Multi-mycotoxins Analysis in Dried Fruit by LC/MS/MS and a Modified QuEChERS Procedure

Ines Azaiez · Federica Giusti · Gianni Sagratini · Jordi Mañes · Mónica Fernández-Franzón

Abstract A sensitive and reliable multi-mycotoxin method was developed for the simultaneous determination of 16 toxicological important mycotoxins, such as aflatoxins B_1, B_2, G_1, and G_2; enniatins A, A_1, B, and B_1; beauvericin; ochratoxin A; fumonisin B_1, B_2, and B_3; diacetoxyscirprenol; HT-2; and T-2 toxin in dried fruits using liquid chromatography combined with electrospray ionization-triple quadrupole tandem-mass spectrometry. Mycotoxins have been extracted from the samples using a modified quick, easy, cheap, effective, rugged, and safe procedure. The method was based on a single extraction with acidified acetonitrile, followed by partitioning with salts, avoiding any further clean-up step. Limits of detections ranged from 0.08 to 15 µg kg^{-1} and limits of quantification ranged from 0.2 to 45 µg kg^{-1}, which were below the legal limit set by the European Union for the legislated mycotoxines. The recoveries in spiked samples ranged from 60 to 135 % except for beauvericin using matrix-matched calibration curves for quantification, with good inter- and intraday repeatability (respective relative standard deviation ≤20 and 9 %). The developed method was applied to 15 commercial dried fruits: raisins, figs, apricots, plums, and dates purchased in local markets from Spain. Among the mycotoxins studied, enniantins and aflatoxins were the most predominant mycotoxins.

Keywords Mycotoxins · QuEChERS · Dried fruits · LC/MS/MS

I. Azaiez · J. Mañes · M. Fernández-Franzón (✉)
Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain
e-mail: monica.fernandez@uv.es

F. Giusti · G. Sagratini
Scuola di Scienze del Farmaco e dei Prodotti della Salute, Università di Camerino, via S. Agostino 1, 62032 Camerino, Italy

Introduction

Mycotoxins, secondary metabolites of filamentous fungi, represent a very large group of different substances produced by different mycotoxigenic species. These compounds with great structural variation result in diverse physicochemical properties (Köppen et al. 2010). Hundreds of mycotoxins have been recognized, although only a few of them present a significant toxic effect and are of major concern. These substances are associated with carcinogenic, teratogenic, nephrotoxic, and hepatotoxic properties; besides toxic effects, mycotoxins can cause tremendous economic losses derived from the contamination of the world's crop production.

Moulds of the genera *Aspergillus* and *Penicillium* occur in different fruits. Fruits become increasingly susceptible to fungal invasion during ripening as the pH of the tissue increases, skin layers soften, soluble carbohydrates build up, and defense barriers weaken (Fernández-Cruz et al. 2010). Furthermore, varying processing and storage conditions can provide mould growth and mycotoxin development. There have been many investigations on the occurrence of mycotoxins in these products. Dried fruits, particularly dried vine fruit (raisins, sultanas and currants), and figs have been of considerable interest as various dried fruits are in great demand in the health food markets (Trucksess and Scott 2008).

Only a few mycotoxins in dried fruits have been regulated by law. The European Union (EU) has imposed vigorous regulations to limit the presence of ochratoxin (OTA) in dried vine fruit (currants, raisins, and sultanas; 10 µg kg^{-1}) as well as aflatoxin (AF)B_1 in dried fruits for human consumption or food ingredient (2 µg kg^{-1}) and total aflatoxins (4 µg kg^{-1} for the sum of AFB_1+AFB_2+AFG_1+AFG_2) (Commission of the European Communities 2006). Also, a relatively new EU regulation (Commission of the European Communities 2010) was set for the total aflatoxins in dried figs (10 µg kg^{-1}).

Nowadays, the general trend is the performance of multi-mycotoxin methods which permit the analysis of a wide range of mycotoxins with varying physicochemical characteristics in a single run. Thus, the combination of liquid chromatography (LC) with tandem-mass spectrometry (MS/MS) has proved to be a powerful tool for the simultaneous determination of different classes of mycotoxins (Capriotti et al. 2012; Tang et al. 2013), and it has become the selected technique in multiresidue analysis.

The analysis of mycotoxins in dried fruits can be an extremely challenging task due to minute amounts usually presents in the samples and the large quantities of co-extracted compounds especially carbohydrates which can adversely affect the method and instrument performance.

The methods proposed for the determination of mycotoxins in dried fruits are mainly based on liquid chromatography fluorescence detector for aflatoxins and OTA (Karbancıoglu-Guler and Heperkan 2008; Reinhold and Reinhardt 2011; Zinedine et al. 2007). Most of these methods use solid–liquid extraction and immunoaffinity column (IAC) for clean-up, but IAC is an expensive and complex purification system which leads to low recoveries for some mycotoxins, because of the complexity of these matrices and its use is limited to a single mycotoxin.

Recently, an extraction procedure defined as "quick, easy, cheap, effective, rugged, and safe" (QuEChERS) has been developed mainly for the extraction of pesticides, but it has also been used for other compounds (Anastassiades et al. 2003).

The QuEChERS method has several advantages over most traditional methods of analysis, such as its simplicity, small solvent usage; waste minimum steps, and effectiveness for cleaning-up complex samples (Sospedra et al. 2010; Rodríguez-Carrasco et al. 2012). Its inherent speed and simplicity are also important when analyses of large number of samples in short time flame are required. It includes two steps: the first one is a partitioning step via salting-out extraction, and the second one is a dispersive solid-phase extraction step that involves further clean-up using combinations of MgSO4 and different sorbents, such as C18 or primary and secondary amine (PSA). QuEChERS based methods have been recently reported for the extraction of different mycotoxins from variety of food matrices such as cereal products (Cunha and Fernandes 2010; Ediage et al. 2011; Ferreira et al. 2012), eggs (Garrido Frenich et al. 2011), beer (Tamzura et al. 2011), spices (Yogendrarajah et al. 2013), and bee pollen (Rodríguez-Carrasco et al. 2013). The main advantages of this extraction procedure were not only the low amount of organic solvent required, but also the fastness and the good recoveries obtained for the tested mycotoxins (Ferreira et al. 2012).

