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A new approach in sample treatment combined with UHPLC-MS/MS for the determination of multiclass mycotoxins in edible nuts and seeds

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ABSTRACT

A sensitive, simple and rapid method for the determination of fourteen mycotoxins in nuts and seeds (including almonds, peanuts, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) has been developed using ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS). The sample treatment comprises a first step based on QuEChERS procedure for the determination of fumonisin B_1, fumonisin B_2, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, sterigmatocystin, zearalenone and ochratoxin A. A subsequent clean-up step based on the dispersive liquid–liquid microextraction (DLLME) was necessary for the determination of aflatoxins (B_1, B_2, G_1 and G_2), since their determination was not possible applying only the QuEChERS-based extraction. The method was validated for peanuts as representative matrix and was subsequently evaluated for the other eight matrices. Quantification limits obtained for aflatoxins, the unique mycotoxins legislated on these matrices, were lower than the maximum levels allowed by the current legislation, while quantification limits obtained for the other mycotoxins were lower than the limits usually permitted by the legislation in other food matrices. Precision of the method was always lower than 11%, and recoveries ranged between 60.7% and 104.3%.

1. Introduction

Nuts and seeds are susceptible to mould growth and consequently to mycotoxin contamination. In recent years, there have been many studies that have revealed a high mycotoxin and fungal contamination in these matrices [1–3]. This fact is reflected in the high number of alerts reported by the Rapid Alert System for Food and Feed (RASFF) in its annual reports; for instance, in 2010 about 500 alerts for contaminations of aflatoxins were reported in nut, nuts products and seeds and up to 320 in 2011. Also, two alerts of ochratoxin A in pistachio were reported in 2010 [4].

The presence of mycotoxins in food may affect human and animal health as they may cause many different adverse effects such as induction of cancer and mutagenicity, as well as oestrogenic, gastrointestinal and kidney disorders. Furthermore, some mycotoxins are also immunosuppressive, reducing resistance to infectious disease [5]. Regarding the contamination of nuts and seeds by mycotoxins, current EU food safety legislation only regulates the content of aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) in these matrices by means of the Regulation (CE) 1881/2006 [6] and its subsequent amendment Regulation (EU) 165/2010 [7], which set maximum permitted levels ranging between 2 and 8 μg kg^{-1} for AFB_1, and between 4 and 15 μg kg^{-1} for the sum of AFB_1, AFB_2, AFG_1 and AFG_2, depending on the kind of nuts or seeds for direct human consumption. Different analytical techniques have been used for the control of mycotoxins in these foods, i.e. HPLC with fluorescence (HPLC-FL) detection in the determination of aflatoxins in nuts [1], tigernuts and beverages [8] or pistachio [9], thin layer chromatography (TLC) for peanuts and pistachios [9,10] or enzyme-linked immunosorbent assay (ELISA) for the determination of AFB_1 in peanuts, pistachios, nuts, sesame and sunflowers seeds [11,12]. In recent years, multiresidue methods able to monitor a high number of compounds in a single run according to the established legislation [13] for many different matrices, are also very attractive. In this sense the application of LC-MS/MS has increased, being used for the control of ochratoxins [14] or aflatoxins [15] in nuts and resulting especially interesting for multiclass analysis although the number of applications is still reduced for this kind of matrixes, as example, applications in nuts [16] or in peanuts and pistachios [17] are found.

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Until now, the most commonly sample treatment used for the determination of mycotoxins in nuts and seeds involved the use of solid–liquid extraction [11,12,16,17] usually followed by a clean-up step using an immunoaffinity column (IAC) [9,10]. The most precious characteristic of IAC columns is their analyte selectivity, however, they present some drawbacks such cost, complexity, inefficiency and high susceptibility to sample pH, therefore suffering from low recoveries for some mycotoxins. Moreover, their selectivity in the extraction would limit the multiclass analysis of these contaminants. Also, in nuts and seeds the aflatoxins have also been determined by using SPE with C18 sorbent [1,8,18], or ochratoxin A (OTA) [14] and aflatoxins [15] using SPME.

In recent years, several methods based on the QuEChERS methodology (Quick, Easy, Cheap, Effective, Rugged and Safe) have been developed for the determination of mycotoxins in different food and drug matrices such as wine [19], cereals [20–25], eggs [26] and medicinal plants [27], obtaining excellent results. QuEChERS methodology presents some advantages, such as its simplicity, minimum steps, and effectiveness for cleaning-up complex samples [28]. It involves two steps: the first one is an extraction step based on partitioning via salting-out extraction, which involves the equilibrium between an aqueous and an organic layer, and the second one usually involves a dispersive SPE (DSPE) step, that involves further clean-up using combinations of $MgSO_4$ and different sorbents such as florisil, carbon black, C18 or primary and secondary amine (PSA) to remove interfering substances. However, dispersive SPE has proven to cause a decrease in the recovery for mycotoxins [27], making necessary to avoid this step or to consider other alternatives for cleaning-up.

