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Evaluation of matrix solid-phase dispersion (MSPD) extraction for multi-mycotoxin determination in different flours using LC–MS/MS

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ABSTRACT

An existing matrix solid-phase dispersion (MSPD) method for aflatoxins (AFs) and ochratoxin A (OTA) extraction was extended by further 14 mycotoxins. After it careful optimization, this method was applied to determine the occurrence of these mycotoxins on commercial flour samples (with different cereals composition) collected from local markets. In a total of 49 samples investigated, 9 mycotoxins were identified. Nivalenol (NIV) and Beauvericin (BEA) were the mycotoxins found most frequently. The samples that presented major contamination were wheat flours and bakery preparations. Despite of the great number of positives finding, only one wheat flour sample exceeded the maximum limits (ML) for OTA established by the European Union (EU). However, it would be interesting to calculate the total ingest of these mycotoxins along the years.

1. Introduction

The contamination of food by the intentional use of chemicals, such as pesticides or veterinary drugs, is a worldwide public health concern [1]. However, extensive contamination of food and drinks with natural toxins as mycotoxins is the main problem over the world since they can also compromise the safety of food and feed supplies and adversely affect health in humans and animals [2,3]. Owing to the toxicity and carcinogenic risk of these mycotoxins, there have been established European maximum levels (MLs) directed toward the control of these toxins in food, but only those toxins that pose a major risk (aflatoxins (AFs), ochratoxin A (OTA) and patulin (PAT)) had been regulated during years.

It was not until 2003, when the European Union started to develop new community legislations which have included, year after year, new groups of mycotoxins. Concretely, in 2006, it was published the Directive (CE) 1881/2006 which establishes MLs for 12 mycotoxins in different food commodities [4]. That directive was followed by other modifications, as the modification of 2007 based on *Fusarium* toxins in maize and maize products [5], the latest decisions for OTA in spices and liquorice [6] and the latest update for AFs in foodstuffs that it was in 2010 [7]. The need to apply these directives makes indispensable the accurate quantification of these mycotoxins in different commodities to evaluate their intake, as well as its associate risks, enabling to establish prevent measures that could protect the health of the consumers [8–15].

There exists around over 400 mycotoxins, but the well-known mycotoxins are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), trichothecenes as deoxynivalenol (DON), nivalenol (NIV), the toxin T-2 (T-2), toxin HT-2 (HT-2) and diacetoxyscirpenol (DAS), aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), ochratoxin A and zearalenone (ZEN), because they are the wide distributed. For this reason these mycotoxins can usually interfere in the safety food of the consumer [1,16].

These mycotoxins are produced for such genera as *Aspergillus*, *Fusarium* and *Penicillium*, but these genera, together to other genera as *Claviceps* and *Alternaria*, can also produce other mycotoxins less studied and less known, named “emergent” or “new mycotoxins”. Focusing on these “emergent mycotoxins” it would be interesting to cite BEA, a mycotoxin generated by *Fusarium* genera and which is starting to be detected in some foods [17].

Nowadays, the trend in mycotoxins analysis is the development of multi-mycotoxin methods able to cover, in a single analysis, all the mycotoxins considered by the EU food legislation [18,19]. However, several difficulties are found to reach this objective. First, the mycotoxins exhibit a wide range of different physicochemical properties in terms of pH stability, solubility, diversity of chemical structure and molecular weight [20]. Second, very different maximum levels are admitted for mycotoxins as a function of their toxicity and type of food. Finally, the matrix food composition under study is also highly variable [21].

In this way, one of the main problems to develop a multi-mycotoxin method is the extraction and purification in a single step of the all mycotoxins from the matrix, owing to the great differences in their physicochemical properties presented for these compounds. In fact, the extraction is the most critical step since it

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should determine the recoveries for all mycotoxins under investigation in a specific food matrix [22].

An attractive alternative is the matrix solid-phase dispersion (MSPD), where the sample and sorbent material are mixed homogenously; this mixture is then packed in cartridge and afterwards elution is performed [23].

Operational steps in MSPD, and efficiency and selectivity of the extraction process, are conditioned by a number of factors, for example: the physical state of the sample, the relative concentrations and properties of analytes, the interferences of the sample, the suitable combination of sorbent, etc. To start the optimization, the attention must fall upon the careful selection of sorbent materials and elution solvents to enhance the yield of the extraction [24].

Despite of its demonstrated advantages, MSPD is not still a mainstream sample-preparation technique in the mycotoxins field, and it use is limited to few reports as determination of AFs in peanuts, chilli powder, olive oil and hazelnuts [25–28], AFs and OTA in coffee, malt and cereal beverage [29], trichothecenes in corn flour [30] and PAT in apple and apple juice [31].

In the present work, a previous MSPD–LC/MS/MS method [29] was further extended and applied for the simultaneous determination of AFs, fumonisins, trichothecenes, OTA, ZEN and BEA in flour. To reach this objective, it was evaluated in detail the performance and features of the methodology in terms of cleanliness of the extracts, efficiency of the extraction (recoveries), analytical performance, matrix effects and sensitivity (limits of detection).

2. Materials and methods

2.1. Chemical and reagents

Acetonitrile, methanol, hexane, ethyl acetate and dichloromethane were supplied by Merck (Darmstadt, Germany). Solid-phases used for MSPD were silica, amino, basic alumina, acid alumina, neutral alumina, celite, phenyl, octy-silica (C_8) (50 μm) and octadecy-silica (C_{18}) (50 μm) bonded silica from Analisis Vinicos S.L. (Tomelloso, Spain). Florisil® (60–100 mesh) was obtained from Aldrich (Steinheim, Germany).

The standards of AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEN, NIV, DON, DAS, FB_1, FB_2, BEA were purchased from Sigma–Aldrich (Madrid, Spain). T-2 and HT-2 toxin stock solution (in acetonitrile), as internal standard Deepoxy-deoxynivalenol (DOM-1) and Aflatoxin M_1 (AFM_1) stock solution (in acetonitrile) were obtained from Biopure referenzsubstanzen GmbH (Tulln, Austria).

The individual stock solutions of AFs, OTA, ZEN, NIV, DON, FB_1, FB_2, BEA at concentration of 500 μg ml^{-1} were prepared in methanol. On the other hand, stock solutions of DAS, T-2 and HT-2 at concentration of 100 μg ml^{-1} were prepared in methanol. Internal standard compounds used were AFM_1 at 0.05 μg ml^{-1} and DOM-1 at 0.150 μg ml^{-1} were prepared by dilution of individual stock solutions in methanol. All these solutions were prepared and kept in safety conditions at −20 °C.

All other working standard solutions were prepared immediately before use by diluting the stock solution with methanol.

Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade reagent formic acid (purity > 98%), and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

Reference material was purchased from Biopure referenzsubstanzen GmbH (Tulln, Austria).

2.2. Matrix solid phase dispersion

Samples (200 g) were prepared using a food processor and mixed thoroughly. Portions of 1 g were weighed and placed into a glass mortar (50 ml) and were gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain homogeneous mixture. For the preparation of fortified samples, 0.2 ml of the standard working solution was added to 1 g of sample. Then, they were allowed to stand at room temperature for 3 h before the extraction for the evaporation of the solvent and the equilibration between the mycotoxins and the flour sample. The homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 20 ml of acetonitrile/methanol (50/50, v/v) 1 mM ammonium formate by applying a slight vacuum.

Then, extract was transferred to 25 ml conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 ml with methanol and filtered through a 13-mm/0.45-μm nylon filter purchased from Análisis Vínicos S.L. (Tomelloso, Spain) before the injection of 20 μl into the LC–MS/MS system.

2.3. Matrix effect measurements

The matrix effect (ME) was assessed by employing matrix-matched standards. MS/MS areas of known amounts of standards (A) were compared with those measured in a blank multicereal flour extract spiked after extraction with the same analyte amount (B). The ratio ($B/A \times 100$) is defined as the absolute matrix effect (ME %). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%. Tests were conducted in triplicate on blank multicereal flour samples, originating from a local supermarket, and spiked to obtain the experimental concentration of each mycotoxin for the HPLC-QqQ-MS/MS analysis. Flour samples were first examined for the presence of possible contaminants.

In order to evaluate the possible differences between flour varieties, three flour samples of different cereal (wheat, rice or corn) composition were used to calculate matrix effects.

2.4. Liquid chromatography–mass spectrometry

The triple quadrupole mass spectrometry detector (QqQ) was equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) that included an autosampler and a quaternary pump. Separation was attained on a Phenomenex (Madrid, Spain) Gemini-NX C_{18} (150 mm × 4.6 mm I.D., 5 μm particle size) analytical column, preceded by a security guard cartridge C_{18} (4 mm × 2 mm I.D.), using a gradient that started at 100% of A (5 mM ammonium formate and 0.1% of formic acid in water) and 0% of B (5 mM ammonium formate in methanol), increased linearly to 100% B in 10 min. After, it was decreased linearly to 80% of B in 5 min and it was gradually decreased to 70% B in 10 min. Afterwards, the initial conditions were maintained for 5 min. Flow rate was maintained at 0.3 ml min^{-1}.

A QqQ mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Ver. 4.1 was used for MS/MS analyses. Parameters were optimized in positive (ESI+) and negative (ESI−) ionization mode by continuous infusion of a standard solution (10 μg ml^{-1}) via syringe pump at a flow rate of 20 μl min^{-1}. Finally, analysis was performed in ESI+ and ESI−. The ESI source values were capillary voltage, 3.50 kV positive ionization mode and 3 kV negative ion mode; extractor, 1 V; RF lens 0.5 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 l h^{-1};

cone gas 50 l h^{-1} (nitrogen 99.99% purity). Cone voltages and collision energies were optimized for each analyte during infusion of the pure standard and the most abundant fragment ion chosen for the selected reaction monitoring. The analyzer setting were: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energies, 0.5; entrance and exit energies, 5 and 3; multiplier, 650; collision gas (argon, 99.99% purity) pressure 3.83×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass spectrometer was operated in scan, product ion scan, and single reaction monitoring (SRM) modes. All the measurements were carried out in triplicate.

2.5. Method validation

The method was validated for linearity, accuracy, precision and sensitivity.

Linearity was evaluated using the standard calibration curves that were constructed for each mycotoxins by plotting the signal intensity versus the analyte concentration and the internal standard calibration curves were constructed from the peak area ratio of each analyte to the corresponding I.S. In the same manner, matrix-matched standards of the studied mycotoxins were prepared using MSPD sample treatment, by adding known amount of working solution to the obtained extracts in order to reach the desired concentration range and the signal intensities obtained were plotted.

Recovery experiments were conducted at two concentration levels between 0.25 and 85.24 μg kg^{-1} (limits of quantification, LOQs) and between 2.5 and 852.4 μg kg^{-1} (10 times LOQs) added before the corresponding extraction procedure. Intraday precision was assessed by calculating the RSD of six determinations per concentration in a single day and interday precision by one determination per concentration on 6 days. In case of calculations with internal standards, areas ratios (area analyte/area internal standard) were used.

Sensitivity was evaluated by limit of detection (LOD) and limit of quantification (LOQ) values. The LOD was estimated from blank extract, spiked with decreasing concentrations of the analytes, where the response of the qualifier ion was equal to 3 times the response of the blank extract. Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LOQ (coefficient of variation $\leq$19% and an accuracy $\geq 70 \pm 19\%$) was preliminarily estimated, in the same way as the LOD, but using the criterion of S/N $\geq$ 10 for the qualifier ion.

2.6. Application to commercial sample

Around 50 flour samples with different cereal composition (wheat, corn, rice, soy, oats, etc.) were collected from several local markets of Valencia (Spain) and analyzed in order to investigate the presence of selected mycotoxins. Samples were stored in plastic (high density polyethylene) containers and stored in the dark at <−18 °C until analyses.

In every sequence of analysis, multicereal flour (blank sample previously analyzed) MSPD extracts were injected by duplicate between two calibration curves. Recoveries were considered satisfactory if they were in the range 70–120% for every analyte.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q) transitions and comparing them with ion-ratios obtained from a reference standard. The sample was considered positive when the experimental ion-ratio fulfilled the tolerance range, according to EU Decision 2002/657/EC [32].

3. Results and discussion

3.1. Optimization of liquid chromatography–mass spectrometry

3.1.1. Optimization of MRM mode

As in the previous work [29], preliminary experiments were conducted to find instrumental conditions that allow unambiguous identification of the analytes in real samples at low levels. The optimization of MS and MS/MS parameters were carried out by infusing individual solutions of the analytes. All the mycotoxins showed acceptable sensitivity in ESI+ to undertake food analysis, excepting ZEN that it was detected better in ESI− than ESI+. The results are summarized in Table 1.

According to Commission Decision 2002/657/EC, two specific transitions must be acquired for each compound reaching the minimum number of identification points (IPs) required for unambiguous confirmation [32]. In the present work, two transitions for reliable confirmation were possible for all the mycotoxins analyzed (Table 1). Given the high number of transitions (28 without I.S. and 32 when the I.S.s were used) to be monitored, SRM detection was separated in eight segments, in order to perform detection with sufficient instrumental sensitivity. Each segment included 1–5 substances, based on its retention time, as it is shown in Table 1.