The aim of the present study is to adapt, apply and evaluate the QuEChERS procedure to the extraction of multiple mycotoxins in dried fruits. To the best of our knowledge, the

QuEChERS procedure has not been used in extraction of mycotoxins from dried fruits. Finally, its application to the determination of the studied mycotoxin in real samples has been accomplished successfully.

Materials and Methods

Reagents and Standards

Acetonitrile and methanol were high performance liquid chromatography grade and supplied by Merck (Darmstadt, Germany). Deionized water (<8 MΩ cm−1 resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, USA).

Solid phases used were Sepra C18-E (50 μm, 65 A°) endcapped silica based C18 from Phenomenex (Torrance, USA) and Sep-Pak®C18 cartridges and Classic CN cartridges from Waters Corporation (Massachusetts USA).

Analytical grade reagent formic acid, acetic acid (purity, >98 %), and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). Polypropylen syringes and filters (25 mm/0.45 μm and 0.22 μm) were from Analysis Vínicos (España). Magnesium sulfate anhydrous 99.5 % powder (MgSO$_4$) was purchased from Alfa Aesar® Gmbh & Co KG (Germany) and sodium chloride (NaCl) from Merck (Darmstadt, Germany).

The standards of aflatoxin AF(B$_1$, B$_2$, G$_1$, and G$_2$), OTA, diacetoxyscirpenol (DAS), fumonisins (FB$_1$ and FB$_2$), beauvericin (BEA), and enniatins Enns (Enn A, Enn A1, Enn B, and Enn B1) were purchased from Sigma Aldrich (Madrid, Spain). T-2 toxin (T-2) and HT-2 toxin (HT-2) stock solutions (in acetonitrile) were acquired from Biopure Referenzsubstanzen GmBH (Tulln, Austria). Fumonisin B$_3$ (FB$_3$) was supplied by the PROMEC unit (Program on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa). Stock standard solutions of all mycotoxins were made in methanol, except FB$_1$, FB$_2$, and FB$_3$ that were diluted in acetonitrile/water 50:50 (v/v). They were all kept in safety conditions at −20 °C. Purity checks of stock solutions are made by UV measurement since their molar absorptivities are known.

From the individual stock standard solutions, a standard mixture was prepared at the following concentrations: Enn B, Enn B$_1$, Enn A, and Enn A$_1$ and AFB$_1$, AFB$_2$, AFG$_1$, and AFG$_2$ (0.5 μg mL^{-1}); OTA and T-2 (2 μg mL^{-1}); BEA, FB$_1$, FB$_2$, and FB$_3$; DAS; and HT-2 (5 μg mL^{-1}). All other working solutions for determine calibration curves, ion suppression, recoveries, and limit of detection were prepared by diluting the standard mixture in methanol or matrix extract.

LC/MS/MS Analysis

Detection and quantification was performed with 3200 QTRAP® LC/MS/MS System (Applied Biosystems, Foster City, CA) with a Turbo V® ionspray ESI source coupled to Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA). The instrument data were collected and processed using the Analyst® version 1.5.2 software.

Chromatographic separation of analyte was performed with a reversed-phase analytical column (Gemini ® C18 column, 3-μm particle size, 150×2 mm, I.D.), equipped with a C18 (4×2 mm, I.D.; 5 μm security guard cartridge) all from Phenomenex, Madrid, Spain. The mobile phases were composed of two eluents both containing 5 mM ammonium formiate, the eluent A was water/formic acid 99:1 (v/v) and the eluent B was methanol/formic acid 99:1(v/v). The flow rate was 0.25 mL min^{-1}. The gradient program started with 90 % A and 10 % B and was kept 3 min, afterwards a linear gradient was applied, reaching 70 % B after 1.5 min (holding time, 3 min). Other linear gradients to 80 % B (6 min) and 90 % B (14 min) were included. Finally, gradient switched back (5 min) to 90 % A.

MS/MS was performed in the selected reaction monitoring (SRM) mode using ESI in positive mode. For each compound, two characteristic product ions were monitored; the first and most abundant one was used for quantification, while the second one was used as a qualifier.

For LC/MS/MS analysis, scheduled SRM was used with a 120 s SRM detection window and 1 s of target scan time. The applied parameters were: ion spray voltage, 5,500 V; source temperature, 450 °C; curtain gas, 20; ion source gas 1 (sheath gas), 50 psi; ion source gas 2 (drying gas), 55 psi. Nitrogen served as nebulizer and collision gas.

Samples

Fifteen samples of dried fruits: plums (3), raisins (3), apricots (3), figs (3), and dates were collected from different local markets and supermarkets from Valencia. They were kept at −18 °C until analysis.

Extraction and Clean-up Optimization

The extraction was performed by a modified version of QuEChERS procedure. In a 50-mL polypropylene centrifuge tube, 5 g±(0.01 g) of homogenized sample were weighed. Sample recovery was investigated with 5 g of the blank dried fruit samples using three different fortification levels: 50, 100, and 200 μL of the standard mix. The spiked samples were shaken for 3 min with a (mechanical mixing) vortex and left

2 h at room temperature to allow the mycotoxins absorption into the matrix.

In this study, in order to optimize the extraction procedure, different volumes of organic solvents as methanol and acetonitrile (10; 22.5 and 32 mL) were assayed along with different proportions of acetic acid (0; 0.5 and 1 %). Then, MgSO$_4$ (7.5 g) and NaCl (3 g) were added in order to obtain a complete dispersion and humidification of the sample, which will permit a better extraction of the mycotoxins. For clean-up step CN, C18 cartridge and C18 powder were proved. From analytical point of view, the best extraction results were obtained via the following steps:

Step I   Five grams of raisins were homogenized with 7.5 mL of water with acetic acid (1%) for 3 min with a vortex. The obtained mash was extracted with 22.5 mL of acetonitrile for 3 min with a vortex.