Dispersive liquid–liquid microextraction (DLLME), an emerging technique introduced for the treatment of liquid samples [29–32], is based on the use of a ternary component solvent system, where an appropriate mixture of a few microliters of an organic extraction solvent (usually with a density higher than water), and a small volume of a disperser solvent (miscible with the extraction solvent and with water), is injected rapidly into an aqueous sample, resulting in the formation of a stable emulsion. The organic analytes present in the aqueous sample are rapidly extracted into the extraction solvent as a result of the large contact surface between the organic and the aqueous phases. Phase separation is performed by centrifugation and an organic phase with the analytes of interest is settled in the bottom of a conical tube and subsequently analysed. This technique has been applied for the determination of OTA in wines [19,33–35], patulin in fruit juices [36], aflatoxins in edible oils after an IAC step [37] and aflatoxins in cereals after extraction with methanol/water 8:2 (v/v) [38]. DLLME has also been optimized and applied in our lab for the multiresidue determination of mycotoxins in the botanical *Silybum Marianum*, after a previous solid–liquid extraction based on QuEChERS [27].

In this paper, we proposed a multiclass method for the simultaneous determination of 14 mycotoxins in different nuts and seeds, based on an extraction using a QuEChERS procedure for determination of fumonisin B1 (FB_1), fumonisin B2 (FB_2), deoxynivalenol (DON), fusarenon-X (F-X), HT-2 toxin (HT-2), T-2 toxin (T-2), ochratoxin A (OTA), citrinin (CIT), sterigmatocystin (STE) and zearalenone (ZEN) with an additional clean-up DLLME step for determination of four aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2). Considering the advantages of UHPLC-MS/MS, which has become very popular in the past years for the multiclass analysis of mycotoxins [25,26,39–42], this technique has been applied in this work for the analysis of nine different nut and seed samples (almonds, peanuts, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts). To the best of our knowledge, this is the first time that the proposed sample treatment has been used for these food matrices, obtaining satisfactory results in combination with UHPLC-MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical reagent grade, solvents were LC-MS grade and mycotoxins were analytical standard grade. Formic acid, used as eluent additive for LC-MS, methanol (MeOH), ammonium formiate and individual standards of each mycotoxin were obtained from Sigma Aldrich (St Louis, MO, USA). Formic acid (analysis grade) was supplied by Merck (Darmstadt, Germany). Sodium chloride and acetonitrile HPLC grade (MeCN), were supplied by Panreac (Madrid, Spain); and chloroform was purchased from VWR BDH Prolabo (West Chester, Pensilvania, USA).

Ultrapure water (18.2 MΩ cm^{-1}, Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout for all the work.

Kits SampliQ QuEChERS consisted of buffered QuEChERS extraction packed (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) were supplied by Agilent Technologies Inc. (Wilmington, DE, USA).

Acrodisc 13 mm syringe filters with 0.2 μm nylon membrane (Pall Corp., MI, USA) were used for filtration of samples prior to the injection into the chromatographic system.

2.2. Instruments and equipment

All experiments were carried out using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump, online degasser, autosampler (5 μL loop), and a column thermostat. The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (AB SCIEX, Toronto, ON, Canada) with electrospray ionization (ESI). A Zorbax Eclipse Plus RRHD (50 × 2.1 mm, 1.8 μm) chromatographic column was used for the separation.

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and an evaporator system (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used in the sample treatment procedure.

The instrumental data were collected using the Analyst® Software version 1.5 with Schedule MRM TM Algorithm (AB Sciex).

2.3. Sample treatment

Nut and seed samples (peanuts, almonds, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) were purchased in local markets from Granada (Spain) and stored at room temperature. After milling and homogenization of the sample, a 2 g portion was weighed into a 50 mL screw cap test tube with conical bottom. A volume of 8 mL of water was subsequently added and the tube was shaken by vortex for 10 s. Subsequently, 10 mL of 5% formic acid in MeCN was added to the tube, which was shaken by vortex for 2 min. QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the tube was vigorously hand-shaken for 1 min. After that, it was centrifuged at 4500 rpm for 5 min.

Then, 2 mL of the upper MeCN layer was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2 μm filter before injection into the chromatographic system for the determination of DON, F-X, FB_1, FB_2, T-2, HT-2, STE, OTA, ZEN and CIT.

For the analysis of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) an additional clean up step based on DLLME was necessary. It consisted of taking 2 mL of the upper MeCN layer (obtained after

extraction/partitioning step) which was transferred into a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). This solution was placed into a 15 mL screw cap test tube with conical bottom. Subsequently, 4 mL of water and 0.21 g of NaCl (4.2%; w/v) were added. The mixture of the disperser solvent (950 μL of MeCN) and the extraction solvent (620 μL of chloroform) was rapidly injected into the test tube with a 2.0 mL syringe. The solution was vigorously shaken for a few seconds, creating a cloudy solution in the tube. In this step, the aflatoxins were extracted into the fine droplets of chloroform. The mixture was centrifuged at 6000 rpm for 3 min and the fine particles were sedimented at the bottom of the tube. The sedimented phase (approximately 400 μL) was removed using a 1 mL syringe, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The solution was filtered and injected into the UHPLC-MS/MS system for the analysis of AFB_1, AFB_2, AFG_1 and AFG_2. Fig. 1 shows a diagram of the sample treatment.