Regarding the chromatographic analysis, due to the different physical–chemical characteristics of the mycotoxins selected, it was necessary to find a compromise to guarantee a sharp peak shape and reproducible retention times. With this goal, several mobile phases which varied in concentration and type of buffer (ammonium formate and ammonium acetate), pH (by addition of formic or acetic acids) and organic solvent (methanol or acetonitrile) were tested. The final selected mobile phase is described in the experimental section.

For the choice of gradient elution programme, results of multiple injections indicated that a gradient that starts with a high percentage of water is required, being more sensible the detection of NIV owing to it high polarity. After this step, the percentage of methanol was increased as it is indicated in experimental section, achieving good peak shape and high sensitivity. Fig. 1 illustrates typical ion chromatograms obtained under selected time-scheduled conditions for multicereal flour spiked with the mycotoxin mixture at concentration of LOQ level, providing evidence that the LC optimized conditions fulfilled the separation requirements.

Our results were according to the literature about multi-mycotoxin methods for food published [20,24,33–36]. However, in none of them MSPD has been applied.

3.2. Optimization of the sample pre-treatment

As this work supposed an expansion of the previous work [29], the authors decided to study the same parameters in this experiment, to get as much information on MSPD as possible. With this objective, the most suitable elution solvents and the polarity of solid-phase were assessed. For the optimization and development of the extraction, all the tests were performed in triplicate. It must be kept in mind that these conditions were a compromise resulting from the chemically diverse set of mycotoxins and may be far from optimal for some compounds.

3.2.1. Solvent extraction selection

In a first series of experiments, different extraction solvents or mixtures of them, and the volume required were tested. Since the study included different compounds (from polar compounds as NIV to apolar compounds as BEA) the extraction solvent was evaluated, checking a variety of solvents with different polarities such methanol, dichloromethane, acetonitrile, ethyl acetate,

Table 1
Product-ions observed in product ion scan mode for selected mycotoxins and MRM optimized parameters.

Mycotoxin	Precursor ion (*m*/*z*)	Time window (range min)	Product ion	Cone (V)	Collision energy (eV)	
NIV	313.0 $[M+H]^+$	1 (10.5–13)	125^Q 177^q	23	12	12
DON	297.2 $[M+H]^+$	2 (11–14)	249^Q 203^q	20	10	10
DOM-1$^{(IS)}$	281.1 $[M+H]^+$		108^Q 137^q	20	14	14
AFB_1	313.2 $[M+H]^+$		241^Q 269^q	47	30	30
AFB_2	315.2 $[M+H]^+$		243^Q 259^q	50	30	30
AFG_1	329.2 $[M+H]^+$	3 (13.5–17)	200^Q 215^q	43	40	30
AFG_2	331.2 $[M+H]^+$		189^Q 217^q	46	45	25
$AFM_1$$^{(IS)}$	329.2 $[M+H]^+$		273^Q 259^q	30	20	20
DAS	384.0 $[M+NH_4]^+$	4 (14.5–16.5)	307^Q 105^q	15	15	45
FB_1	722.0 $[M+H]^+$	5 (14–18)	334^Q 352^q	50	25	20
FB_2	706.4 $[M+H]^+$		336^Q 318^q	50	30	30
OTA	404.2 $[M+H]^+$		358^Q 239^q	24	15	15
HT-2	442.2 $[M+NH_4]^+$	6 (15.5–18)	263^Q 215^Q	10	12	13
T-2	484.5 $[M+NH_4]^+$		305^Q 215^q	10	12	15
ZEN	317.1 $[M-H]^-$	7 (16.5–18.5)	131^Q 175q	25	25	25
BEA	784.4 $[M+H]^+$	8 (18.5–22)	244^Q 262^q	35	25	25

Q, Quantification transition.
q, Confirmation transition.

and mixtures of them. As sorbent, C_{18} was applied to this experiment in view of the good results obtained in the previous study.

An illustration of these tests appears in Fig. 2, representing the intensities of target analytes obtained using the different solvents. In this study, aqueous mixtures were discarded owing to the interaction between water and flour, leading a doughy consistency that makes the pass of the analytes difficult.

When dichloromethane was used, only AFB_2, OTA, FB_2 and ZEN were extracted and giving low mean recoveries for the other mycotoxins. Employing ethyl acetate, no acceptable recoveries were obtained for AFG_1, AFG_2 and FB_1 (<40%). The mixture methanol:acetonitrile provided the best results for all the compounds: while using methanol the fumonisins signal improved about 20%, using acetonitrile the recoveries of AFs (principally of AFG_1) and trichothecenes type A were improved.