Step II   Anhydrous MgSO$_4$ (7.5 g) and NaCl (3 g) were added into the mixture and the homogenizates were extracted under continuous mechanic shaking for 1 h in order to facilitate the extraction and partitioning of the mycotoxins compounds into the organic layer.

Step III   The extract was centrifuged for 10 min at 5,000 rpm, 5 °C and subsequently the supernatant layer was collected.

Step IV   The eluate was evaporated to dryness under a nitrogen stream at 40 °C, using a multisample Turbovap LV Evaporator (Zymark, Hoptkinton, USA) and was dissolved with 1 mL of 5 mM ammonium formiate aqueous solution/methanol (50/50, v/v) acidified with 1 % of formic acid. Each sample was filtrated through a 0.22-μm PTFE filter prior to the LC/MS/MS analysis.

Results and Discussion

Optimization of Chromatographic Conditions

The main aim of this study was to develop a single run multimethod for mycotoxin on dried fruits subject to current and future EU legislation. The MS/MS method was based on a previous reported LC/MS/MS method performed in our laboratory, selecting those mycotoxins that gave better sensitive in positive ionization (Rubert et al. 2012). The optimization of MS parameters was performed by flow injection analysis for each compound; entrance potential (EP) was set at 10 V, for all analytes. The quantification and qualification ion transitions of the respective mycotoxins, the declustering potential, collision energy, and collision cell exit potential programmed are shown in Table 1, as well as the retention times.

Table 1 Optimized MS\MS parameters for each mycotoxin

Analyte	Rt (min)	Precursor ion (m/z)	Product ions (m/z)	DP (V)	CE (V)	CXP (V)
AFB1	8.4	313.0 [M+H]$^+$	241.1^Q	46	39	4
			128.0^q		41	4
AFB2	8.4	315.0 [M+H]$^+$	287.0^Q	81	33	6
			259.0^q		39	6
AFG1	8	329.0 [M+H]$^+$	243.0^Q	76	39	6
			200.0^q		29	6
AFG2	7.9	331.1 [M+H]$^+$	313.0^Q	61	27	6
			189.0^q		39	4
BEA	16.7	801.2 [M+NH$_4$]$^+$	784.1^Q	116	27	10
			244.1^q		39	6
DAS	8.2	384.0 [M+NH$_4$]$^+$	307.2^Q	66	15	16
			105.0^q		63	12
ENN A	17.9	699.4 [M+NH$_4$]$^+$	210.1^Q	76	35	14
			228.2^q		59	16
ENN A1	17.1	685.4 [M+NH$_4$]$^+$	210.2^Q	66	37	8
			214.2^q		59	10
ENN B	15.4	657.3 [M+NH$_4$]$^+$	196.1^Q	51	39	8
			214.0^q		59	10
ENN B1	16.2	671.2 [M+NH$_4$]$^+$	214.1^Q	66	61	10
			228.1^q		57	12
FB1	8.8	722.4 [M+H]$^+$	334.3^Q	101	51	20
			352.3^q		45	26
FB2	10.4	706.4 [M+H]$^+$	336.2^Q	131	50	16
			318.3^q		50	16
FB3	9.6	706.5 [M+H]$^+$	336.3^Q	100	45	16
			318.4^q		45	16
HT-2	9.2	442.2 [M+NH$_4$]$^+$	215.1^Q	61	19	4
			263.0^q		19	8
OTA	11.2	404.0 [M+H]$^+$	102.0^Q	91	27	6
			239.0^q		97	6
T-2	9.9	484.2 [M+NH$_4$]$^+$	185.1^Q	76	29	4
			215.1^q		22	4

Q quantification, *q* qualification, *DP* the potential difference, *CE* collision energy, *CXP* collision cell exit potential

Tuning experiments favored the choice of the ESI(+) mode since EU limits for AFB$_1$, OTA on dried fruits were met. Even for HT-2, T-2, DAS, BEA, FBs, and Enns the ionization in positive mode was successful, whereas FUS X, DON, and NIV gave no or very weak signals in the positive ion mode (Sulyok et al. 2006) (these mycotoxins were not included in this study). The majority of mycotoxins have a higher tendency to ionize in ESI+ forming protonated molecular ions. Moreover, it has been observed that a limited ionization of trichothecenes as protonated or deprotonated molecular ions was obtained with ESI. This problem was overcome by monitoring ammonium adducts (Garrido Frenich et al. 2009; Sulyok et al. 2010).

LC mobile phases commonly used for mycotoxins analysis are usually water, acetonitrile and methanol, with or without addition of salts, acids, or bases. Methanol as organic solvent provides the best sensitivities for all compounds. The addition of ammonium formiate to the aqueous mobile phase clearly enhanced the sensitivity for T-2, HT$_2$, DAS, and the other mycotoxins detected under their ammonium adduct [M+NH$_4$]$^+$, whereas formic acid in both mobile phases increased the overall sensitivity for the acidic compounds, i.e., FB$_1$, FB$_2$, and OTA (Desmarchelier et al. 2010). In negative mode, the formiate buffer is preferred to formic acid, which makes it impractical to switch polarity during the same injection even when technically possible in the MS. Therefore, it was decided to maintain the positive mode in the multimethod for the entire run.

Extraction and Clean-up Optimization

Owing to the great chemical diversity of mycotoxins and the complexity of dried fruits matrices validated in this study, the

solvent composition applied for extraction was an important parameter during the development of the multi-mycotoxin method. Optimization studies were performed with prune samples spiked with Enns and aflatoxins (10 μg kg^{-1}); T-2 and OTA (40 μg kg^{-1}); and BEA, fumonisins, DAS, and HT-2 (100 μg kg^{-1}).

Different mixtures of methanol, or acetonitrile and water with or without organic acids were evaluated. Several extractions with methanol/water (80:20) mixtures were firstly carried out. Although mycotoxins extraction was satisfactory, increased amounts of matrix components such as polysaccharides and brown pigments were also extracted (Ediage et al. 2011), which could result in column spoilage (Knudsen et al. 2011) and polluted MS equipment causing MS damage. Consequently, acetronitrile was considered to be the best choice for this type of matrix.

