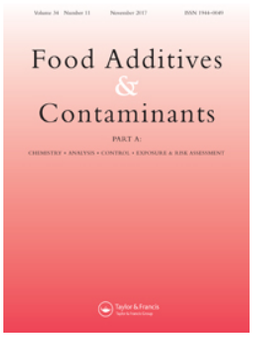

Taylor & Francis
Taylor & Francis Group

Food Additives & Contaminants: Part A

ISSN: 1944-0049 (Print) 1944-0057 (Online) Journal homepage: https://www.tandfonline.com/loi/tfac20

Determination of Fusarium toxins in functional vegetable milks applying salting-out-assisted liquid–liquid extraction combined with ultra-high-performance liquid chromatography tandem mass spectrometry

Ahmed M. Hamed, Natalia Arroyo-Manzanares, Ana M. García-Campaña & Laura Gámiz-Gracia

To cite this article: Ahmed M. Hamed, Natalia Arroyo-Manzanares, Ana M. García-Campaña & Laura Gámiz-Gracia (2017) Determination of Fusarium toxins in functional vegetable milks applying salting-out-assisted liquid–liquid extraction combined with ultra-high-performance liquid chromatography tandem mass spectrometry, Food Additives & Contaminants: Part A, 34:11, 2033-2041, DOI: 10.1080/19440049.2017.1368722

To link to this article: https://doi.org/10.1080/19440049.2017.1368722

FOOD ADDITIVES & CONTAMINANTS: PART A, 2017
VOL. 34, NO. 11, 2033–2041
https://doi.org/10.1080/19440049.2017.1368722

Taylor & Francis
Taylor & Francis Group

ARTICLE

Determination of Fusarium toxins in functional vegetable milks applying salting-out-assisted liquid–liquid extraction combined with ultra-high-performance liquid chromatography tandem mass spectrometry

Ahmed M. Hamed[a,b], Natalia Arroyo-Manzanares [a], Ana M. García-Campaña [a] and Laura Gámiz-Gracia [a]

[a]Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Granada, Spain; [b]Department of Dairy Science, Faculty of Agriculture, Cairo University, Giza, Egypt

ABSTRACT

Vegetable milks are considered as functional foods due to their physiological benefits. Although the consumption of these products has significantly increased, they have received little attention in legislation with regard to contaminants. However, they may contain mycotoxins resulting from the use of contaminated raw materials. In this work, ultra-high-performance liquid chromatography tandem mass spectrometry has been proposed for the determination of the most relevant *Fusarium* toxins (fumonisin B_1 and B_2, HT-2 and T-2 toxins, zearalenone, deoxynivalenol and fusarenon-X) in different functional beverages based on cereals, legumes and seeds. Sample treatment consisted of a simple salting-out-assisted liquid–liquid extraction with no further clean-up. The method provided limits of quantification between 3.2 and 57.7 μg L^{-1}, recoveries above 80% and precision with RSD lower than 12%. The method was also applied for studying the occurrence of these mycotoxins in market samples of vegetable functional beverages and deoxynivalenol was found in three oat-based commercial drinks.

ARTICLE HISTORY
Received 13 June 2017
Accepted 7 August 2017

KEYWORDS
Fusarium toxins; vegetable milks; salting-out-assisted liquid–liquid extraction; ultra-high-performance liquid chromatography; mass spectrometry

Introduction

Mycotoxins are highly toxic natural secondary metabolites produced by filamentous fungi belonging mainly to *Aspergillus*, *Penicillium*, and *Fusarium* genera that grow in a wide range of agricultural goods before, during and after the harvest process. Specifically, *Fusarium* species can produce several mycotoxin groups, the most important being trichothecenes, such as T-2 and HT-2 toxins; deoxynivalenol (DON); fusarenon-X (F-X); fumonisins as fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2); and zearalenone (ZEA). They are commonly found worldwide on cereals such as wheat, rye, barley, oat and maize, and subsequently in derived products. Moreover, the coexistence of different *Fusarium* spp. in the same crop is frequent, making possible the co-ocurrence of several mycotoxins in the same commodity (Yazar and Omurtag 2008; Ferrigo et al. 2016).

Due to the high occurrence of *Fusarium* mycotoxins and their toxic effects in animals and humans, maximum levels (MLs) for these contaminants in foodstuffs have been established worldwide. In particular, European legislation has established MLs for ZEA, DON and fumonisins, and made a recommendation for monitoring the presence of T-2 and HT-2 toxins, including indicative levels in cereals and cereal-based products (Commission of the European Communities 2006a, 2007, 2013). However, at the moment no legislation exists in relation to other products such as leguminous plants or seeds, such as soybean or bird seeds, although the risk of contamination by fungi and mycotoxins should be considered (Barros et al. 2011; Bhat et al. 2010).