2.3.1. UHPLC-MS/MS analysis

The chromatographic method for the determination of these 14 mycotoxins has been previously developed in our laboratory [27]. UHPLC separations were performed in a C18 column (Zorbax Eclipse Plus RRHD 50 × 2.1 mm, 1.8 μm) using a mobile phase consisting of 0.3% aqueous formic acid solution with 5 mM ammonium formiate (solvent A), and MeOH with 0.3% formic acid and 5 mM ammonium formiate (solvent B) at a flow rate of 0.4 ml min^{-1}.

The eluent gradient profile was as follows: 0 min: 5% B; 1 min: 50% B; 2 min: 72% B; 4 min: 80% B; and 6 min: 90% B. Finally it was back to 5% B in 0.2 min and maintained for 1.8 min for column equilibration. This gradient was optimized in order to obtain the best peak separation, which was not adequate using a linear gradient. The temperature of the column was 35 °C and the injection volume was 5 μL (full loop).

The MS was working with ESI in positive mode under the multiple reaction monitoring (MRM) conditions shown in Table 1. The ionization source parameters were: source temperature 500 °C; curtain gas (nitrogen) 30 psi; ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi.

3. Results and discussion

3.1. Extraction and sample preparation

Peanut was selected as representative matrix for the optimization of the sample treatment. A portion of 2 g was taken and different solvents were checked for solid–liquid extraction: (a) 10 mL of MeOH with 5% formic acid and (b) 10 mL of MeCN with 5% formic acid. However, good results were not obtained in terms of extraction efficiency so we considered the use of the first step of the QuEChERS procedure based on partitioning via salting-out extraction, checking

