in the aforementioned studies. IfUsing acidified water was used as the extraction solvent, it is no necessary to do any change of

the solvent for a clean-up process using a SPE cartridge ~~could be applied, without having to change the solvent~~. Prior to ~~sample extraction~~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 ~~analytes~~ mycotoxins at ~~10~~25 µg L^{-1} for T-2, ~~50~~125 µg L^{-1} for DON, 3AcDON and 15AcDON, and ~~100~~500 µg L^{-1} for HT-2 and DON3G, was loaded into the previously conditioned cartridge. The ~~analytes~~ mycotoxins were then eluted with three sequential fractions of MeOH: a first fraction of 3 mL, ~~and~~ a second fraction of 2 mL and a third of 2 mL ~~each~~. Most of the mycotoxins eluted at the first 3 mL. The second fraction also contained some ~~analytes~~mycotoxins, with a recovery ~~of over~~up to 10%.~~,~~ ~~whereas,~~But in the third fraction, the ~~analytes~~ 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% ~~(%Rec SPE std)~~. Further tests were performed~~,~~ in order to discard interactions between the cartridge and the matrix.~~,~~ ~~and~~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 ~~obtained~~ (%Rec SPE matrix) were lower than in water solution, decreasing equally in both cartridges.~~,~~ ~~thus~~However, recoveries were slightly higher for OASIS HLB ~~and all these results are~~ (detailed in Table 2). ~~In conclusion, the obtained recoveries denote the high selectivity of the chosen method.~~

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 Section 2.4. To do so, 1 g of homogenized maize sample was poured into a stainless steel extraction cell with DE, as explained in Section 2.4, and two different SWE temperatures were examined: 80ºC and 100ºC. Both temperatures provided suitable results in a similar order of magnitude, so a temperature of 80ºC 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 ~~analytes~~ 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 μg kg^{-1} (for T-2), at 5 μg kg^{-1} (for DON, 3AcDON and 15AcDON) and at 20 μg kg^{-1} (for HT-2 and DON3G). The other concentration group was at 15 μg kg^{-1} (for T-2), at 75 μg kg^{-1} (for DON, 3AcDON and 15AcDON) and at 200 μg kg^{-1} (for HT-2 and DON3G). The ~~extraction recovery (~~%Rec ~~PLE~~SWE+SPE~~)~~ was calculated by comparing the concentration~~s~~ obtained ~~when~~ from~~the~~ samples ~~were~~ spiked before the ~~SWE~~extraction process with th~~e~~~~ose~~ concentration obtained ~~with the pure standard~~from samples spiked after the extraction process. ~~It should be noted that, in these experiments, ME was not considered just to evaluate the extraction recovery values.~~ The obtained recovery values were similar at both tested groups, and just values when the sample was spiked at the lower concentration ~~from the lower concentration group~~ are shown in Table 2. As can be seen, the recovery values (%Rec ~~PLE~~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 ~~PLE~~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 ~~trichothecenes~~ mycotoxins from cereals.

~~As expected, the extraction recoveries from maize were lower in comparison with the values obtained from standards, which must be due to ME. Therefore~~ In addition, ME were evaluated and the values were obtained by comparing the concentration obtained when the samples were spiked after the ~~SPE~~whole extraction process with the concentration obtained with the pure standard, ~~when calculating this way~~and considering: ME=0 (no matrix effect), ME>0 (ion enhancement) and ME<0 (ion suppression). The obtained ME values are shown in Table 2, and it can be observed that all of the ~~analytes~~mycotoxins, except the acetylated forms of DON, are highly affected by ion suppression due to the complexity and composition of the maize samples.~~, since it is the first eluting compound, usually co-eluting with several ions in the matrix.~~ ~~Moreover, DON is the mycotoxin which is more affected by ion suppression, reported previously (Beltran et al. 2013).~~ 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 ~~analytes~~ mycotoxins were diluted in a re-suspension ~~re-suspended with~~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. ~~Moreover~~Even, in the case of some ~~analytes~~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, ~~I~~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 ~~trichothecenes~~mycotoxins, in the same way and concentrations as the ~~validation~~ 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 ~~recoveries from s~~esame matrices, the recovery was not calculated since ~~results were unsuitable~~ there was an interference which masked the mycotoxin and it was not possible to quantify it~~. These results were obtained for all~~ . Thus,~~replicates~~, thereby they are not collected in Table 3. With regard~~s~~ 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 was higher for the same analytes ~~reduced~~ due to the use of a ~~more~~less selective extraction solvent. ~~, namely water, in comparison to previous research based on the use of PLE with organic solvents (Kokkonen and Jestoi 2009).~~ ~~Conclusively~~Thus, using water as extraction solvent could be a suitable alternative because extract the ~~analytes~~ 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.