Different analytical approaches have been proposed for determination of *Fusarium* toxins in cereal and cereal-based products, including immunological methods such as enzyme-linked immunosorbent assay (ELISA) (Li et al. 2014; Coronel et al. 2016), or gas chromatography coupled with mass spectrometry (GC-MS) (Cunha and Fernandes 2010; Ibáñez-Vea et al. 2011; Ferreira et al. 2012). However, for determination of multiple mycotoxins, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS)

CONTACT Laura Gámiz-Gracia lgamiz@ugr.es Department of Analytical Chemistry, Faculty of Sciences, University of Granada, Av Fuentenueva s/n, 18071 Granada, Spain

is considered to be the method of choice (Silva et al. 2009; Lattanzio et al. 2014; Bolechova et al. 2015) due to its several advantages such as selectivity, sensitivity and ability to cover a wide range of mycotoxins. For extraction and clean-up, liquid extraction followed by a solid phase extraction (SPE) using immunoaffinity columns (IACs) has typically been proposed for mycotoxin determination. However, IACs present some disadvantages such as rather high cost, cross-reactivity, limited lifetime and, most important, they are limited to a reduced number of compounds, not allowing a multi-class mycotoxin determination (Castegnaro et al. 2006; Senyuva and Gilbert 2010). Therefore, alternative sample treatments have been proposed for mycotoxin determination in cereal and cereal-based products. An extensive revision of analytical methodologies as well as sample treatments on this topic has been published (Pereira et al. 2014). The QuEChERS procedure was found to be increasingly adopted as a simple methodology based on extraction/partitioning in the presence of salts followed by dispersive-SPE (d-SPE) for clean-up. It has been widely applied for mycotoxin determination in food samples including cereals and cereal-based products due to its low solvent consumption and low cost, together with its versatility and flexibility (Cunha and Fernandes 2010; Desmarchelie et al., 2010; Sospedra et al. 2010; Tamura et al. 2011; Ferreira et al. 2012; Arroyo-Manzanares et al. 2014, 2015; Koesukwiwat et al. 2014; Jettanajit and Nhujak 2016). When there is no need for sample clean-up, salting-out-assisted liquid–liquid extraction (SALLE) is a simple and efficient strategy for sample treatment, carried out by adding an appropriate amount of salt to the mixture of aqueous sample and water-miscible organic solvent, which induces the separation of the solvent from the mixture containing the target analytes (Tang and Weng 2013). This strategy has been previously used for multi-mycotoxin determination in pig urine (Song et al. 2013).

However, despite the considerable number of publications devoted to determination of mycotoxins in cereal and cereal-based products, including studies of occurrence in different commodities (Pereira et al. 2014), little attention has been paid to beverages based on cereals, legumes or seeds, commonly called vegetable milks. Most common vegetable milks are based on soybean, rice or oat and they may be contaminated by mycotoxins resulting from the use of contaminated raw materials. Moreover, during the last decade, both supply and consumption of vegetable milks have significantly increased due to lactose intolerance, the decision to avoid consumption of animal products, or the health claims attributed to these products, considered as functional foods. In any case, they should be considered as a potential source for ingestion of mycotoxins, as for other cereal-based products. New analytical methodologies are required for these matrices, as very few articles have been published on this topic (Beley et al. 2013).

The goal of this study was therefore to develop a sensitive method based on the application of SALLE combined with UHPLC–MS/MS for the simultaneous determination of most relevant *Fusarium* toxins (FB_1, FB_2, T-2, HT-2, ZEA, DON and F-X) in different types of functional beverages produced from oat, soybean, rice and bird seeds. From our point of view, the proposed method for *Fusarium* toxins determination in these types of matrices could contribute to food safety and the study of the relationship between mycotoxin-contaminated raw material (cereals, leguminous plants or seeds) and their derived products. The method was characterised in terms of matrix effect (ME), linear dynamic ranges, limits of detection (LODs) and quantification (LOQs), precision and trueness. Finally, it was applied to control of mycotoxins in different commercial functional beverages.

Materials and methods

Chemicals, reagents and standard solutions

Ultrapure water (18.2 MΩ/cm, Milli-Q Plus system, Millipore Bedford, MA, U.S.A.) was used throughout all the work. All reagents were of analytical reagent grade and solvents were LC-MS grade. Methanol (MeOH), acetonitrile (MeCN), ammonium formate, formic acid (analysis grade) and chloroform ($CHCl_3$) were supplied by VWR International Eurolab, S.L. (Barcelona, Spain). Magnesium sulphate ($MgSO_4$), tri-sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and sodium chloride (NaCl) were purchased from Panreac Química (Barcelona, Spain). Potassium dihydrogen phosphate and disodium hydrogen citrate sesquihydrate were supplied by Merck (Darmstadt, Germany). Formic acid eluent additive for LC-MS was obtained from Sigma Aldrich (St. Louis, MO, U.S.A.).

Nylon syringe filters, 0.22 µm × 25 mm (Agela Technologies, New York, U.S.A.) were used for filtration of samples prior to the injection into the chromatographic system. Individual standard solutions (10 µg mL^{-1} in MeCN) of FB_1, FB_2, HT-2, T-2, ZEA, DON and F-X were obtained from Techno Spec (Barcelona, Spain) and stored in glass vials at −20°C.