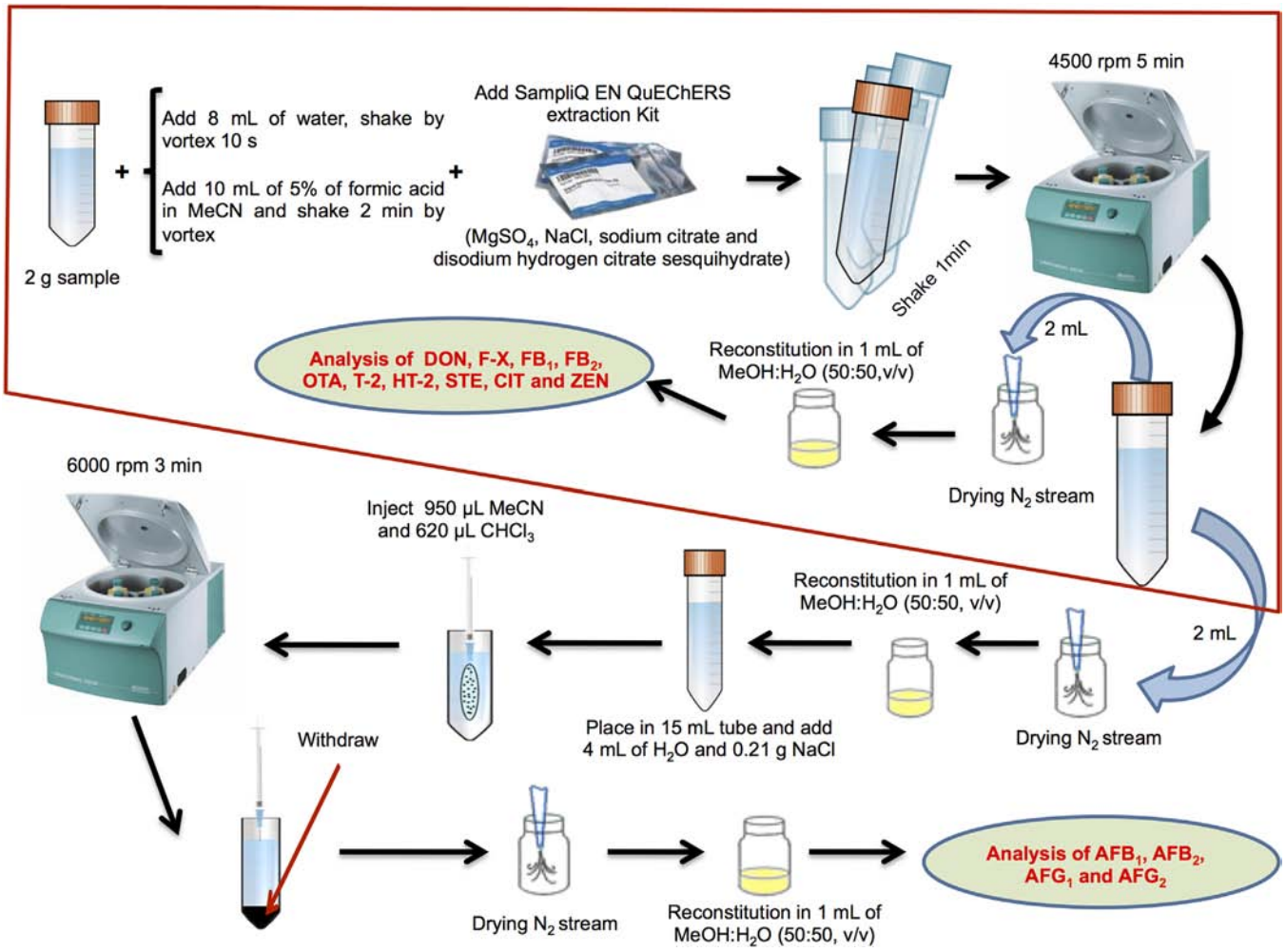

Fig. 1. Diagram of the proposed sample treatment.

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

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CE[a]	CXP[a]
DON	1.32	297.1	$[M+H]^+$	36.0	5.5	16.0	249.2 (Q)	17.0	4.0
							161.0 (I)	29.0	4.0
F-X	1.50	355.1	$[M+H]^+$	26.0	12.0	18.0	174.7 (Q)	23.0	4.0
							137.1 (I)	31.0	4.0
AFG_2	1.86	331.1	$[M+H]^+$	61.0	6.0	42.0	245.1 (Q)	39.0	4.0
							313.1 (I)	27.0	6.0
AFG_1	1.95	329.0	$[M+H]^+$	76.0	9.5	16.0	243.1 (Q)	39.0	6.0
							311.1 (I)	29.0	6.0
AFB_2	2.07	315.1	$[M+H]^+$	81.0	4.0	34.0	286.9 (Q)	33.0	6.0
							259.0 (I)	39.0	8.0
AFB_1	2.18	313.1	$[M+H]^+$	46.0	12.0	26.0	241.0 (Q)	41.0	4.0
							284.9 (I)	39.0	4.0
CIT	2.31	251.2	$[M+H]^+$	26.0	11.0	18.0	233.0 (Q)	23.0	23.0
							204.8 (I)	73.0	10.0
HT-2	2.70	442.0	$[M+NH_4]^+$	21.0	5.5	21.0	262.8 (Q)	22.0	8.0
							215.4 (I)	19.0	4.0
FB_1	2.93	722.2	$[M+H]^+$	71.0	10.0	30.0	334.2 (Q)	51.0	6.0
							352.2 (I)	47.0	6.0
T-2	3.11	484.0	$[M+NH_4]^+$	21.0	10.0	22.0	215.0 (Q)	22.0	4.0
							185.0 (I)	29.0	4.0
ZEN	3.53	319.0	$[M+H]^+$	26.0	8.0	20.0	282.9 (Q)	19.0	4.0
							301.0 (I)	15.0	10.0
OTA	3.59	404.0	$[M+H]^+$	41.0	7.5	16.0	238.9 (Q)	31.0	6.0
							102.1 (I)	91.0	6.0
STE	3.68	325.1	$[M+H]^+$	66.0	3.5	26.0	281.0 (Q)	43.0	4.0
							310.0 (I)	37.0	4.0
FB_2	3.76	706.2	$[M+H]^+$	71.0	10.5	20.0	336.3 (Q)	43.0	14.0
							318.3 (I)	45.0	12.0

[a] Declustering potential (DP), Entrance potential (EP), Collision Cell Entrance Potential (CEP), Collision Cell Exit Potential (CXP) and Collision Energy (CE). All expressed in voltage.
[b] Product ions: (Q) transition used for quantification, (I) Transition employed to confirm the identification.

different solvents with different extraction kits: (c) 8 mL of H_2O+10 mL of MeCN with 5% formic acid+QuEChERS Extraction packed (4 g $MgSO_4$, 1 g NaCl); (d) 8 mL of H_2O+10 mL of MeCN with 5% formic acid+buffered QuEChERS extraction tubes (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate); (e) 8 mL of 30 mM NaH_2PO_4 buffer pH 7.1+10 mL of MeCN with 5% formic acid+buffered QuEChERS extraction tubes. The best results were obtained with (d) so it was selected as extraction step. This simple procedure provided adequate efficiency in the extraction and determination of DON, F-X, FB_1, FB_2, T-2, HT-2, STE, OTA, ZEN and CIT. However, aflatoxins could not be determined at low concentrations due to lack of sensitivity produced by a strong ion suppression, thus a subsequent clean-up step was required in order to enable the quantification of these four analytes. The second step of the QuEChERS procedure, involving sample clean-up by DSPE with different sorbents (C18, $MgSO_4$ and PSA) was checked but it was discarded because it caused a significant loss of the analytes, decreasing the recoveries for all the mycotoxins, being higher if PSA was included (i.e., fumonisins disappeared completely). For this reason we choose a DLLME step, which was previously optimized and used in our laboratory for the analysis of targeted mycotoxins in botanicals [27], obtaining satisfactory results in terms of cleaning up of the matrix with adequate extraction efficiency. This procedure is described in detail in Section 2.3.