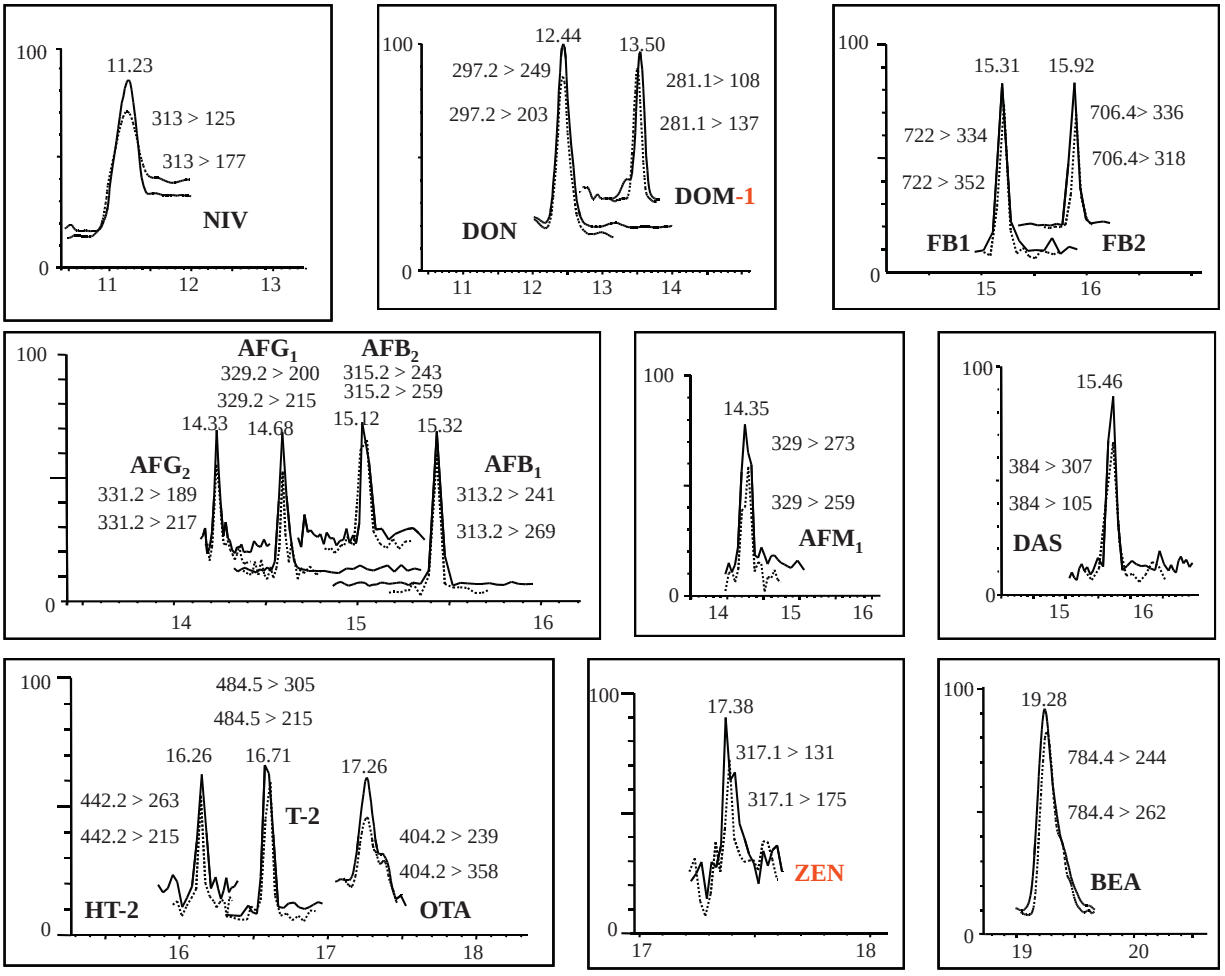

Fig. 1. Chromatogram obtained under optimized conditions of spiked multicereal flour at LOQ levels.

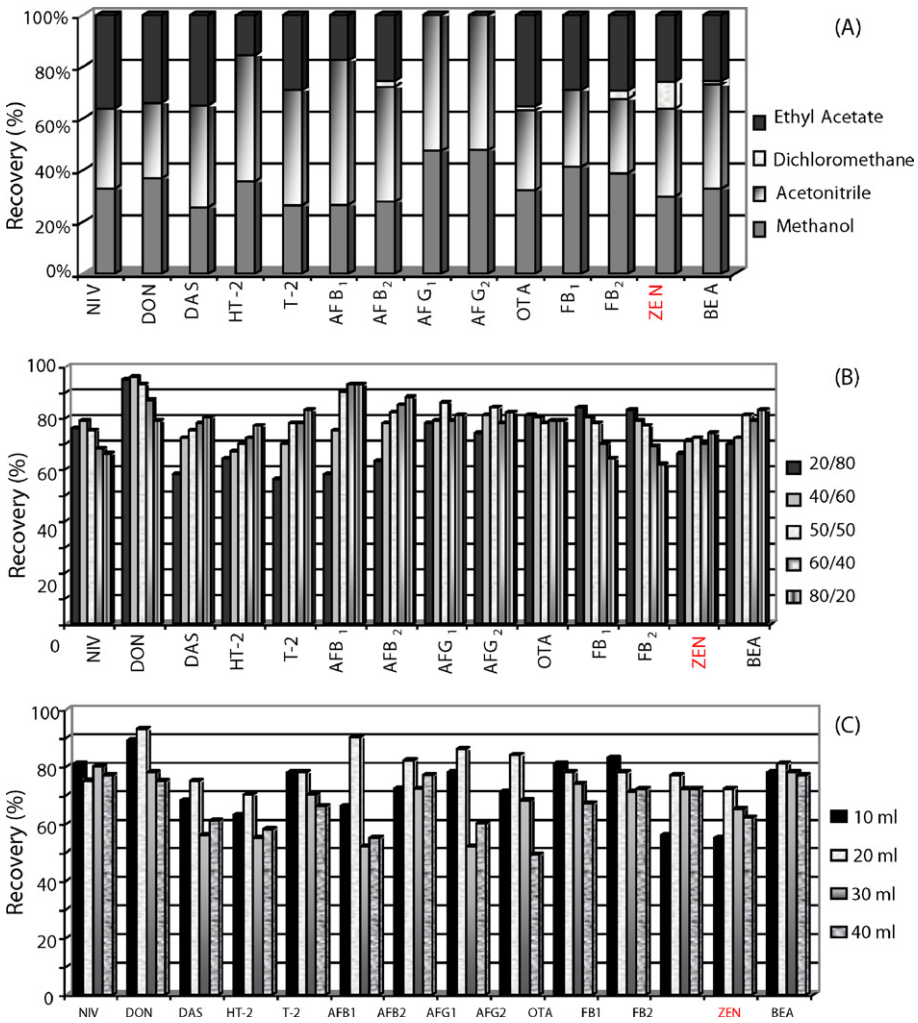

Fig. 2. Optimization of the solvents parametres. (A) Recoveries obtained using different solvents. (B) Recoveries obtained by different methanol:acetonitrile mixture rates. (C) Study of the optimum extraction volume.

For this reason, mixtures of methanol:acetonitrile at different ratios were checked (Fig. 2B). Although the results were very similar, a good compromise between all the mycotoxins was observed when the percentage 50:50 (v/v) of mixtures was used (recoveries ranged from 70% for HT-2 to 93% for DON).

On the other hand, to study the effect of pH and ionic strength, several assays were performed employing different acids (formic and acetic) and different salts (ammonium acetate and formate). For both tests, the same procedure as it is explained above was carried out; C_{18} was the sorbent and methanol:acetonitrile (50/50) was the eluting solvent.

In the case of the pH study, the addition of formic acid only improved the extraction of fumonisins, and it was dramatically detrimental to other compounds such AFs or ZEN. The addition of acetic acid not demonstrated any improvement in the extraction and for this reason pH variation was rejected.

The addition of salts in extraction solvents is known as "salting out" and it may enhance the extraction efficiency of compounds and improve sensitivity and precision of the analysis. The combination of this salting effect with MSPD is simple, fast, and results in extracts that are in an organic solvent that can be evaporated [35]. In this study, the addition of ammonium formiate improved the extraction of HT-2, DAS, and maintained constant the recoveries of the other mycotoxins. For this objective, different amounts of this salt were evaluated, obtaining the best results when 1 mM of this salt was used.