Firstly a modification of QuEChERS method was applied by adding acid to the extraction solvents. Initially mixtures of acetonitrile/water without and with 0.5, and 1 % of acetic acid were checked. The results are presented in Fig. 1.

Comparison of the results from each of the samples indicated that acidified (1 %) aqueous acetonitrile provided the best overall performance. Without acidification, FBs could not be extracted, and low recoveries were obtained especially for OTA, HT2, and T2. At low pH, the four carboxylic groups of FBs are protonated leading to less interaction and binding to the sample matrix so acid solvents are the best choice (Garrido Frenich et al. 2011; Knudsen et al. 2011).

Afterwards, extractions with four different volumes and proportions of acetonitrile/water mixture with 1 % of acetic acid were evaluated (volume of water was kept almost fixed (7.5/8 mL) and acetonitrile volume was varied). The results are shown in Fig. 2.

For the majority of mycotoxins, unsatisfactory recoveries were obtained when only acetonitrile was used as extraction solvent. Low recoveries were also obtained with the mixture of acetonitrile/water (55:45). It could be assumed that smaller solvent-sample ratios can result in a saturation of the extraction solvent when samples with a large amount of solutes are extracted such as samples with a high content of sugar. The best recovery results were obtained using the proportion acetonitrile/water (75:25).

In order to improve the recoveries obtained, we optimized the ratio sample/water, increasing the amount of water required to obtain a suitable homogenized dried fruit sample. "Wetting" prior to the extraction is recommended in the Document SANCO/12495/2011 whenever the matrix contains less than 40 % of moisture. Therefore, water/solvent/salt amount was increased and better recoveries were obtained with the high volumes tested.

Several types of agitations were put into practice in order to improve extractions' performances. Ultra-sonication, horizontal shaker, manual and vortex agitation were evaluated. The best results in terms of recoveries were obtained using vortex agitation (data no shown).

Finally, a clean-up procedure for reducing co-extracted compounds and matrix interferences was evaluated. Original QuEChERS usually used PSA but this step has been avoided as fumonisins are strongly retained because of the ionic affinity between the primary and secondary ammine of the PSA and the carboxylic groups of fumonisins (Desmarchelier et al. 2010). Conventional SPE cartridges (C18 and CN) and C18 powder were evaluated. The obtained results are presented in Fig. 3. Lower recovery rates were obtained for most of the mycotoxins tested, when using C18 powder clean-up. It was assumed that they were not well recovered for being
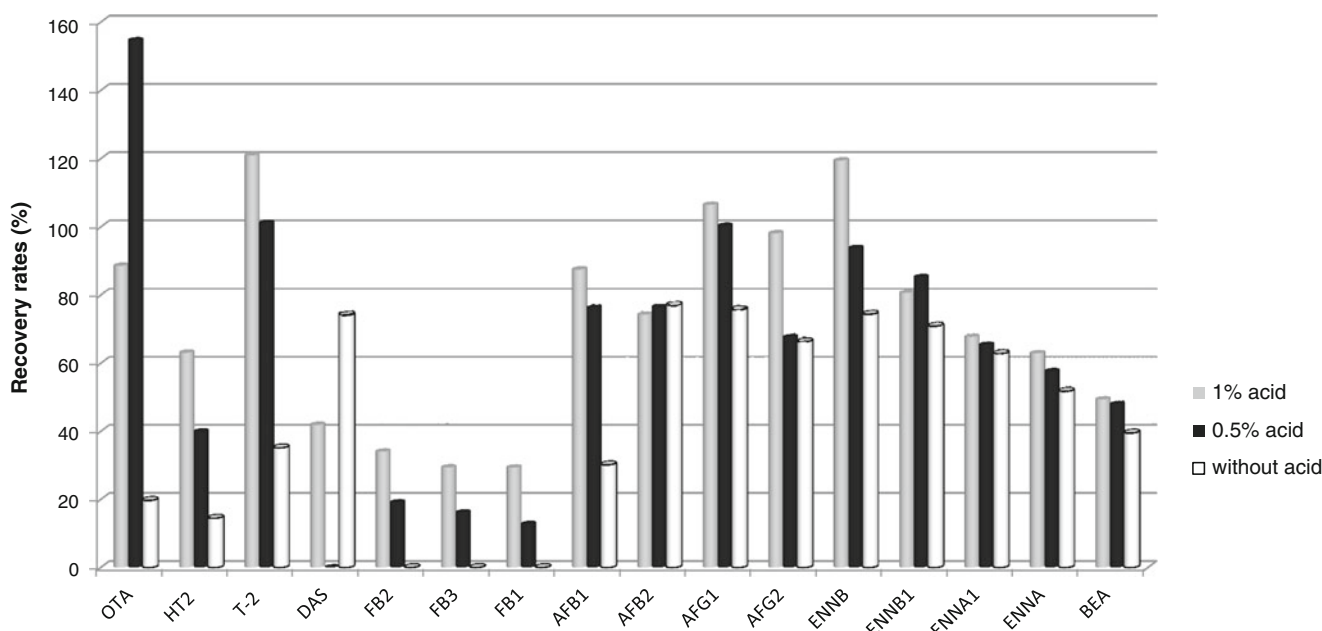