3.2. Characterization of the method

In order to check the suitability of the method for the determination of the target mycotoxins in nuts and seeds, a full validation was carried out in peanut, which, together with pistachios, are responsible of most of the mycotoxin contamination alerts. Moreover, most of the proposed official methods of determination of aflatoxins in this kind of foods are validated for this matrix [43].

Table 2
Matrix effect calculated as 100 × (signal of a spiked extract−signal of standard solution)/signal of standard solution.

	Level 1	Level 2	Level 3
AFB_1	−56.0	−57.8	−59.3
AFB_2	−54.8	−50.3	−52.7
AFG_1	−55.1	−52.1	−57.4
AFG_2	−54.1	−85.0	−56.2
OTA	−60.7	−68.7	−67.6
FB_1	−12.1	−15.7	−17.3
FB_2	−11.9	−19.3	15.6
T-2	−16.1	−19.5	−16.3
HT-2	−20.2	−27.4	−26.4
STE	−54.4	−40.7	−55.4
CIT	−17.7	−13.6	−19.9
F-X	−55.8	−59.7	−50.9
DON	−54.8	−50.9	−51.7
ZEN	−56.1	−58.3	−61.3

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1}; F-X: 200 μg kg^{-1}.
Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X: 2500 μg kg^{-1}.
Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X: 5000 μg kg^{-1}.

With this purpose linear dynamic range, limits of detection and quantification, and precision were evaluated. Recovery studies were performed in all the selected matrices to check the trueness of the method.

3.2.1. Calibration curves and performance characteristics

Table 2 shows the values of matrix effect at three concentration levels for each mycotoxin, calculated as 100 × [(signal of a spiked extract−signal of standard solution)/signal of standard solution].

As can be seen, matrix effect was significant for all the compounds (even for aflatoxins after the clean-up step) and therefore matrix-matched calibration curves were necessary for quantification purposes.

Matrix-matched calibration curves were established by spiking peanut blank samples at five different concentrations of each mycotoxin, and considering the peak areas as analytical signal. Each concentration level was prepared in duplicate and injected in triplicate. Spiked concentrations ranged from 1 to 50 μg kg^{-1} for AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE; 2 to 50 μg kg^{-1} for CIT; 10 to 500 μg kg^{-1} for FB_1, FB_2, T-2 and HT-2 toxin; 50 to 2000 μg kg^{-1} for DON; 200 to 5000 μg kg^{-1} for F-X; and 25 to 500 μg kg^{-1} for ZEN.

Table 3
Statistical and performance characteristics of the proposed method.

Analyte	Linear dynamic range (μg kg^{-1})	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
AFB_1	0.71–50	0.9988	0.21	0.71
AFB_2	0.97–50	0.9994	0.29	0.97
AFG_1	0.61–50	0.9986	0.18	0.61
AFG_2	0.95–50	0.9975	0.29	0.95
OTA	0.57–50	0.9989	0.17	0.57
FB_1	1.57–500	0.9994	0.47	1.57
FB_2	0.65–500	0.9977	0.19	0.65
T-2	4.74–500	0.9980	1.42	4.74
HT-2	3.77–500	0.9997	1.13	3.77
STE	0.61–50	0.9959	0.18	0.61
CIT	1.72–50	0.9993	0.52	1.72
F-X	150–5000	0.9978	45.1	150
DON	32.6–2000	0.9975	9.68	32.6
ZEN	20.2–500	0.9979	6.05	20.2

The statistical parameters were calculated by least-square regression and are presented in Table 3. The determination coefficients (R^2) were higher than 0.99, showing that mycotoxin analytical responses were linear over the studied ranges.

Limits of detection (LODs) and limits of quantification (LOQs) were estimated as $3 \times S/N$ ratio and $10 \times S/N$ ratio, respectively, and are presented in Table 3. The low LOQs obtained by this

Table 4
Precision study (% RSD of peak areas).