Finally, the volume of this extraction mixture was evaluated, and it was observed that when 20 ml of the mixture methanol:acetonitrile 1 mM ammonium formate were employed, reproducible results and good recoveries were obtained (Fig. 2C).

3.2.2. Optimization of the solid phase

Classic applications of the MSPD technique employ octadecyl-silica (C_{18}) and octyl-silica (C_8). However normal-phase as florisil, amine, phenyl and silica, have been also proposed as sorbent in many MSPD applications.

In this work, eight widely used solid phases were tested: (1) C_{18}, (2) C_8, (3) celite, (4) silica, (5) florisil, (6) phenyl, (7) alumina (acidic, neutral and basic) and (8) amine. Fig. 3 depicts the recoveries obtained by using these solid-phases expressed as percentage between the spiked sample after and before the extraction method.

Fumonisins were only extracted when C_{18} and C_8 were used. This circumstance limited the selection of the solid phase sorbent for this expanded experiment.

In the case of trichothecenes, they are divided into two groups according to their chemical structures: type A (such DAS, T-2 and HT-2) that are characterized by an oxygen function different from a carbonyl group at the C-8 position, and type B (NIV and DON) that posses a carbonyl function at this position. As a consequence, the polarity of these trichothecenes varies considerably (from the polar type B (NIV) to the less polar type A (T-2)). While NIV and DON reached acceptable recoveries with all the solid phases, DAS,

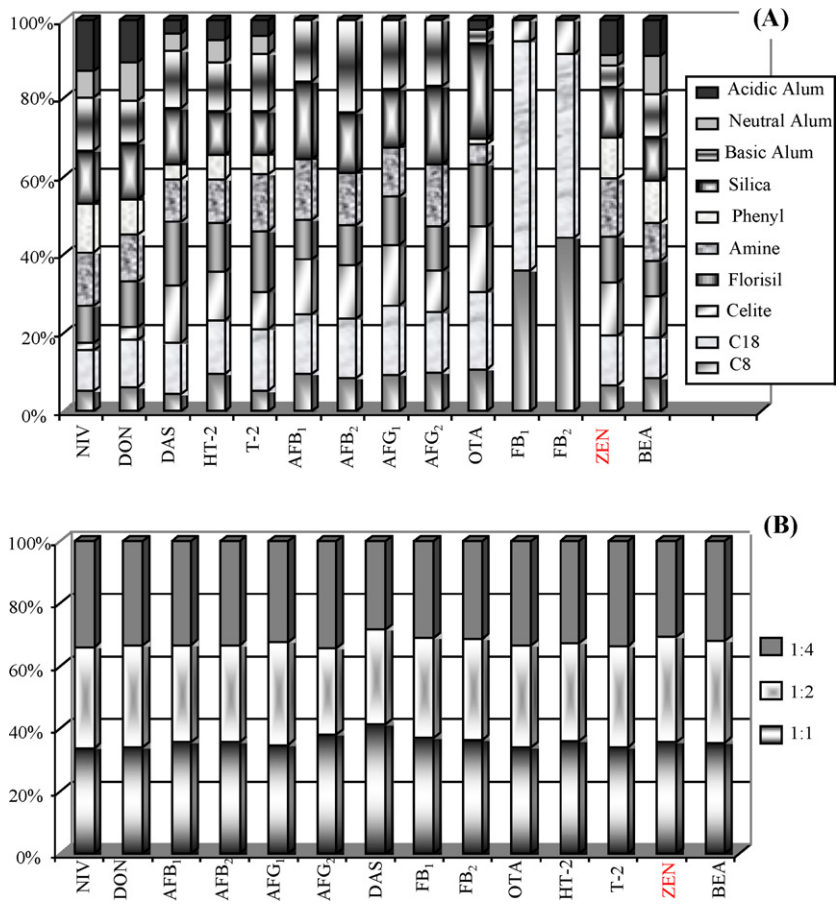

Fig. 3. Representation of the recoveries obtained by MSPD evaluating different solid sorbents (A) and different ratios (B) from a multicereal spiked sample at LOQ levels.

T-2 and HT-2 only reached recoveries > 70% when silica or acidic alumina were used. Even though using C_{18} the best results were not obtained, a compromise had to be made for the optimal extraction procedure for all mycotoxins.

As it was explained in the previous work [29], the presence of polar groups in the structure of AFs, could explain the interactions between these compounds and the polar solid phase as phenyl (Rec. 0%) or florisil (Rec. < 62%) that not allow their elution when medium polar solvent as acetonitrile or methanol were used. As a conclusion, and according to our previous studies [29], C_{18} proved to be a good solid support for the extraction of AFs.

Paying attention to the other mycotoxins (OTA, ZEN and BEA), all of them were extracted from the flour employing almost all the solid phases studied. The results obtained by C_{18} were into the acceptable range (88%, 72% and 81%, respectively).

In light of the above results, it was decided to use C_{18} alone, since acceptable recoveries were obtained for all the studied mycotoxins from multicereal-flour samples (from 72% of ZEN to 93% of DON).

3.2.3. Ratio of sample-to-sorbent

Another critical parameter in the MSPD is the ratio between matrix and sorbent material. This ratio depends on the sample nature, although the ratios often applied are 1–4. For further optimization different amounts of C_{18} (1, 2, and 4 g) were added to a glass mortar and blended with 1 g of sample, and then elution was performed with 20 ml of the mixture methanol:acetonitrile 1 mM ammonium formate. Fig. 3B shows that there were no remarkable differences between the recoveries obtained with the different ratios. In order to minimize the use of inorganic material sorbent, 1:1 ratio (1 g of multicereal-sample and 1 g of C_{18}) was selected for this study.

3.3. Matrix effects

Despite the elimination of some interfering components during the extraction, complex matrix as flour could cause analytical errors due to the presence of interferents, leading to inaccurate results. Indeed, the residual components of the matrix can promote either ion suppression or enhancement of the analyte signal in the ES interface.

In order to evaluate the influence of the flour matrix in this experiment, the obtained slopes by matrix-matched standards calibration were compared with those obtained by solvent-based standards calibration, calculating matrix/solvent slope ratios for each mycotoxin.

When different samples of a similar matrix are analyzed, some authors suggest performing a calibration plot for a standard matrix similar to that of the samples to be investigated, but free from analytes [36]. In this study, multicereal flour was used as a "standard matrix", owing to the fact that multicereal flour contains all the main cereals and therefore it could present all the selected mycotoxins.

Blank flour extracts were spiked at six concentration levels between LOQ and 10 LOQ to obtain a matrix-matched standard calibration and all the standards were prepared in triplicate. Based on empirical results presented in Table 2, the impact of matrix interferences was different for each compound. For example, significant suppression (<70%) was only observed for AFs and trichothecens type A.