Instrumentation

Separation was performed on an Agilent 1290 Infinity LC using a C18 Zorbax Eclipse Plus Rapid Resolution High Definition (RRHD) (50 × 2.1 mm, 1.8 µm) as chromatographic column. The measurements were performed on a triple quadrupole (QqQ) mass spectrometer API 3200 (AB Sciex, Darmstadt, Germany) with electrospray ionisation (ESI). The instrumental data were collected using the Analysts Software version 1.5 with Schedule MRM™ Algorithm (AB Sciex).

In addition, a centrifuge (Universal 320 model from Hettich, Leipzig, Germany), a vortex (Genie 2 model from Scientific Industries, Bohemia, NY, U.S.A.), a bench mixer multi-tube vortex agitator (model BV1010, Edison, NJ 08818, U.S.A.), a nitrogen evaporator (System EVA-EC from VLM GmbH, Bielefeld, Germany), and a pH-meter with a resolution of ±0.01 pH unit (Crison model pH 2000, Barcelona, Spain) were used during the sample preparation procedure.

Sample preparation

Oat milk (14% oat), soybean milk (15% soy), rice milk (15% rice) and bird seed milk (15% bird seeds) were purchased in local markets from Granada (Spain) and stored at 4°C. An aliquot of 5 mL of sample and 5 mL of 50 mM potassium dihydrogen phosphate at pH 7.0 were placed into a 50-mL screw cap test tube with a conical bottom and shaken by vortex for 10 s. Subsequently, 10 mL of MeCN with 5% formic acid was added, and the mixture was shaken again using the bench mixer for 2 min. Then, 4 g $MgSO_4$, 1 g NaCl, 1 g tri-sodium citrate and 0.5 g disodium hydrogen citrate sesquihydrate were added and the tube was shaken for 1 min using the bench mixer and centrifuged at 4500 rpm for 5 min. After that, 2 mL of the upper MeCN layer was transferred to a glass vial, evaporated to dryness under a gentle stream of nitrogen and reconstituted with 500 µL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2 µm filter prior their injection in the UHPLC–MS/MS system.

UHPLC–MS/MS analysis

For the determination of *Fusarium* toxins, the UHPLC–MS/MS was adapted from a method previously developed in our laboratory for multiclass mycotoxin determination in cereals and cereal-based products (Arroyo-Manzanares et al. 2014, 2015). Separation was performed in a C18 column (50 × 2.1 mm, 1.8 µm), using a mobile phase consisting of 0.3% aqueous formic acid solution with 5 mM ammonium formate (solvent A), and MeOH with 0.3% formic acid and 5 mM ammonium formate (solvent B) at a flow rate of 0.4 mL min^{-1}. The eluent gradient profile was as follows: 0 min 5% B; 0.5 min 50% B; 1.5 min 72% B; 2.5 min 80% B and 4.5 min 90% B. Finally, it was returned to 5% B in 0.2 min and maintained for 2 min for column equilibration. The temperature of the column was set at 35°C and the injection volume was 5 µL. The UHPLC system was coupled to a mass spectrometer with ESI operating in positive ion mode, under the multiple-reaction monitoring (MRM) conditions shown in Table 1, previously reported in references (Arroyo-Manzanares et al. 2014, 2015).

The ionisation source parameters were: dry gas temperature, 500°C; curtain gas (nitrogen), 30 psi; ion spray voltage, 5000 V; collision gas, 5 and dry gas pressure (GS 1 and GS 2, both of them nitrogen) 50 psi.

Results and discussion

Optimisation of sample preparation

A very simple methodology based on SALLE was explored as sample treatment for the determination of *Fusarium* toxins in the selected vegetable milks. During the optimisation, oat milk sample was used as representative matrix and different extraction solvents and mixtures of salts were tested. As initial conditions, the extraction was carried out using 5 mL of sample plus 5 mL of 50 mM potassium dihydrogen phosphate, pH 7.0. In addition, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium hydrogen citrate sesquihydrate were

Table 1. Monitored ions of the target analytes and MS/MS parameters.

Analyte	Precursor ion(m/z)	Rt	Molecular ion	DP	EP	CEP	Product ion*	CE	CXP
DON	297.1	1.0	[M + H]+	36.0	5.5	16.0	249.2 (Q)	17.0	4.0
							161.0 (I)	29.0	4.0
F-X	355.1	1.1	[M + H]+	26.0	12.0	18.0	174.7 (Q)	23.0	4.0
							137.1 (I)	31.0	4.0
HT-2	442.0	1.9	[M+NH4] +	21.0	5.5	21.0	262.8 (Q)	22.0	8.0
							215.4 (I)	19.0	4.0
FB_1	722.2	2.0	[M + H]+	71.0	10.0	30.0	334.2 (Q)	51.0	6.0
							352.2 (I)	47.0	6.0
T-2	484.0	2.1	[M+NH4] +	21.0	10.0	22.0	215.0 (Q)	22.0	4.0
							185.0 (I)	29.0	4.0
ZEA	319.0	2.3	[M + H]+	26.0	8.0	20.0	282.9 (Q)	19.0	4.0
							301.0 (I)	15.0	10.0
FB_2	706.2	2.5	[M + H]+	71.0	10.5	20.0	336.3 (Q)	43.0	14.0
							318.3 (I)	45.0	12.0