Fig. 1 Effect of the acetic acid addition on the recovery results obtained for the extraction of spiked prune samples
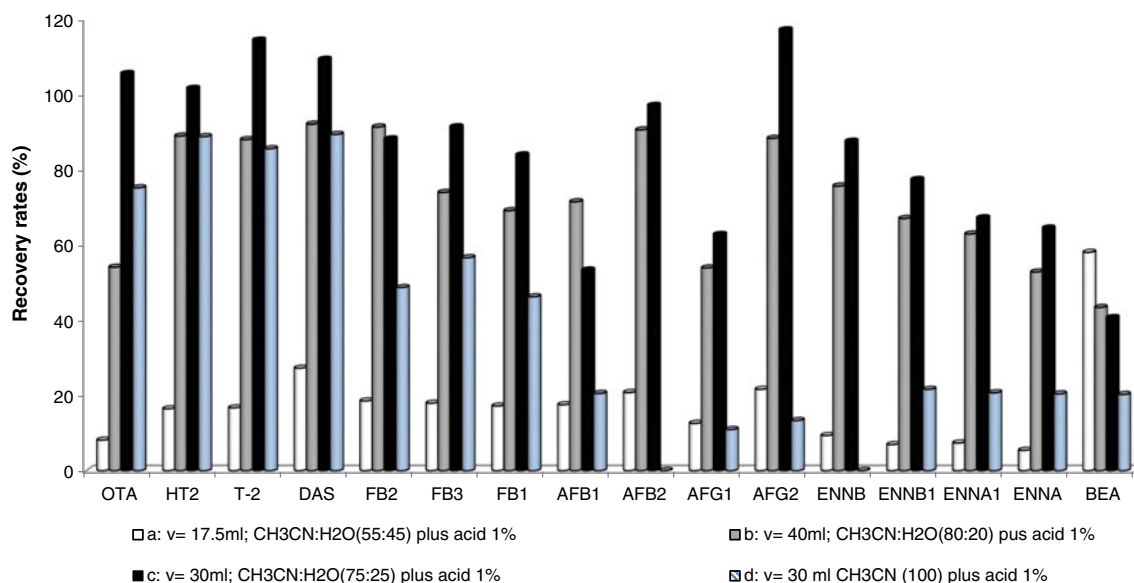
Fig. 2 Effect of the solvents proportion and volume on the extraction recovery of mycotoxins in prunes

partially absorbed by C18 powder. In order to improve recovery rates, clean-up was carried out using solid-phase extract (SPE) cartridges C18. The recovery rates of HT_2, DAS, FBs, AFB_1 and AFG_2 have improved while those corresponding to OTA, T2, Enns and BEA have decreased. To reduce the retention of mycotoxins with the solid phase, the cartridges were washed with 2 mL of methanol at the end of each elution. Recoveries data showed a slightly improvement for all mycotoxins. Similar recovery rates were obtained while using (SPE) cartridges of CN. It can be clearly observed that poorer results were obtained when clean-up procedure was applied. Therefore, no clean-up was used for further experiments.

Method Validation

Method validation was performed in terms of linearity, intra- and interday repeatability, matrix effect, limit of detection (LODs), limits of quantifications (LOQs) and recovery. Validation parameters were studied on prune samples except for recoveries that were studied also on apricots, figs, raisins and dates.

Results of the evaluation are summarized in Table 2. To compare the efficiency of each extraction procedure, absolute recoveries were determined by spiking blank extract samples (standards added to blank samples of each matrix studied) at
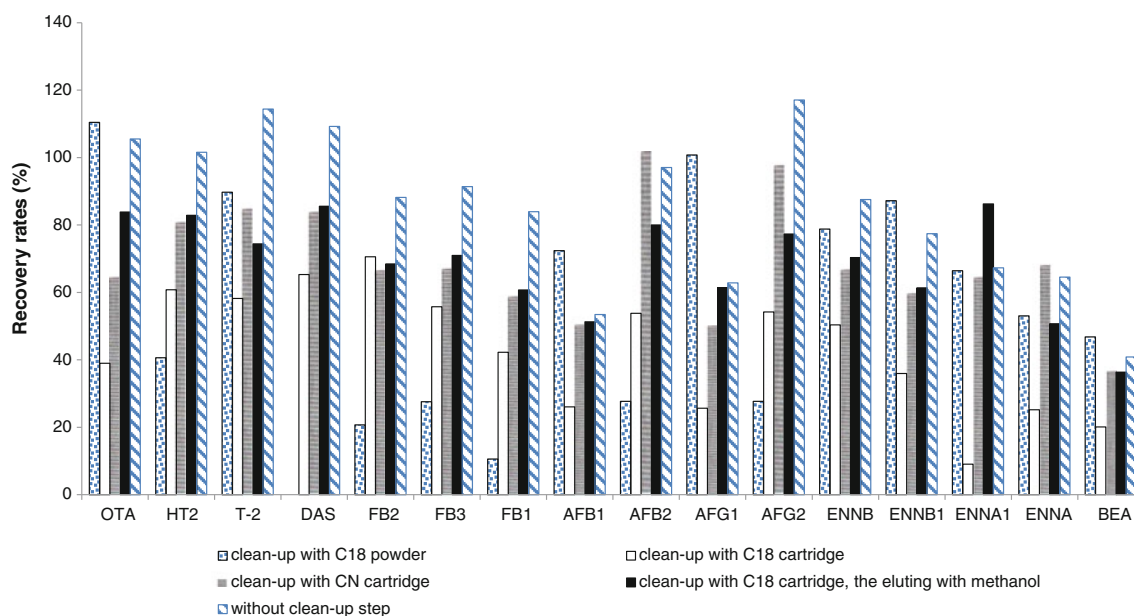

Fig. 3 Recovery rates obtained applying a clean-up step using C18 powder, C18 cartridge, CN cartridge, and without clean-up

Table 2 LOQ and LOD (micrograms per kilogram), average recoveries (percent), and repeatability (%RSD), of the selected mycotoxins

Mycotoxins	LODs (µg/kg)	LOQs (µg/kg)	Recovery (%)					Repeatability (%)	
			Dates	Apricots	Raisins	Figs	Prunes	Intraday	Interday
AFB1	0.08	0.2	89±3	84±2	62±8	83±3	60±4	9	12
AFB2	0.08	0.2	99±15	101±14	86±6	92±8	97±13	4	13
AFG1	0.16	0.5	83±4	78±1	70±8	96±12	63±20	5	10
AFG2	0.3	0.9	89±7	118±16	85±4	83±2	117±20	4	14
T-2	1.60	5	135±8	90±13	130±12	83±6	114±5	2	17
HT-2	5	15	107±4	85±4	92±10	101±11	101±7	3	20
DAS	15	45	94±4	93±4	96±15	81±7	109±16	4	20
Enn A	0.3	1	62±3	71±7	61±11	63±8	64±19	3	16
Enn A1	0.3	1	60±3	81±6	79±10	61±7	67±1	2	17
Enn B	0.3	1	64±5	86±4	78±6	68±6	87±1	4	20
Enn B1	0.3	1	60±4	81±7	71±7	66±8	77±2	2	20
FB1	5	15	62±2	72±9	64±1	64±4	84±1	3	12
FB2	5	15	73±3	100±15	69±2	84±3	88±5	6	13
FB3	5	15	62±2	81±9	76±3	107±5	91±2	5	9
OTA	3	9	87±3	90±12	75±4	81±1	105±5	4	18
BEA	7	20	43±4	49±5	42±8	45±6	41±1	3	20