Table 2
Evaluation of the matrix effects of mycotoxins in multicereal flour.

Compound	Without IS							With IS						
		Slope	r^2		Slope	r^2	ME (%)		Slope	r^2		Slope	r^2	ME (%)
NIV[1]	(a)	0.27	0.999	(b)	0.28	0.998	103.7	(a)	1×10^{-4}	0.999	(b)	1×10^{-4}	0.999	100
				(c)	0.21	0.995	75.0				(c)	1×10^{-4}	0.994	100
DON[1]	(a)	3.63	0.999	(b)	3.08	0.998	84.8	(a)	17×10^{-4}	0.999	(b)	14×10^{-4}	0.999	82.3
				(c)	2.80	0.998	90.9				(c)	13×10^{-4}	0.998	92.8
DOM-1[(IS)]	(a)	33.24	0.999	(b)	27.81	0.998	83.7							
				(c)	20.86	0.998	75.0							
AFB_1[2]	(a)	340.70	0.992	(b)	217.61	0.996	63.8	(a)	0.76	0.999	(b)	0.62	0.996	81.1
				(c)	205.10	0.994	95.9				(c)	0.59	0.996	94.9
AFB_2[2]	(a)	15.78	0.995	(b)	9.85	0.994	62.4	(a)	0.24	0.994	(b)	0.20	0.996	83.9
				(c)	8.89	0.998	90.2				(c)	0.19	0.993	92.1
AFG_1[2]	(a)	296.07	0.993	(b)	168.11	0.992	56.8	(a)	0.50	0.997	(b)	0.47	0.994	92.5
				(c)	142.21	0.994	84.6				(c)	0.44	0.996	93.7
AFG_2[2]	(a)	85.29	0.999	(b)	49.46	0.992	57.9	(a)	0.24	0.999	(b)	0.16	0.992	67.6
				(c)	43.19	0.996	87.3				(c)	0.15	0.997	93.7
AFM_1[(IS)]	(a)	84.35	0.995	(b)	57.63	0.996	68.3							
				(c)	44.89	0.993	90.8							
DAS[1]	(a)	16.35	0.998	(b)	10.91	0.999	66.7	(a)	75×10^{-4}	0.998	(b)	50×10^{-4}	0.999	66.6
				(c)	10.10	0.991	92.5				(c)	39×10^{-4}	0.990	78.0
FB_1[1]	(a)	4.07	0.990	(b)	5.05	0.990	107.4	(a)	21×10^{-4}	0.994	(b)	23×10^{-4}	0.990	109.5
				(c)	4.17	0.994	82.6				(c)	16×10^{-4}	0.998	69.6
FB_2[1]	(a)	10.96	0.996	(b)	12.85	0.990	117.2	(a)	51×10^{-4}	0.997	(b)	59×10^{-4}	0.992	115.6
				(c)	9.22	0.993	71.1				(c)	43×10^{-4}	0.995	72.9
OTA[1]	(a)	8.54	0.993	(b)	10.84	0.997	106.9	(a)	54×10^{-4}	0.997	(b)	49×10^{-4}	0.996	90.7
				(c)	10.03	0.992	92.5				(c)	49×10^{-4}	0.997	100
HT-2[1]	(a)	3.57	0.990	(b)	2.41	0.998	67.5	(a)	17×10^{-4}	0.999	(b)	11×10^{-4}	0.997	64.7
				(c)	2.26	0.994	93.7				(c)	9×10^{-4}	0.998	81.8
T-2[1]	(a)	8.98	0.994	(b)	5.29	0.991	58.9	(a)	43×10^{-4}	0.997	(b)	25×10^{-4}	0.990	58.1
				(c)	4.03	0.995	76.3				(c)	23×10^{-4}	0.994	92.0
ZEN[1]	(a)	3.01	0.991	(b)	3.07	0.994	102	(a)	14×10^{-4}	0.992	(b)	14×10^{-4}	0.996	100.1
				(c)	2.60	0.993	84.7				(c)	12×10^{-4}	0.994	85.7
BEA[1]	(a)	341.56	0.997	(b)	208.07	0.997	60.9	(a)	0.14	0.998	(b)	0.08	0.995	59.9
				(c)	156.02	0.995	74.9				(c)	0.07	0.991	93.9

(a) Standard in pure solvent (solvent calibration).
(b) Standard spiked after extraction (matrix-matched calibration).
(c) Standard spiked before extraction (fortified sample).
Internal standard used: [(1)] DOM; [(2)] AFM_1.

In these cases, and as the European guide SANCO [37] recommends, the matrix-matched calibration standard was evaluated to minimize these matrix effects; the slope of fortified samples calibration is compared with the slope obtained by matrix matched calibration. In light of the results included in the same Table 2, using the matrix matched calibration, the matrix effect was compensate for all the studied mycotoxins in multicereal flour (ME% between 71.1% and 95.5%).

Although this method showed to be efficient, in the last years there is a trend toward the use of other methods to minimize the matrix effects: for example, internal standard calibration approach [27,35,38–40]. Usually, an isotopically labelled internal standard is preferred to correct analytes signal suppression or enhancements resulting from matrix interferents. Ideally, each analyte would be corrected by own isotope-labelled molecule. However, this ideal situation is problematic in a multiresidue method due to economical restrictions to acquire a large number of these compounds.

An alternative normally applied within the mycotoxins field is the use of established internal standard, as DOM-1 and AFM_1 since they are metabolites of DON and AFB_1, which could not be present in cereal products [26,39].

To obtain more information about accurate quantification, these two compounds were checked for correction of matrix effects since, only AFs and trichothecenes presented suppression problems. NIV, DON, DAS, T-2, HT-2 were quantified using DOM-1 and AFB_1, AFB_2, AFG_1, AFG_2 using AFM_1.The dilemma was selected an IS for the emergent mycotoxin BEA. In this study, it was decided to calibrate with DOM-1, owing to the similar polarities between the trichothecenes group B and this mycotoxin.

As it can be deduced from the results in Table 2, suppression effects were also compensated by use of these internal standards. Moreover, it was interesting to prove that normalized ME using IS were close to those obtained by matrix matched standard calibration, confirming that these two methods were suitable to compensate the matrix effects presented in this study.

The linear regression coefficient of all calibration curves are also presented in this Table 2, showing that good results were achieved, with corresponding correlation coefficients (r^2) higher than 0.990.

3.3.1. Matrix effect from flour to flour

To obtain more accurate results, matrix effects were evaluated in different main cereal flours: corn, rice and wheat were selected since they are the three principal cereals world wide-used.