*(Q) Transition used for quantification, (I) Transition employed to complete the identification.
Rt: retention time. DP: declustering potential. EP: entrance potential. CEP: collision cell entrance potential. CE: collision energy. CXP: collision exit potential.

added for partitioning. Acid media can help the extraction process by breaking interactions between the toxins and sample constituents such as proteins (Rahmani et al. 2009). Moreover, the addition of formic acid to the extraction solvent could help the simultaneous extraction of fumonisins from cereals (Zitomer et al. 2008). Thus, the extraction of *Fusarium* mycotoxins from vegetable milks was carried out using acidic conditions. On the other hand, MeCN is the preferred extraction solvent as it extracts the widest range of mycotoxins and least amount of matrix components (Bolechova et al. 2015). Therefore, MeCN with different percentages (between 0 and 10%) of formic acid was tested as extraction solvent. When 0% formic acid was used, low recoveries (aprox. 65%) were obtained for FB_1, FB_2 and ZEA from some samples. The recoveries significantly increased when formic acid was added, being slightly better with 5% formic acid; therefore this mixture was selected for further experiments.

Subsequently, different combinations of solvents and salts were tested for extraction/partitioning: (a) 5 mL of H_2O, 4 g $MgSO_4$ and 1 g NaCl; (b) 5 mL of H_2O, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate; (c) 5 mL of 50 mM phosphate buffer at pH 7.0, 4 g $MgSO_4$ and 1 g NaCl; and (d) 5 mL of 50 mM phosphate buffer at pH 7.0, 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium hydrogen citrate sesquihydrate. In all cases a volume of 10 mL of MeCN with 5% formic acid was added as extractant. Combination (a) gave the lowest average recovery, being as low as 52% for HT-2 toxin, while the best results in terms of recovery were obtained using the buffered conditions (5 mL of 50 mM phosphate buffer at pH 7.0) and the salt mixture of 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium hydrogen citrate sesquihydrate. After that, different concentrations of phosphate buffer (50, 100 and 150 mM) were tested. Although no significant differences were observed, slightly better results in terms of recovery and %RSD were obtained using 50 mM phosphate buffer and it was therefore selected for the rest of the work. Finally, the effect of the volume of reconstitution solvent was studied using 500 and 1000 µL. Similar recoveries were obtained in both cases and a volume of 500 µL of MeOH:H_2O (1:1 v/v) was selected to obtain the best enrichment factor for the studied mycotoxins. With this SALLE approach, no further clean-up was necessary, as extracts were clean enough for quantification and identification purposes.

A typical chromatogram corresponding to a spiked oat-based beverage (75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg L^{-1} for T-2, HT-2 and ZEA) submitted to the proposed method is shown in Figure 1.

Method characterisation

According to EU regulation requirements, the suitability of the proposed method for *Fusarium* mycotoxin determination in different cereal and legume-based milks (oat, soybean, rice and bird seed) was evaluated in terms of matrix effect (ME), linear dynamic ranges, limits of detection (LODs) and quantification (LOQs), precision and trueness.

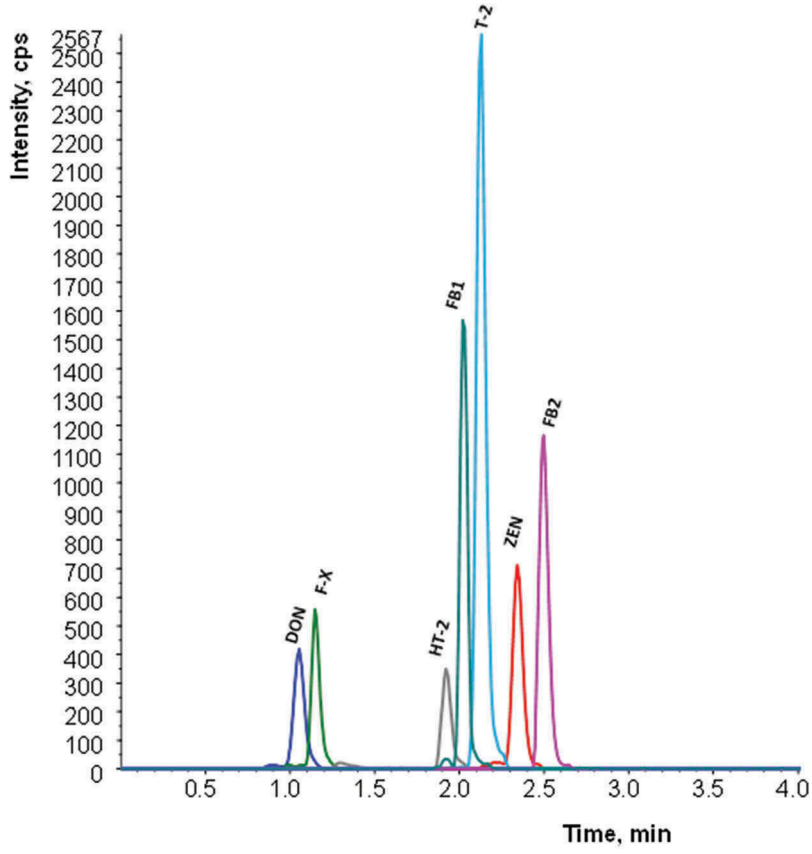

Figure 1. Chromatogram of a spiked oat milk sample applying the proposed method (75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg L^{-1} for T-2, HT-2 and ZEA).