	Repeatability ($n=9$)			Intermediate precision ($n=15$)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	5.9	5.4	5.1	6.3	9.6	8.1
AFB_2	6.6	6.4	4.6	7.2	9.5	8.4
AFG_1	3.0	7.2	4.1	6.4	9.1	8.9
AFG_2	8.8	6.5	6.3	8.1	8.6	10.4
OTA	8.6	1.4	3.3	9.2	9.9	9.7
FB_1	8.3	4.0	5.0	6.3	8.6	9.9
FB_2	7.7	2.9	2.7	7.5	9.3	9.8
T-2	7.2	3.6	3.5	9.8	6.0	9.6
HT-2	7.8	2.9	5.0	9.6	8.5	6.4
STE	7.4	7.3	5.9	8.0	7.0	9.7
CIT	8.9	2.9	4.9	8.1	7.8	9.8
F-X	5.3	5.2	4.2	9.5	8.4	9.6
DON	6.9	3.9	3.7	10.6	10.1	10.2
ZEN	8.5	2.2	0.6	10.5	7.8	10.4

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1}; F-X: 200 μg kg^{-1}.
Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X: 2500 μg kg^{-1}.
Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X: 5000 μg kg^{-1}.

Table 5
Intervals for the recovery values in the studied foods obtained by spiking blank samples at three different levels (Intervals of % RSD of peak areas for the three levels).

	Peanut ($n=9$)	Pumpkin seed ($n=6$)	Pistachio ($n=6$)	Hazelnut ($n=6$)	Walnut ($n=6$)	Macadamia nut ($n=6$)	Pine nuts ($n=6$)	Sunflower seed ($n=6$)	Almond ($n=6$)
AFB_1	71.8–75.4 (5.1–5.9)	75.7–80.4 (1.0–8.9)	72.9–95.0 (2.4–9.3)	82.1–83.0 (8.4–9.3)	76.2–86.8 (4.1–7.0)	73.0–86.0 (8.4–8.7)	74.7–85.7 (3.8–8.7)	72.5–84.2 (4.2–8.2)	71.3–82.6 (2.7–8.1)
AFB_2	80.3–91.6 (4.6–6.6)	99.3 -80.1 (3.6–4.8)	85.3–94.1 (4.3–6.5)	82.2–94.8 (5.2–6.5)	81.5–93.4 (5.9–9.0)	65.8–73.3 (2.0–8.2)	74.9–85.3 (2.9–5.6)	79.4–87.3 (6.2–9.6)	78.2–87.0 (3.8–8.1)
AFG_1	71.4–74.4 (3.0–7.2)	79.3–91.7 (1.5–7.5)	72.8–84.9 (3.4–8.3)	76.2–91.5 (8.0–9.8)	73.0–78.2 (8.2–9.2)	71.0–83.9 (4.3–9.8)	71.1–79.3 (6.4–9.6)	69.4–77.7 (3.3–5.5)	77.4–86.5 (4.9–7.6)
AFG_2	84.3–94.0 (6.3–8.8)	73.3–98.9 (2.4–6.5)	85.8–96.5 (4.8–7.5)	74.4–92.1 (5.1–7.0)	80.4–88.1 (2.2–9.4)	71.6–80.7 (2.4–9.0)	73.7–88.7 (6.1–7.8)	73.3–84.4 (6.3–8.3)	89.1–97.9 (8.1–9.2)
OTA	83.6–89.0 (1.4–8.6)	82.6–84.5 (7.7–9.5)	81.4–94.6 (2.6–6.8)	86.8–93.1 (5.0–9.7)	82.6–93.6 (5.9–7.2)	78.5–84.8 (5.8–7.9)	81.1–89.4 (5.5–8.4)	90.6–97.0 (3.5–8.0)	88.4–97.8 (3.0–9.8)
FB_1	66.9–77.1 (4.0–8.3)	83.5–86.1 (0.5–5.5)	76.9–92.1 (2.2–3.1)	84.3–93.0 (1.9–3.5)	81.8–88.5 (1.0–6.8)	78.1–94.8 (1.8–8.0)	85.0–96.2 (2.3–9.4)	74.8–88.3 (1.3–8.6)	76.0–78.1 (2.2–5.0)
FB_2	78.1–91.2 (2.7–7.7)	75.5–90.8 (1.1–4.5)	95.5–101.3 (1.2–4.6)	83.7–100.5 (1.8–6.6)	86.7–98.7 (1.9–4.6)	81.4–98.7 (0.7–5.4)	86.6–96.3 (1.2–5.0)	89.9–94.8 (1.9–7.8)	84.7–87.5 (1.0–3.3)
T-2	93.1–96.1 (3.5–7.2)	92.0–99.3 (1.3–7.7)	93.0–99.5 (1.4–3.1)	99.1–104.3 (1.7–4.1)	95.3–99.6 (3.4–5.6)	90.4–100.4 (3.8–8.7)	61.9–72.4 (2.1–8.5)	94.2–98.8 (1.3–5.2)	90.7–97.4 (2.9–6.5)
HT-2	79.0–83.4 (2.9–7.8)	77.6–79.0 (0.7–5.3)	70.3–98.9 (1.3–4.6)	92.0–101.9 (2.43.5)	90.2–97.6 (1.1–6.6)	86.4–99.0 (3.3–7.2)	78.6–92.7 (3.2–5.1)	96.7–101.3 (2.0–5.2)	92.6–99.0 (2.1–4.2)
STE	70.0–85.1 (5.9–7.4)	74.0–80.0 (4.7–8.1)	68.5–74.5 (4.9–9.9)	70.9–89.3 (5.1–8.6)	73.4–81.8 (3.1–8.5)	69.5–77.9 (5.6–9.0)	74.8–81.6 (4.1–7.1)	89.3–96.8 (3.6–6.5)	60.7–65.6 (5.4–9.9)
CIT	69.5–80.1 (2.9–8.9)	77.7–87.3 (2.6–7.3)	80.8–95.7 (4.4–8.5)	77.5–85.6 (3.1–8.0)	89.9–98.5 (4.6–7.7)	87.6–94.2 (3.3–7.3)	77.6–86.9 (5.5–9.0)	87.9–101.6 (4.6–10.2)	92.1–100.0 (3.0–7.4)
F-X	73.0–89.3 (4.2–5.3)	69.9–75.2 (1.8–9.8)	95.7-92.5 (1.4–6.9)	75.1–86.1 (2.0–7.5)	87.6–98.6 (2.5–6.6)	88.6–95.6 (2.5–8.7)	86.1–94.1 (1.4–8.0)	78.4–93.7 (3.2–9.6)	88.0–92.6 (1.3–9.1)
DON	82.8–93.0 (3.7–6.9)	86.4–102.5 (1.5–3.7)	71.6–80.5 (2.3–9.2)	76.0–88.0 (2.2–5.4)	82.5–92.6 (5.3–7.6)	92.1–100.0 (2.6–7.4)	80.3–93.5 (2.8–8.5)	80.6–89.2 (4.3–9.8)	81.7–86.1 (2.1–7.5)
ZEN	81.2–94.2 (0.6–8.5)	89.5-79.4 (3.5–6.4)	98.6-73.7 (2.8–7.6)	79.9–89.0 (1.9–8.2)	94.9-81.7 (2.8–7.6)	76.8–87.8 (2.4–7.3)	94.6-85.3 (3.1–7.6)	87.5–95.8 (2.3–6.9)	81.8–99.3 (1.3–3.8)