This part of the work was divided in two steps: in the first phase, it was evaluated ME (%) using the standard calibration in pure solvent (A). In view of the results (Table 3), statistically significant variations were presented. For example, in wheat flour analysis, a general suppression effect existed except for OTA, T-2 and HT-2 which suffer an enhancement in their signal. Moreover, in the analysis of corn flour and rice flour, the matrix effects were very variable.

Once evaluated the existence of ME, the second phase of this study was based on minimizing the matrix effects by different strategies: the matrix-matched calibration of the studied flour (B),

Table 3
Matrix effects (%) in different cereal flours (A) slope matrix matched in cereal flour/slope of standard in solvent, (B) slope spiked cereal flour/slope of matrix matched cereal flour, (C) slope spiked cereal flour/slope of matrix matched multicereal flour, (D) area ratios with IS.

Compound	Wheat flour				Corn flour				Rice flour			
	ME $\%^{(A)}$	ME $\%^{(B)}$	ME $\%^{(C)}$	ME $\%^{(D)}$	ME $\%^{(A)}$	ME $\%^{(B)}$	ME $\%^{(C)}$	ME $\%^{(D)}$	ME $\%^{(A)}$	ME $\%^{(B)}$	ME $\%^{(C)}$	ME $\%^{(D)}$
NIV[1]	42.6	77.8	80.1	90.1	62.6	79.8	82,3	97.6	66.6	73.2	97.6	99.6
DON[1]	56.3	76.6	81.0	89.2	77.7	85.4	84.9	92.3	73.5	84.6	83.2	90.3
AFB_1[2]	56.5	77.7	80.3	80.1	68.4	81.9	83.6	88.9	69.1	70.1	76.5	80.5
AFB_2[2]	64.5	76.8	81.2	81.3	66.6	82.7	88.6	89.7	69.8	72.4	75.7	83.4
AFG1[2]	67.1	72.3	75.4	79.8	67.2	82.1	84.2	88.2	68.1	79.9	81.0	81.5
AFG2[2]	63.2	74.3	81.2	77.6	69.5	80.6	87.6	91.1	70.2	78.4	78.5	80.3
DAS[1]	58.3	88.2	78.2	71.2	89.4	80.2	83.2	93.1	83.3	92.2	88.3	72.3
FB_1[1]	67.9	77.2	84.3	93.2	89.4	97.7	96.7	105.3	93.6	94.4	99.1	101.2
FB_2[1]	79.8	74.3	76.2	89.2	91.3	104.3	106.2	112.3	96.7	93.9	92.3	105.2
OTA[1]	124.4	90.2	92.3	94.4	89.4	93.2	94.1	96.7	92.4	94.5	93.5	94.8
HT-2[1]	101.4	80.2	81.5	70.2	74.6	89.2	88.6	88.9	77.8	92.2	87.4	84.3
T-2[1]	120.7	67.7	70.6	72.4	81.2	88.1	78.2	79.6	87.1	79.6	70.9	72.8
ZEN[1]	90.2	100.2	98.7	99.8	108.3	106.4	102.1	103.2	101.2	102.9	99.3	101.2
BEA[1]	48.6	69.0	71.1	74.2	82.3	78.9	78.2	70.3	77.9	80.3	78.5	77.3

$ME\%^{(A)}$: slope matrix matched selected flour/slope standard in solvent × 100.
$ME\%^{(B)}$: slope spiked selected flour/slope matrix matched selected flour × 100.
$ME\%^{(C)}$: slope spiked selected flour/slope matrix matched multicereal flour × 100.
$ME\%^{(D)}$: correction with IS × 100.
Internal standard used: (1) DOM-1; (2) AFM_1.

employing the matrix matched calibration of multicereal flour as a "standard model" (C) for all the samples and the internal standard calibration approach (D). The results are presented also in Table 3.

When, according to other authors [40,41], matrix-matched calibration of the selected flour was applied, matrix effects were compensated. However, it means that each matrix should be evaluated in each analysis, what complicates the analysis and makes it very tedious. To simplify the dependence of matrix effect on variety of samples, and according to the literature, careful study about the selection of a "standard matrix" was carried out. As it was cited before, multicereal flour was selected with this aim and the results (Table 3) demonstrated that this approach resulted in further reductions of the matrix effects and it allowed accurate quantification of the selected mycotoxin in different cereal flours. Comparable slopes, intercepts and response linearity were observed for each mycotoxin among the calibration curves in these three matrices (Table 2).

3.4. Validation of the method

The performance of the method was evaluated according to EU guidelines [32,42]. All these parameters were calculated in multicereal flour.

LODs and LOQs were calculated analysing fortified flour sample and the results obtained are shown in Table 4. In the same table are indicated the maximum levels recommended by the European Union for multicereal flour or cereal derivates [5] to compare the results obtained. It is important to emphasize those only AFs, fumonisins, OTA, ZEN and DON are regulated by the European legislation in this food commodity. In all these cases, LOQs were always lower than the MLs established by EU.

Although no limits are set for NIV, DAS T-2, HT-2 and BEA in multicereal flour, results presented in Table 4 indicate that the developed method was suitable for the detection of these mycotoxins at convenient concentration level (comparing with the MLs established by EU for other mycotoxins).

Table 4
Maximum levels and performance parameters (recovery values (%) and relative standard deviations given in brackets (%)).

Compound	ML (EU) ($\mu g\,kg^{-1}$)	LOD ($ng\,g^{-1}$)	Intra-day precision[d]				Inter-day precision[e]			
			Low level (LOQs)		High level (10 × LOQs)		Low level (LOQs)		High level (10 × LOQs)	
			Concentration	Recovery	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery
NIV		75	85.24	76.6 (6)	852.4	72.6 (4)	85.24	74.5 (4)	852.4	76.3 (5)
DON	750[a]	20	31.25	89.3 (4)	312.5	85.6 (8)	31.25	79.8 (8)	312.5	84.6 (7)
DAS		3	5	77.4 (3)	50	81.9 (6)	5	78.6 (11)	50	80.0 (9)
HT-2		10	35.5	72.6 (4)	355	73.1 (4)	35.5	68.7 (5)	355	72.8 (7)
T-2		5	12.5	79.2 (9)	125	82 (12)	12.5	88.4 (14)	125	84.3 (11)
AFB_1	2	0.1	0.25	76.7 (12)	2.5	83.3 (7)	0.25	72.7 (13)	2.5	81.1 (11)
AFB_2		1	1.5	68.8 (11)	15	76.1 (9)	1.5	69.1 (13)	15	74.5 (9)
AFG_1	4[b]	0.1	0.25	80.3 (4)	2.5	75.4 (5)	0.25	71.0 (9)	2.5	73.8 (6)
AFG_2		0.5	0.75	78.7 (6)	7.5	81.7 (5)	0.75	77.1 (12)	7.5	77.9 (10)
OTA	3	1	3	71.1 (11)	30	83.5 (8)	3	76.7 (14)	30	78.2 (12)
FB_1	1000[c]	40	83.33	81.3 (6)	833.3	83.9 (5)	83.33	77.2 (11)	833.3	80.1 (12)
FB_2		60	83.75	89.6 (10)	837.5	87.5 (8)	83.75	85.2 (4)	837.5	87.6 (7)
ZEN	75[a]	6	12.5	79.4 (14)	125	77.1 (6)	12.5	78.6 (9)	125	77.4(7)
BEA		0.5	1	74.1 (11)	10	79.8 (8)	1	73.1 (10)	10	80.4 (9)