Matrix effect

Matrix effects are caused by co-eluting sample components that cause an increase or suppression of the analyte signal compared with the signal of the same analyte when injected in solvent. As this can cause systematic errors in the quantification process, ME was evaluated in this work at two different concentration levels (75 and 300 µg L^{-1} for FB_1, FB_2, DON and F-X; 10 and 50 µg L^{-1} for T-2, HT-2 and ZEA) in the different samples. Peak areas of the most abundant product ions were considered as analytical signals and ME was calculated as 100 × [(signal of spiked extract – signal of standard solution)/signal of standard solution]. Table 2 shows the results and, as can be seen, the MEs ranged between −7 and −39, depending on the mycotoxin. Thus, a moderate ME (< 20%) was obtained for FB_1, FB_2, T-2 and ZEA in all matrices. However, higher MEs were obtained for HT2, F-X and DON. As a consequence, in order to compensate ME, procedural calibration curves were established.

Table 2. ME% for all samples studied.

	Oat milk		Soy milk		Rice milk		Bird seed milk	
Analyte	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2	Level 1	Level 2
FB_1	−11	−8	−11	−12	−9	−14	−11	−8
FB_2	−9	−7	−9	−7	−8	−13	−8	−13
T-2	−14	−16	−12	−16	−11	−14	−18	−19
HT-2	−21	−25	−13	−26	−21	−20	−13	−13
F-X	−26	−29	−28	−33	−23	−34	−23	−23
DON	−37	−29	−36	−28	−37	−31	−30	−39
ZEA	−18	−18	−10	−12	−8	−8	−13	−13

Level 1: 75 µg L^{-1} for FB_1, FB_2, DON and F-X and 10 µg L^{-1} for T-2, HT-2 and ZEA.
Level 2: 300 µg L^{-1} for FB_1, FB_2, DON and F-X and 50 µg L^{-1} for T-2, HT-2 and ZEA.

Calibration curves and analytical performance characteristics

Procedural calibration curves were used for quantification purposes, established by spiking blank samples at five concentration levels (ranging from 75 to 750 µg L^{-1} for FB_1, FB_2, DON and F-X; and from 10 to 100 µg L^{-1} for T-2, HT-2 and ZEA) before the extraction process. Each level was prepared in duplicate following the proposed QuEChERS-based extraction procedure and injected in

triplicate. Performance characteristics of the method are shown in Table 3. The statistical parameters were calculated by least-square regression, and LODs and LOQs were considered as 3×signal/noise and 10×signal/noise ratio, respectively. These values were provided by the Analysts Software, considering the intensity of the signal for the lowest concentration level of calibration and the intensity of the noise at the retention time of each analyte. For all analytes, the response was linear with a coefficient of determination (R^2) higher than 0.99. As can be seen, low LOQs were obtained, showing the suitability of the proposed method for the determination of very low concentrations of these toxins in the selected matrices. Although there are no specific maximum levels for mycotoxin content in this kind of products, EU legislation establishes a maximum content for these mycotoxins in cereals (1250–1750 μg kg^{-1} for DON and 100–350 μg kg^{-1} for ZEA in unprocessed cereals, and 4000 μg kg^{-1} sum of FB_1 and FB_2 in unprocessed maize) (European Commission 2007), and a recommendation for T-2 and HT-2 content (100–1000 μg kg^{-1} for unprocessed cereals) (European Commission 2013). Taking into account the cereal, legume or seed content in the studied vegetable milk samples (14–15%), the proposed method could also provide an estimation of the mycotoxin contamination of the raw materials used for the processing of these beverages.

Precision study

The precision of the whole method was evaluated in terms of intraday precision (repeatability) and interday precision (intermediate precision). Intraday precision was assessed by application of the proposed SALLE-UHPLC–MS/MS method in samples spiked at two different concentration levels (75 and 300 μg L^{-1} for FB_1, FB_2, DON and F-X; 10 and 50 μg L^{-1} for T-2, HT-2 and ZEA). Each sample was prepared in triplicate (experimental replicates) and injected in triplicate (instrumental replicates) on the same day. A similar procedure was carried out during three consecutive days in order to evaluate intermediate precision. The results, expressed as %RSD of peak areas, are shown in Table 4. Good precision (RSD lower than 12%) was obtained in all cases, being in agreement with current legislation (European Commission 2006b).

Table 3. Statistics and performance characteristics of the QuEChERS-UHPLC–MS/MS method for the determination of *Fusarium* mycotoxins in vegetable milks. LOD and LOQ are expressed as μg L^{-1}.