3.3 Method validation

~~V~~Method validation parameters, such as linear range, ~~limits of detection (MDL~~LOD~~),~~ ~~and quantification (MQL)~~LOQ, ~~linear range,~~ 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 ~~method validation~~ range was assessed ~~within~~from LOQs ~~and~~to 40 µg kg^{-1} for T-2, to 200 µg kg^{-1} for DON and its acetylated forms, and to 400 µg kg^{-1} for DON3G and HT-2. The linearity was acceptable with ~~the~~ r^2 higher than 0.990. ~~MDL~~LODs and ~~MQL~~LOQs were obtained in the same way as in the case of instrumental limits described above in Section 3.1. The ~~MDL~~LODs obtained were 0.05 µg kg^{-1} for T-2, between 0.5 µg kg^{1} and 1.0 µg kg^{-1} for DON, 3AcDON, 15AcDON and HT-2 and 4.0 µg kg^{-1} for DON3G. With respect to ~~MQL~~LOQs, they ranged between 0.4 µg kg^{-1} and 1.0 µg kg^{-1} for DON, 3AcDON, 15AcDON and T-2, 4.0 µg kg^{-1} for HT-2 and 20 µg kg^{-1} for DON3G. ~~The linearity was acceptable with the r^2 higher than 0.990, and the linear range was within MQLLOQs and 40 µg kg^{-1} for T-2, 200 µg kg^{-1} for DON and its acetylated forms, and 400 µg kg^{-1} for DON3G and HT-2.~~ The regulation for maize samples permits a maximum level for DON of 1750 µg kg^{-1} (EC 2007), recommends a maximum level for the sum of T-2 and HT-2 of 100 µg kg^{-1} (EC 2013) and recommends a maximum level for 3AcDON and 15AcDON of 1 µg kg^{-1} (JECFA 2011). Taking into account these regulated levels and using them as reference values, the obtained ~~MQL~~LOQs are acceptable because they are below them. In some ~~analytes~~ mycotoxins such as DON, HT-2 and T-2, ~~MQL~~LOQs 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~~s~~. There is in the literature ~~a~~previous researches which analyze diverse mycotoxins, ~~which uses~~by PLE with organic solvents and LC-MS/MS (Kokkonen and Jestoi 2009; Desmarchelier et al. 2010). In these~~at~~ 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. ~~Furthermore, obtained MDL and MQL values were lower with those reported in the literature using PLE with the target compounds (Kokkonen and Jestoi 2009; Desmarchelier et al. 2010).~~

~~Method~~ repeatability (intra-day, n=5) and reproducibility (inter-day, n=5) were obtained from different trichothecene concentration tests: T-2 at 1~~0~~ µg kg^{-1}, DON, 3AcDON and 15AcDON at 5~~0~~ µg kg^{-1},

and HT-2 and DON3G at 10~~0~~ µg kg^{-1}. ~~The obtained results were below 9% and 18%, respectively,~~ 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.

3.4 Application to different samples

~~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 trichothecenes, in the same way and concentrations as the validation 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 recoveries from sesame matrices, results were unsuitable. Thus, they are not collected in Table 3. With regards to ME for all matrices, they were considerably reduced due to the use of a more selective extraction solvent, namely water, in comparison to previous research based on the use of PLE with organic solvents (Kokkonen and Jestoi 2009).~~

~~One of the most important facts of the obtained results is that it was possible to extract these six different trichothecenes without using organic solvents at very low concentrations and with low ME. One study, where an organic solvent is used as extraction solvent in PLE is not able to extract these trichothecenes at concentrations as low as those used in the present research (Kokkonen and Jestoi 2009).~~

Once the method was successfully applied to ~~different types of~~maize samples, the natural presence of trichothecenes was studied using three different commercial brands of each cereal, pseudocereal and oilseed (~~n~~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 ~~method~~curve and applying the total recovery values (~~considering ME~~recovery explained in section 3.2). 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, ~~(also taking into account maize samples),~~ 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. ~~As expected,~~ DON was ~~detected~~found in all the samples at low level, except in sesame samples.~~, but at low level displaying the greatest trichothecene incidence ratio.~~ DON was detected in spelt and quinoa samples and quantified in maize in values up to 17.8 µg kg^{-1}, in oat up to 64.5 µg kg^{-1} and in millet up to 8.09 µg 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 µg kg^{-1} and in oat up to 10.6 µg kg^{-1}. With regard to the oat matrix, mycotoxin co-exposure is common, as identified in the previous studies (Schollenberger et al. 2005). As such, three more mycotoxins were quantified in oat: DON3G up to 8.71 µg kg^{-1}, HT-2 up to 35.2 µg kg^{-1} and T-2 up to 4.51 µg kg^{-1}. The concentration found in these samples is similar to those described in a previous study~~ies~~ (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 µg kg^{-1}, DON3G at 91 µg kg^{-1}, 3AcDON at 4 µg kg^{-1} and 15AcDON at 5 µg kg^{-1}, the quantitative transition MRM chromatograms of which are shown in Figure 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.