Recovery and repeatability data was obtained with spiked sample at 5 µg kg^{-1} of aflatoxins and Enns; 20 µg kg^{-1} of T-2 and OTA; 50 µg kg^{-1} of BEA; FB$_1$, FB$_2$, and FB$_3$; DAS; and HT-2

three concentration levels: Enn B, Enn B1, Enn A and Enn A1, and AFB1, AFB2, AFG1, and AFG2 (20, 10, 5 µg kg^{-1}); T-2 and OTA (80, 40, 20 µg kg^{-1}); BEA, FB$_1$, FB$_2$ and FB$_3$, DAS and HT-2 (200, 100, 50 µg kg^{-1}). As can be observed in Table 2, the recoveries were higher than 60 % for all analytes, except for BEA, which yielded recoveries bellow 41 % (at the lower spiked level). The low recovery does not prevent a reliable determination of these compounds in the real samples because of the other validation parameters, especially repeatability (Relative standard deviations (RSD) <20 %) and sensitivity (limits of quantification (LOQs) <20 µg kg^{-1}), were acceptable. Recoveries were higher on apricots and prunes than in dates, figs and

Table 3 Evaluation of matrix effect: comparison of the calibration curves slopes correlation coefficient (r^2) and calculation of signal suppression/enhancement SSE (percent) for the selected mycotoxins

Mycotoxins	Slope		R^2		SSE (%)
	Solvent	Matrix matched	Solvent	Matrix matched	
Afla B1	4×10^6	2×10^6	0.9992	0.9981	50
Afla B2	3×10^6	1×10^6	0.9987	0.9979	33
Afla G1	2×10^6	1×10^6	0.9997	0.9983	50
Afla G2	2×10^6	424,159	0.9984	0.9934	21
T-2	85,880	68,453	0.9998	0.9933	80
HT2	59,262	47,490	0.9997	0.9911	80
DAS	203,231	74,318	0.9983	0.9959	37
Enn A	2×10^7	1×10^7	0.9981	0.9904	50
Enn A1	3×10^7	2×10^7	0.9987	0.9922	67
Enn B	4×10^7	5×10^7	0.9997	0.9931	125
Enn B1	9×10^6	8×10^6	0.9995	0.9904	89
FB1	841,347	989,609	0.9992	0.9973	118
FB2	1×10^6	2×10^6	0.9992	0.9995	200
FB3	696,155	1×10^6	0.9996	0.9976	144
OTA	185206	156125	0.9995	0.9904	84
BEA	2×10^7	3×10^7	0.9999	0.9861	150

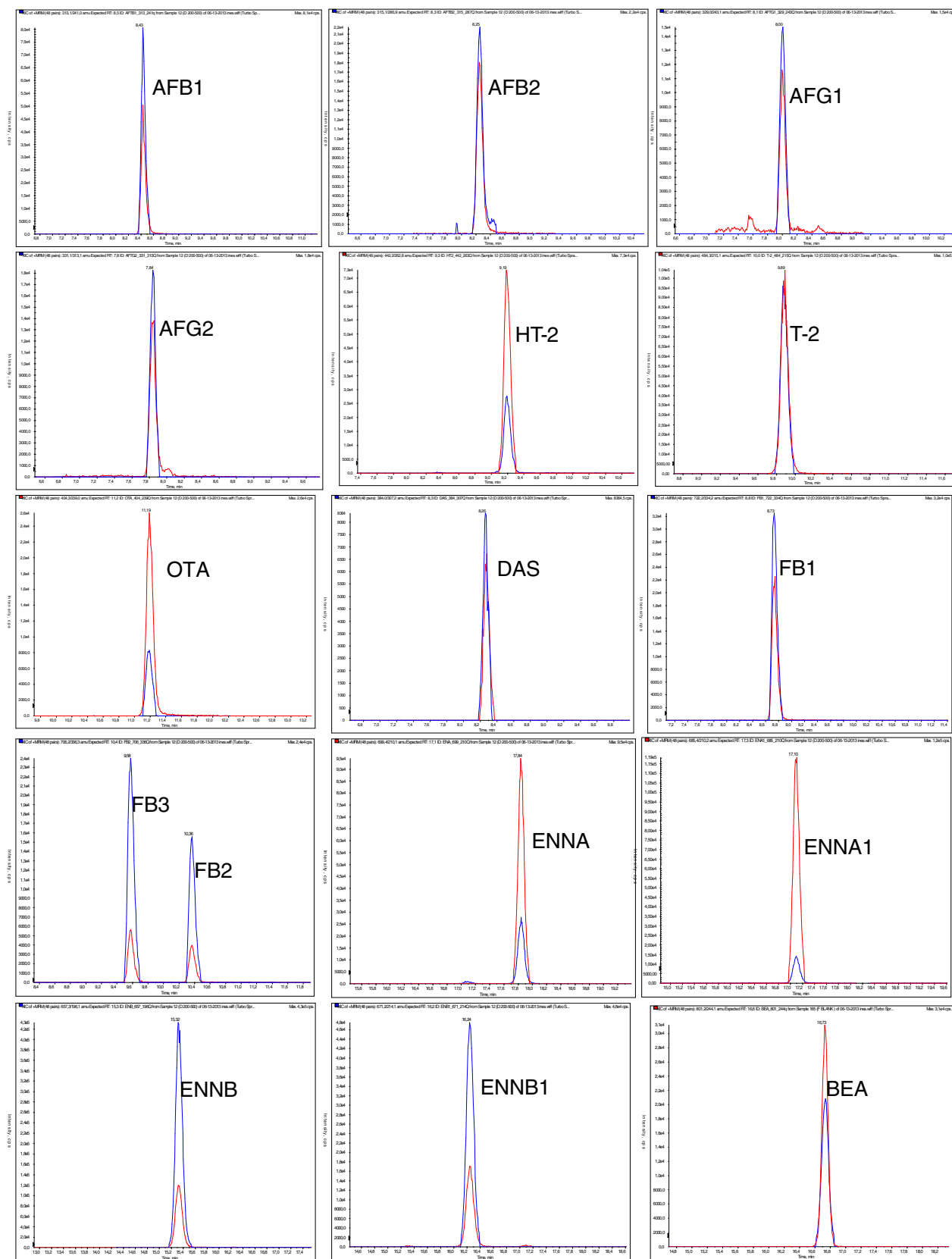**Fig. 4** LC/ESI (+)/MS/MS chromatogram of a spiked blank plum sample (20 mg kg^{-1} of AFs and ENNs; 80 µg kg^{-1} of OTA and T-2 and 200 µg kg^{-1} of DAS, HT-2, and FBs and BEA)