	Oat milk					Soy milk					Rice milk					Bird seed milk				
Analyte	Slope (s_b)	Intercept (s_a)	R^2	LOD	LOQ	Slope (s_b)	Intercept (s_a)	R^2	LOD	LOQ	Slope (s_b)	Intercept (s_a)	R^2	LOD	LOQ	Slope (s_b)	Intercept (s_a)	R^2	LOD	LOQ
FB_1	40.2 (0.4)	−510 (171)	0.998	17.3	57.7	42.4 (0.2)	348 (95)	0.999	12.4	41.2	47.6 (0.6)	411 (243)	0.997	10.7	35.7	42.2 (0.3)	612 (129)	0.999	9.0	30.0
FB_2	33.6 (0.2)	105 (299)	0.999	11.8	39.5	44.6 (0.4)	459 (174)	0.998	17.2	57.3	59.1 (0.6)	771 (251)	0.998	9.0	30.1	44.7 (0.5)	972 (212)	0.998	10.0	33.3
T-2	404 (6)	200 (324)	0.996	1.8	5.9	672 (8)	2178 (444)	0.997	1.3	4.2	601 (9)	2682 (534)	0.995	1.6	5.3	338 (3)	1396 (188)	0.998	1.0	3.2
HT-2	60.9 (0.7)	−72 (40)	0.998	2.7	9.1	87 (1)	333 (66)	0.997	2.2	7.4	71.2 (0.6)	−106 (38)	0.998	2.3	7.7	83 (2)	135 (90)	0.993	1.5	5.1
F-X	13.0 (0.1)	182 (64)	0.997	8.7	28.8	9.2 (0.1)	77 (45)	0.997	12.2	40.5	11.6 (0.1)	259 (55)	0.998	9.8	32.6	3.83 (0.05)	203 (22)	0.996	14.1	46.9
DON	13.6 (0.2)	157 (89)	0.996	16.1	53.6	11.0 (0.1)	369 (49)	0.997	12.9	42.9	14.1 (0.2)	287 (75)	0.997	11.3	37.5	6.27 (0.09)	835 (38)	0.996	14.2	47.5
ZEA	124 (1)	234 (77)	0.998	1.4	4.5	124 (1)	224 (76)	0.997	2.4	8.0	211 (2)	−292 (110)	0.998	2.4	8.0	115.4 (0.9)	345 (52)	0.999	2.3	7.6

Table 4. Recoveries % (%R), intraday (%RSD-r) and interday precision (%RSD-R) from different spiked samples (n = 9).

	Oat milk						Soy milk						Rice milk						Bird seed milk					
	Level 1			Level 2			Level 1			Level 2			Level 1			Level 2			Level 1			Level 2		
Analyte	%R	%RSD r	%RSD R	%R	%RSD r	%RSD R	%R	%RSD r	%RSD R	%R	%RSD r	%RSD R	%R	%RSD r	%RSD R	%R	%RSD r	%RSD R	%R	%RSD r	%RSD R	%R	%RSD r	%RSD R
FB_1	83	7	6	98	4	8	85	6	7	91	9	5	89	8	7	98	3	6	84	5	8	98	6	4
FB_2	80	4	7	80	5	5	80	6	6	95	5	5	81	5	7	96	5	8	83	3	9	97	4	6
T-2	97	7	11	80	7	7	95	8	9	89	9	9	97	5	11	98	7	8	92	7	11	83	6	8
HT-2	83	9	10	96	7	8	86	8	10	91	7	8	96	5	10	85	9	9	87	9	11	99	8	7
F-X	90	7	8	82	8	9	90	9	6	81	5	8	92	9	10	93	8	10	90	7	8	91	7	9
DON	87	9	9	84	9	5	82	6	10	87	9	9	82	9	12	90	7	9	83	5	10	89	4	7
ZEA	81	6	12	81	5	5	80	4	11	81	6	7	82	6	10	82	7	9	80	4	9	85	9	9

Level 1: 75 μg L^{-1} for FB_1, FB_2, DON and F-X and 10 μg L^{-1} for T-2, HT-2 and ZEA.
Level 2: 300 μg L^{-1} for FB_1, FB_2, DON and F-X and 50 μg L^{-1} for T-2, HT-2 and ZEA.

Trueness assessment

In order to check the trueness of the proposed method, recovery experiments were carried out in the selected commercial beverages previously analysed in order to detect the presence of mycotoxins. None of them gave a positive result above the LODs of the method. These samples were spiked at two different concentration levels (75 and 300 μg L^{-1} for FB_1, FB_2, DON and F-X; 10 and 50 μg L^{-1} for T-2, HT-2 and ZEA), processed, and injected in triplicate into the UHPLC–MS/MS system. The results are shown in Table 4, and as can be seen, very good recoveries were obtained, ranging between 80 and 99%.

Application to the analysis of commercial samples

Different samples of commercial vegetable milks, including 8 oat milk, 8 soybean milk (7 natural and 1 light), 5 rice milk and 1 bird seed milk, purchased in local markets from the south of Spain (Granada, Córdoba and Jaén) and the north of Morocco (Tangier) were analysed in order to monitor the presence of *Fusarium* toxins. All of them were supplied as 1 L packs and were stored at 4°C.