% RSD of peak areas is given in parentheses.
Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1}; F-X: 200 μg kg^{-1}.
Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X: 2500 μg kg^{-1}.
Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X: 5000 μg kg^{-1}.

methodology for aflatoxins, the only mycotoxins regulated in nut and seed matrices, allow their quantification at concentrations lower than the maximum level established by current legislation [6,7]. In the same way, the rest of mycotoxins can be determined at concentrations lower than the maximum levels established for these contaminants in different foodstuff [6].

3.2.2. Precision

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by application of the whole procedure on the same day to three peanut samples (experimental replicates) spiked at three concentration levels of mycotoxins. Each sample was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, with five samples analysed in different days. The results, expressed as RSD of peak areas, are shown in Table 4. As can be seen precisions lower than 11% were obtained in all cases, in agreement with current legislation [13].

3.2.3. Sample analysis and recovery studies

It must be highlighted that before performing the recovery studies to check the trueness of the method, all the samples (peanuts, almonds, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) were previously analysed and three of them were found to be positive for several mycotoxins. In these cases, the concentration of each mycotoxin was assessed by the standard addition methodology. Specifically, the sunflower seed sample gave a positive results in STE (3.7 μg kg^{-1}; $y=300.56x+1122.6$, $R^2=0.9929$), the walnut sample in ZEN (221.8 μg kg^{-1}; $y=57.898x+12844.0$, $R^2=0.996$) and DON (345.5 μg kg^{-1}; $y=43.471x+15018.0$, $R^2=0.994$) and the macadamia nut sample in F-X (2465.5 μg kg^{-1}; $y=1.346x+3318.6$, $R^2=0.990$).

Recovery experiments were carried out in all the selected matrices. With this purpose, two samples of each product (except peanuts, where three samples were analysed) were spiked at three different concentration levels, processed and injected in triplicate in the UHPCL-MS/MS system. The spiked concentration levels as well as the obtained results are summarized in Table 5 (the recovery values for the full study are included in the Supplementary material). As can be seen, recoveries ranging from 60.7% to 104.3% were obtained for all the analytes. Thus, current legal requirements for the determination of legislated mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, DON, ZEN, FB_1, FB_2, T-2, HT-2) were fulfilled [13]. A typical chromatogram corresponding to the separation of the analytes in spiked extracts of peanut samples under developed conditions is shown in Fig. 2, showing the effectiveness of the DLLME step in the determination of aflatoxins.