~~Apart from the studied samples indicated above, some maize samples were stored under temperature and humidity conditions that are favourable for fungi growth. After 90 days letting the maize age, at temperatures between 25ºC and 30ºC and humidity higher than 90%, these~~

~~samples were analyzed according to the sample procedure described above. The results obtained showed the presence of DON at 164 µg kg^{-1}, DON3G at 91 µg kg^{-1}, 3AcDON at 4 µg kg^{-1} and 15AcDON at 5 µg kg^{-1}, the quantitative transition MRM chromatograms of which are shown in Figure 1. If these concentrations are compared with those quantified in initial maize, it can be observed that, for example, DON has increased in concentration more than 5-fold. Therefore, it has been shown how adverse storage conditions promote trichothecene growth.~~

4. 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 ~~the~~ 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 ~~analytes~~ 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, ~~also~~ by using less organic solvents and becoming more alternative, apart from broaden the applicability of the method by including more mycotoxins in different type of samples.~~.~~

5. 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 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.

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.

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 (2013) Deoxynivalenol in food and feed: occurrence and exposure. EFSA J 11:3379–3434.

European Food Safety Authority, EFSA-Q-2013-00721 (2013). 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 (accessed 12 March 2017).

~~European Food Safety Authority, EFSA (2013) 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.

Joint FAO/WHO Expert Committe on Food Additives, JECFA (2011) Evaluation of certain contaminants in food. World Heal Organ Tech Rep Ser 959:1-105.

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.

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, 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.

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.

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, Rüfle 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.

Start time (min)	Mycotoxin	Retention Time (min)	Molecular weight (g mol^{-1})	Precursor ion (m/z)		Dwell time (ms)	Product ion (q/Q ratio)	CE (eV)
0	DON	2.9	296.3~~19~~	297.1	$[DON + H]^+$	180	248.9 231.1 (66) 203.1 (59)	8 10 8

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

	DON3G	3.6	458.4~~6~~	297.1	[DON3G - 3G + H]$^+$		248.9 231.1 (66) 203.1 (59)	8 10 8
4.4	3AcDON	9.8	338.3~~56~~	339.2	[3AcDON + H]$^+$	85	231.0 203.0 (39) 175.0 (23)	8 24 18
	15AcDON	10.1	338.3~~56~~	356.2	[15AcDON + NH_4]$^+$		339.1 321.0 (42) 136.9 (36)	16 12 4
5.8	HT-2	14.7	424.4~~9~~	442.2	[HT-2 + NH_4]$^+$	85	215.1 262.9 (90) 196.9 (31)	8 8 20
	T-2	15.1	466.5~~27~~	484.2	[T-2 + NH_4]+		214.9 304.9 (80) 185.0 (66)	16 12 10

Table 2. Trichothecene recoveries from SPE (%Rec SPE) and extraction recoveries from ~~PLE~~ SWE and SPE (%Rec ~~PLE~~SWE+SPE) of both tested cartridges. Matrix effects from OASIS (%ME) are also detailed, from a dilution of 1 and 2 mL. Matrix was the maize sample spiked with the ~~analytes~~ mycotoxins mixture, which concentrations are specified in the text.

	ISOLUTE ENV+			OASIS HLB				
	%Rec SPE std	*%Rec SPE matrix*	*%Rec ~~PLE~~SWE+SPE*	*%Rec SPE std*	*%Rec SPE matrix*	*%Rec ~~PLE~~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 ~~PLE~~SWE+SPE. Results obtained from a final resuspension of 2 mL

Spelt	Millet	Oat	Quinoa	Sesame

	%Rec SWE ~~PLE~~+ SPE	%ME	%Rec ~~PLE~~S WE+ SPE	%ME	%Rec ~~PLE~~S WE+ SPE	%ME	%Rec ~~PLE~~S WE+ SPE	%ME	%Rec ~~PLE~~S WE+ 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

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

n=3, RSD lower than 20%

Table 4. Maximum trichothecene concentration (µg kg^{-1}) extracted from the studied samples.

	Maize	Spelt	Millet	Oat	Quinoa	Sesame
DON	15.6 to 17.8	<~~MDL~~LOD to <~~MQL~~LOQ	<~~MQL~~LOQ to 8.09	<~~MQL~~LOQ to 64.5	<~~MDL~~LOD to <~~MQL~~LOQ	-
DON3G	<~~MQL~~LOQ to 2.94	-	-	<~~MDL~~LOD to 8.71	-	-
3AcDON	<~~MQL~~LOQ	-	-	-	<~~MDL~~LOD to <~~MQL~~LOQ	~~<MDL to <MQL-~~
15AcDON	15.8 to 16.7	<~~MDL~~LOD to <~~MQL~~LOQ	<~~MQL~~LOQ to 3.26	<~~MQL~~LOQ to 10.6	-	-
HT-2	<~~MDL~~LOD to 7.43	-	-	<~~MQL~~LOQ to 35.2	<~~MDL~~LOD to <~~MQL~~LOQ	-
T-2	<~~MDL~~LOD to 2.12	-	<~~MDL~~LOD to <~~MQL~~LOQ	<~~MQL~~LOQ to 4.51	-	-

n=3,
(-) Not detected

Figure caption

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

Figure

Click here to download Figure Fig1.pptx