Three oat milk samples (all of them from the same brand but purchased in different cities) gave a positive result for DON at concentrations of 191, 221 and 270 μg L^{-1}. These results were confirmed by standard addition calibration, obtaining concentrations of 192 μg L^{-1} ($y = 14.537x + 3163$; $R^2 = 0.997$), 218 μg L^{-1} ($y = 14.523x + 3163$; $R^2 = 0.998$) and 263 μg L^{-1} ($y = 14.532x + 3163$; $R^2 = 0.996$). These results provide evidence for the transfer of *Fusarium* mycotoxins from raw cereals to derived beverages, suggesting the need for further investigations on the mycotoxin fate along the process from raw materials to vegetable milk beverages. None of the other samples analysed showed any contamination above the LODs by *Fusarium* toxins.

Figure 2 shows the extracted ion chromatogram for DON of a contaminated oat milk sample applying the proposed method.

Conclusions

A SALLE-based preparation procedure followed by an UHPLC–MS/MS analytical method has been proposed and successfully applied to the determination of the most relevant *Fusarium* toxins in functional

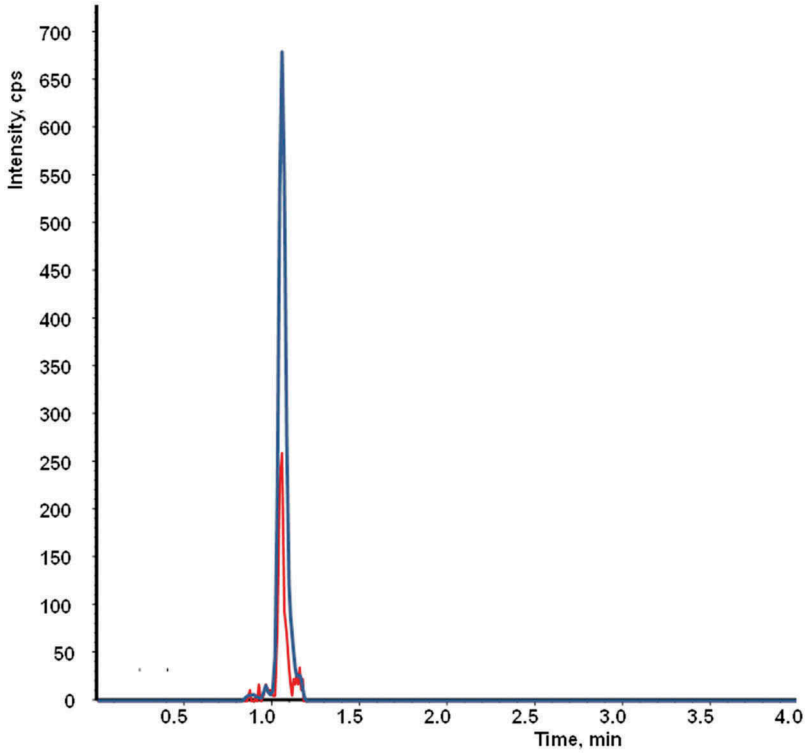

Figure 2. Extracted ion chromatogram for DON from a contaminated oat milk sample applying the proposed method.

beverages based on cereals, leguminous plants and seeds, such as oat, soybean, rice and bird seed milk samples. This offers a relevant analytical strategy for the control of these important toxins in this relatively new product, not included in the present legislation. The proposed method showed low LODs and LOQs, and both recovery and precision studies meet the performance criteria required for mycotoxin analytical methods in foodstuffs. By applying the method on commercial samples, DON with concentrations between 191 and 270 μg L^{-1} was found in three oat milk samples from Spain, indicating that contamination from the raw oat used for milk processing can transfer to the beverages. The data reveal a matter of concern, indicating that proper control should be applied to these increasingly consumed functional food products.

Acknowledgements

AMH thanks the 'Erasmus Mundus – Al Idrisi II' programme for a doctoral grant (2013 – 2401 /001 – 001 – EMA2).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

The authors gratefully acknowledge the financial support of the Spanish Ministry of Economy and Competitiveness (Secretary of State of Research, Development and Innovation) [Project ref: AGL2015-70708-R].

ORCID

Natalia Arroyo-Manzanares http://orcid.org/0000-0001-9882-6270
Ana M. García-Campaña http://orcid.org/0000-0002-3191-3350
Laura Gámiz-Gracia http://orcid.org/0000-0002-8880-5000

References

Arroyo-Manzanares N, Huertas-Pérez JF, Gámiz-Gracia L, García-Campaña AM. 2015. Simple and efficient methodology to determine mycotoxins in cereal syrups. Food Chem. 177:274–279.

Arroyo-Manzanares N, Huertas-Pérez JF, García-Campaña AM, Gámiz-Gracia L. 2014. Simple methodology for the determination of mycotoxins in pseudocereals, spelt and rice. Food Control. 36:94–101.

Barros GG, Oviedo MS, Ramirez ML, Chulze SN. 2011. Safety aspect in soybean food and feed chains: fungal and mycotoxins contamination. In: Ng TB, editor.