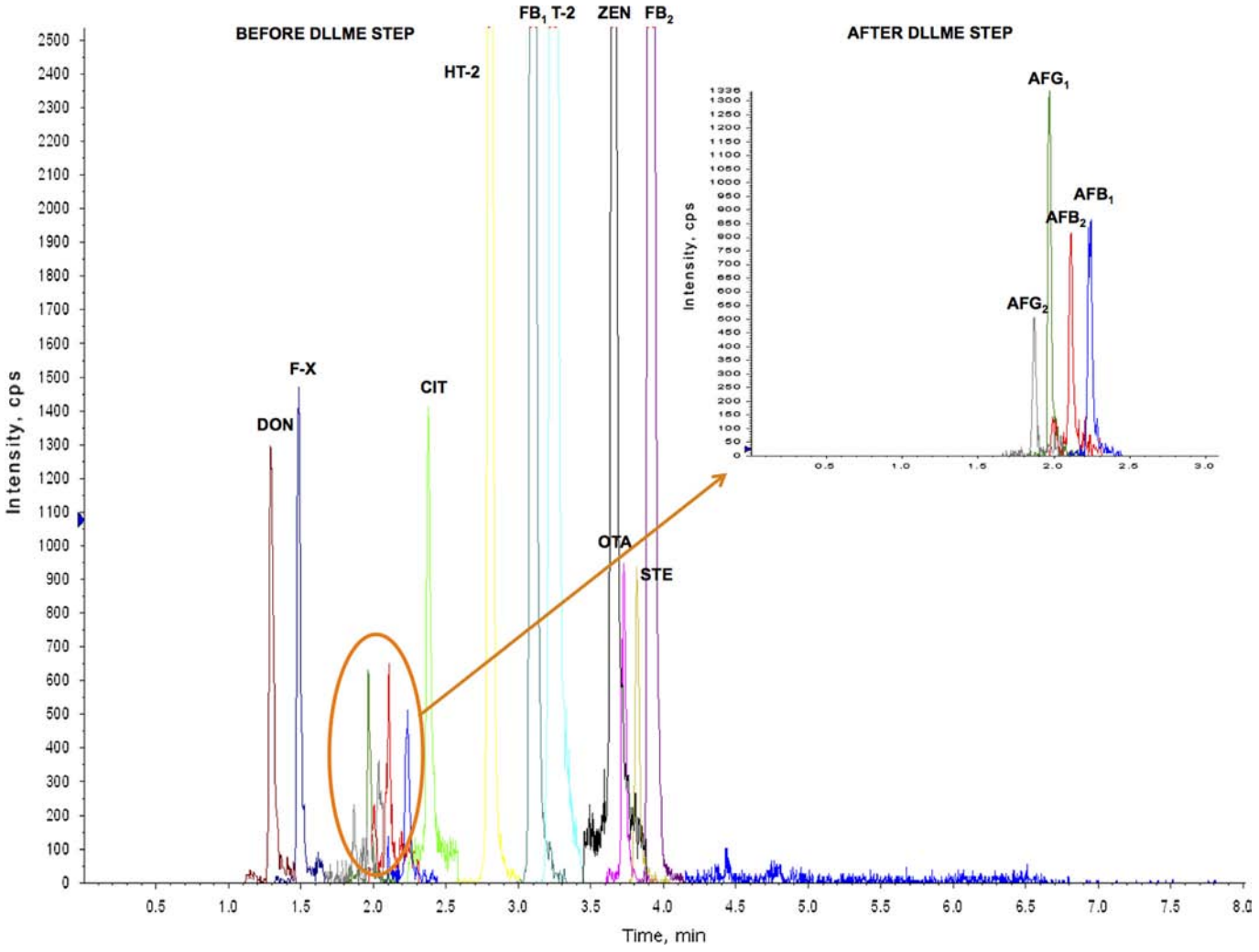

Fig. 2. Chromatogram of a spiked peanut sample (AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X: 2500 μg kg^{-1}).

4. Conclusions

A sample treatment based on QuEChERS and DLLME has been proposed for the determination of 14 mycotoxins in a great variety of nuts and seeds. The first step of the sample treatment, based on an extraction/partitioning, allows the determination of FB_1, FB_2, OTA, T-2, HT-2, STE, CIT, ZEN, DON and F-X. In order to determine the aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) an additional clean-up step using DLLME (proposed as an alternative to dispersive SPE in order to avoid losses of mycotoxins in terms of recovery) is required. This method has shown to be a suitable choice for the sample treatment of this kind of fruits in multiclass mycotoxin determination, reducing extraction time, providing good recoveries and precisions and always fulfilling the current EU legislation. This treatment and the use of UHPLC-MS/MS technology, make possible the determination of the most important mycotoxins in nine different matrices. Furthermore, the use of expensive and tedious IAC is avoided. Matrix-matched calibration curves were established and LODs and LOQs were below the usual maximum limits established by EU regulation in food in all cases. The precision (repeatability and intermediate precision) was lower than 11% in all cases, and recoveries were between 60.7% and 104.3%. Some of analysed samples were positive for some mycotoxins; STE was found in sunflower seed, F-X in macadamia nut and ZEN and DON were found in walnut samples (all purchased in local markets).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.04.024.

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