raisins. These differences come out because the recovery of these mycotoxins strongly depends on the matrix type, and acetonitrile extract is efficient only for some matrixes. Moreover, analysis methods for a wide range of compounds usually require a compromise because the selected extraction and clean-up conditions could not be the optimum for all the mycotoxins.

For repeatability and intermediate precision studies of the LC/MS/MS procedure, five replicate determinations on the same day and on five different days of a standard solution were carried out. RSDs ranged from 2 to 9 % for run-to-run precision and from 9 to 20 % for the day-to-day precision. The limit of detection (LODs) and LOQs for each mycotoxin were obtained from the signal-to-noise ratio. The LOD achieved for this method range from 0.08 to 15 μg/kg and the LOQ range from 0.2 to 45 μg/kg. The levels of LOQ are lower than the maximum residue limits set by EU for the studied mycotoxins. The results obtained demonstrated a good linearity for all the mycotoxins within the tested interval, with correlation coefficients higher than 0.998 for the entire target compounds, as shown in Table 3.

Figure 4 shows a LC/ESI (+)/MS/MS chromatograms of a spiked blank plum sample.

Matrix effect depends on the analyte as well as the matrix, which makes it necessary to determinate ion suppression/enhancement for every matrix–mycotoxin combination. Matrix compounds could interfere the quantitative analysis of the compounds at trace levels, as well as greatly affect the method accuracy and reproducibility. In the majority of the published multi-mycotoxin methods that employ ESI, significant matrix effects, have been observed (Desmarchelier et al. 2010; Yogendrarajah et al. 2013). Table 3 shows the calibration curves obtained using six concentration levels, between LOQ and 100 times LOQ. The slope of the standard addition plot was compared with the slope of standard calibration plot

to evaluate the matrix effects. The signal suppression/enhancement (SSE) was calculated according to Eq. 1 defined by Sulyok et al. 2006 for each mycotoxin (see Table 3).

$$SSE = (\text{slope matrix-matched calibration}/ \qquad (1)$$
$$\text{slope standard calibration in solvent}) \times 100$$

To evaluate this parameter, a commercial sample of prunes was used as being representative of the complexity of dried fruits.

Significant signal suppression (SSE, 21–89 %) was observed for most mycotoxins whereas five of the mycotoxins showed a significant signal enhancement (SSE, 118–200 %).

Analysis of Commercially Available Samples

A total of 15 domestic and imported dried fruits (three of each fruit) available in Spanish markets were analyzed using our developed method. The results are summarized in Table 4. In none of the dried plum samples tested were any of the target mycotoxins detected and they were not included in Table 4. In the rest of the analyzed samples, AFG2, OTA, HT2, Enns (B, B1, and A1) and BEA were detected. Out of the 15 analyzed samples, 8 were found to be contaminated with at least one mycotoxin. Enn B (seven positive samples) and Enn A1 (four positive samples) were the most commonly detected mycotoxins. The highest level of mycotoxins was detected in figs (227.7 μg kg^{-1} of Enn B).

Very little literature is available on emerging *Fusarium* mycotoxins in fruits although *Fusarium* strains (*Fusarium ramigenum, Fusarium solani, Fusarium proliferatum,* and *Fusarium oxysporum*) were isolated from dried fruits in several investigations (Heperkan et al. 2012; Moretti et al. 2010). The results obtained in the present study are comparable to those obtained by Tolosa et al. (2013) in which the incidence

Table 4 Mycotoxins detected in analyzed samples

Samples		Mycotoxin concentration (μg kg^{-1})						
		AFG2	OTA	HT2	Enn B	Enn B1	Enn A1	BEA
Dates	D1							
	D2				18.5			
	D3				53.1		2.2	
Raisins	R1				21.4			
	R2	4.3	4.9	10.2				
	R3							
Figs	F1				227.7	39.9	8.1	18.2
	F2				150.3	30	6.9	131.8
	F3							
Apricots	A1				43.6			
	A2							
	A3				53.3		2.7	

of Enn A, A1, B, and B1) and BEA in different dried fruits (13 samples) were assayed and 52.7 % of the samples were found to be contaminated with at least one of the four Enns with a mean ranging from 0.025 to 0.666 mg kg^{-1} for Enns and 0.006 mg kg^{-1} for BEA.

Conclusions

The liquid chromatography coupled to mass spectrometry/mass (LC/MS/MS) analysis allowed to identify unambiguously and quantify the 16 studied mycotoxins. The optimized QuEChERS extraction procedure was proved to be a fast and efficient extraction method for the analysis of mycotoxins in dried vine fruits, apricots, figs and plums. The results are so far satisfactory and a fully validated extraction and analysis method have been set in this study.