Soybean - Biochemistry, Chemistry and Physiology. Rijeka: InTech; p. 7–20.
Beley MAJ, Teves FG, Madamba MRSB. 2013. Isolation of fungal species and detection of aflatoxin from soy milk products using ELISA method. Int Res J Biol Sci. 2:45–48.
Bhat R, Rai RV, Karim AA. 2010. Mycotoxins in food and feed: present status and future concerns. Compr Rev Food Sci Food Saf. 9:57–81.
Bolechova M, Benesova K, Belakova S, Caslavsky J, Pospíchalova M, Mikulíkova R. 2015. Determination of seventeen mycotoxins in barley and malt in the Czech Republic. Food Control. 47:108–113.
Castegnaro M, Tozlovanu M, Wild C, Molini A, Sylla A, Pfohl-Leszkowicz A. 2006. Advantages and drawbacks of immunoaffinity columns in analysis of mycotoxins in food. Mol Nutr Food Res. 50:480–487.
Coronel MB, Vicente S, Resnik SL, Alzamora SM, Pacin A. 2016. Fumonisins in maize and gluten meal analysed in Argentinean wet milling industrial plants by ELISA compared with HPLC-FLD method. Food Control. 67:285–291.
Cunha SC, Fernandes JO. 2010. Development and validation of a method based on a QuEChERS procedure and heart-cutting GC-MS for determination of five mycotoxins in cereal products. J Sep Sci. 33:600–609.
Desmarchelier A, Oberson JM, Tella P, Gremaud E, Seefelder W, Mottier P. 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 Commission. 2006a. Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union. L364:5–24.
European Commission. 2006b. Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off J Eur Union. L70:12–34.
European Commission. 2007. Regulation (EC) 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.
European Commission. 2013. Recommendation of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. Off J Eur Union. L91:12–14.
Ferreira I, Fernandes JO, Cunha SC. 2012. Optimization and validation of a method based in a QuEChERS procedure and gas chromatography-mass spectrometry for the determination of multi-mycotoxins in popcorn. Food Control. 27:188–193.
Ferrigo D, Raiola A, Causin R. 2016. *Fusarium* toxins in cereals: occurrence, legislation, factors promoting the appearance and their management. Molecules. 21:627–662.
Ibáñez-Vea M, Lizarraga E, Gonzáles-Peñas E. 2011. Simultaneous determination of type-A and type-B trichothecenes in barley samples by GC-MS. Food Control. 22:1428–1434.
Jettanajit A, Nhujak T. 2016. Determination of mycotoxins in brown rice using QuEChERS sample preparation and UHPLC-MS-MS. J Chromatogr Sci. 54:720–729.
Koesukwiwat U, Sanguankaew K, Leepipatpiboon N. 2014. Evaluation of a modified QuEChERS method for analysis of mycotoxins in rice. Food Chem. 15:44–51.
Lattanzio VMT, Ciasca B, Powers S, Visconti A. 2014. Improved method for the simultaneous determination of aflatoxins, ochratoxin A and *Fusarium* toxins in cereals and derived products by liquid chromatography-tandem mass spectrometry after multi-toxin immunoaffinity clean up. J Chromatogr A. 1354:139–143.
Li Y, Luo X, Yang S, Cao X, Wang Z, Shi W, Zhang S. 2014. High specific monoclonal antibody production and development of an ELISA method for monitoring T-2 toxin in rice. J Agric Food Chem. 62:1492–1497.
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.
Rahmani A, Jinap S, Soleiman F. 2009. Qualitative and quantitative analysis of mycotoxins. Compr Rev Food Sci Food Saf. 8:202–252.
Senyuva HZ, Gilbert J. 2010. Immunoaffinity column clean-up techniques in food analysis: A review. J Chromatogr B. 878:115–132.
Silva L, Fernández-Franzón M, Font G, Pena A, Silveira I, Lino C, Mañes J. 2009. Analysis of fumonisins in corn-based food by liquid chromatography with fluorescence and mass spectrometry detectors. Food Chem. 112:1031–1037.
Song S, Ediage EN, Wu A, De Saeger S. 2013. Development and application of salting-out assisted liquid/liquid extraction for multi-mycotoxin biomarkers analysis in pig urine with high performance liquid chromatography/tandem mass spectrometry. J Chromatogr A. 1292:111–120.
Sospedra I, Blesa J, Soriano JM, Mañes J. 2010. Use of the modified quick easy cheap effective rugged and safe sample preparation approach for the simultaneous analysis of type A- and B-trichothecenes in wheat flour. J Chromatogr A. 1217:1437–1440.
Tamura M, Uyama A, Mochizuki N. 2011. Development of a multi-mycotoxin analysis in beer-based drinks by a modified QuEChERS method and ultra-high-performance liquid chromatography coupled with tandem mass spectrometry. Anal Sci. 27:629–635.
Tang YQ, Weng N. 2013. Salting-out assisted liquid-liquid extraction for bioanalysis. Bioanalysis. 5:1583–1598.
Yazar S, Omurtag GZ. 2008. Fumonisins, trichothecenes and zearalenone in cereals. Int J Mol Sci. 9:2062–2090.
Zitomer NC, Glenn AE, Bacon CW, Riley RT. 2008. A single extraction method for the analysis by liquid chromatography/tandem mass spectrometry of fumonisins and biomarkers of disrupted sphingolipid metabolism in tissues of maize seedlings. Anal Bioanal Chem. 391:2257–2263.