Conflict of Interest   Ines Azaiez has no conflict of interest. Federica Giusti has no conflict of interest. Gianni Sagratini has no conflict of interest. Jordi Mañes has no conflict of interest. Mónica Fernández has no conflict of interest. This article does not contain any studies with human or animal subjects.

References

Anastassiades M, Lehotay SJ, Stajnbaher D, Schenck FJ (2003) Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J AOAC Int 86:412–431

Capriotti AL, Caruso G, Cavaliere C, Foglia P, Samperi R, Laganà A (2012) Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/mass spectrometry. Mass Spectrom Rev 31:466–503

Commission of the European Communities (2006) Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off J Eur Union L364:5–24

Commission of the European Communities (2010) Commission Regulation (EU) No 1058/2012 of 12 November 2012 amending Regulation (EC) No 1881/2006 as regards maximum levels for aflatoxins in dried figs. Off J Eur Union L313:14–15

Cunha SC, Fernandes JO (2010) Development and validation of a method based on a QuEChERS procedure and heartcutting GC-MS for determination of five mycotoxins in cereal products. J Sep Sci 33:600–611

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 multi-mycotoxin analysis in complex biological matrices using LC-ESI/MS: experimental study. J Agric Food Chem 13:7510–7519

Document SANCO/12495/2011 Method validation and quality control procedures for pesticide residues analysis in food and feed. Available from http://ec.europa.eu/food/plant/protection/pesticides/docs/qualcontrol_en.pdf. Accessed on 28 Mar 12)

Ediage EN, Di Mavungu JD, Monbaliu S, Peteghem VC, De Saeger S (2011) A validated multianalyte LC-MS\MS method for quantification of 25 mycotoxins in casava flour, peanut cake and maize samples. J Agric Food Chem 59:5173–5180

Fernández-Cruz ML, Mansilla ML, Tadeo JL (2010) Mycotoxins in fruits and their processed products: analysis, occurrence and health implications. JAR 1(2):113–122

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(1):188–193

Garrido French A, Vidal Martínez JL, Romero-González R, Aguilera-Luiz M (2009) Simple and high-throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high performance liquid chromatography/tandem mass spectrometry. Food Chem 117:705–712

Garrido Frenich AG, Romero-González R, Gómez-Pérez ML, Vidal JL (2011) Multi-mycotoxin analysis in eggs using a QuEChERS-based extraction procedure and ultra-high-pressure liquid chromatography coupled to triple quadrupole mass spectrometry. J Chromatogr A 1218(28):4349–4356

Heperkan D, Karbancioglu Güüler F, Oktay HI (2012) Mycoflora and natural occurrence of aflatoxin, cyclopiazonic acid, fumonisin and ochratoxin A in dried figs. Food Addit Contam A 29:277–286

Karbancıoglu-Guler F, Heperkan D (2008) Natural occurrence of ochratoxin A in dried figs. Anal Chim Acta 617:32–36

Knudsen PB, Mogensen JM, Larsen TO, Nielsen KF (2011) Occurrence of fumonisins B(2) and B(4) in retail raisins. J Agric Food Chem 59(2):772–776

Köppen R, Koch M, Siegel D, Merkel S, Maul R, Nehls I (2010) Determination of mycotoxins in foods: current state of analytical methods and limitations. Appl Microbiol Biotechnol 86:1595–1612

Moretti A, Ferracane L, Somma S, Ricci V, Mulèè G, Susca A, Ritieni A, Logrieco AF (2010) Identification, mycotoxin risk and pathogenicity of *Fusarium* species associated with fig endosepsis in Apulia, Italy. Food Addit Contam A 27:718–728

Reinhold L, Reinhardt K (2011) Mycotoxins in foods in Lower Saxony (Germany): results of official control analyses performed in 2009. Mycotoxin Res 27:137–143

Rodríguez-Carrasco Y, Berrada H, Font G, Mañes J (2012) Multi-mycotoxin analysis in wheat semolina using an acetonitrile-based extraction procedure and gas chromatography-tandem mass spectrometry. J Chromatogr A 1270:28–40

Rodríguez-Carrasco Y, Font G, Mañes J, Berrada H (2013) Determination of mycotoxins in bee pollen by gas chromatography-tandem mass spectrometry. J Agric Food Chem 61(8):1999–2005

Rubert J, James KJ, Mañes J, Soler C (2012) Applicability of hybrid linear ion trap-high resolution mass spectrometry and quadrupole-linear ion trap-mass spectrometry for mycotoxin analysis in baby food. J Chromatogr A 1223:84–92

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

Sulyok M, Berthiller F, Krska R, Schuhmacher R (2006) Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Commun Mass Spectrom 20:2649–2659

Sulyok M, Krska R, Schuhmacher R (2010) Application of an LC-MS/MS based multi-mycotoxin method for the semi-quantitative determination of mycotoxins occurring in different types of food infected by moulds. Food Chem 119:408–416

Tamzura 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 YY, Lin HY, Chen YC, Su WT, Wang SC, Chiueh LC, Shin YC (2013) Development of a quantitative multi-mycotoxin method in rice, maize, wheat and peanut using UPLC-MS/MS. Food Anal Methods 6(3):727–736

Tolosa J, Font G, Mañes J, Ferrer E (2013) Nuts and dried fruits: natural occurrence of emerging *Fusarium* mycotoxins. Food Control 33(1): 215–220

Trucksess MW, Scott PM (2008) Mycotoxins in botanicals and dried fruits: a review. Food Addit Contam A 25:181–192

Yogendrarajah P, Van Poucke C, De Meulenaer B, De Saeger S (2013) Development and validation of a QuEChERS based liquid chromatography tandem mass spectrometry method for the determination of multiple mycotoxins in spices. J Chromatogr A 1297:1–11

Zinedine A, Soriano JM, Juan C, Mojemmi B, Moltó JC, Bouklouze A, Cherrah Y, Idrissi L, El Aouad R, Mañes J (2007) Incidence of ochratoxin A in rice and dried fruits from Rabat and Saléé area, Morocco. Food Addit Contam A 24:285–291