[a] EC 1126/2007 amending EC 1881/2006.
[b] Expressed as the sum of the four aflatoxins ($AFB_1 + AFB_2 + AFG_1 + AFG_2$).
[c] Expressed as the sum of fumonisins ($FB_1 + FB_2$).
[d] Number of replicates: 6.
[e] Different days: 6.

Table 5
Occurrence of mycotoxins in analyzed flours.

Sample	Wheat flour		Corn flour		Rice flour		Oats flour		Bakery preparation	
	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)
NIV	9	<LOQ–105	1	92	–	–	–	–	1	76
DON	5	45–367	–	–	–	–	1	153	2	32.5–180
AFB_2	1	2	–	–	–	–	1	1.60	–	–
AFG_1	2	0.53–0.72	–	–	–	–	–	–	–	–
AFG_2	1	1	–	–	–	–	–	–	2	<LOQ–1.2
FB_2	–	–	2	230–468	–	–	–	–	1	<LOQ
OTA	3	<LOQ–3.5	–	–	–	–	–	–	1	<LOQ
ZEN	1	39.3	1	70.5	–	–	–	–	–	–
BEA	6	150–720	–	–	3	327–575	2	226–325	3	115–705

<LOQ = only detected, below the LOQ level.

Recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision) were determined at two spiked levels (LOQ and 10 times LOQ). Results are also summarized in Table 4.

Precision, calculated as relative standard deviation percentages (RSD) was between 3% and 14% for the intra-day test and from 4% to 14% for the inter-day test. The recovery ranges in low and high spiked levels were 68.8–89.6% and 72.6–87.5%, respectively for the intra-day test and 68.7–88% and 72.8–87.6% for the inter-day test at LOQ and 10 times LOQ, respectively.

Therefore, the method was considered "acceptable" according to the EU criteria [32]; an average recovery ($n = 5$) between approximately 70% and 120% and a repeatability (RSD) lower than 20%.

Similarly to matrix effects, recoveries and its repeatability were studied in the three varieties of cereal flour (wheat, corn and rice) by three replicates. Recoveries were satisfactory (between 70% and 120%) in all matrix tested. To overcome matrix effect problems and for accurate quantification, matrix-matched multicereal flour was used in all the experiments as it has been explained in Table 3.

To demonstrate the efficiency of the developed method, it was applied to the analysis of reference certified material: DON in wheat flour. The measured concentration was $973 \pm 12\ \mu g\,kg^{-1}$ ($n = 3$): this concentration showed satisfactory concordance with the certificated values ($1062 \pm 110\ \mu g\,kg^{-1}$).

Table 6
LC–MS/MS ion ratios (A qualifying ion$^{(q)}$/A quantifying ion$^{(Q)}$) for mycotoxin into matrix-matched sample and matrix sample.

Mycotoxin	Ion ratio expected[a,c] (RSD %)	Ion ratio observed[b,c] Multicereal flour (RSD %)
NIV	0.86 (4)	0.79 (11)
DON	0.72 (5)	0.75 (8)
AFB_1	0.45 (2)	0.48 (5)
AFB_2	0.74 (3)	0.76 (6)
AFG_1	0.21 (3)	0.25 (11)
AFG_2	0.67 (7)	0.59 (12)
DAS	0.81 (6)	0.83 (9)
FB_1	0.71 (8)	0.66 (10)
FB_2	0.78 (7)	0.82 (8)
OTA	0.39 (3)	0.36 (7)
HT-2	0.83 (9)	0.79 (12)
T-2	0.68 (7)	0.76 (5)
ZEN	0.85 (5)	0.90 (6)
BEA	0.58 (2)	0.65 (14)

[a] Ratio determined in matrix-matched solution at LOQ level ($n = 5$).
[b] Ratio determined in fortified multicereal flour sample at LOQ level ($n = 5$).
[c] The EU guidelines [32] sets criteria for the observed ratio as follows; expected ratio >0.5. Observed ratio should be within (20%). expected ratio 0.2–0.5. Observed ratio should be within (25%); expected ratio 0.1–0.2. Observed ratio should be within (30%; expected ratio <0.1. Observed ratio should be within (50%).

3.5. Application to commercial samples

The applicability of the MSPD developed method was evaluated carrying out a survey of fourteen selected mycotoxins in around 50 commercial flours produced by different companies and which were purchased in several valencian supermarkets. These samples included wheat flour (25 samples), corn flour (9 samples), rice flour (3 samples), soy flour (1 sample), oats flour (3 sample) and including different bakery preparations that consist on several mix of different cereal flours for muffins, bread, pizza or similar (8 samples). The obtained results are summarized in Table 5.

According to the European Commission [32], the ion ratio of the primary and secondary product ions were monitored. The results are summarized in Table 6. In light of these results the trueness of the method was assessed and it was in good agreement with this European Commission performance criterion for qualitative analysis.

An internal quality control was carried out for every batch of samples to check if the system was under control, and it implied a matrix-matched calibration, a matrix blank and a fortified multicereal flour blank sample at LOQ levels.

Among these 49 tested samples, NIV, DON, AFB_2, AFG_1, AFG_2, FB_2, OTA, ZEN and BEA were detected in 25 different samples. All these mycotoxins were detected in wheat flour samples. It was not surprising since most of the flour samples (50%) belong to this cereal; it is the wide-used flour in Europe and there exist different commercial samples [43–45]. To our surprise, one of these samples exceeded the maximum tolerable level established for the EU for OTA in this food commodity [46].

NIV and BEA were presented in a high percentage of samples. While the first one has been reported in the literature [47,48], the second one has been scarcely cited [43,49]. These results showed the necessity to study and analyze this mycotoxin in deep as a future trend.

Despite of the great number of positives finding, all of them were set below the tolerable levels established by the EU, however, it would be interesting to calculate the total ingest of these mycotoxins along the years. Moreover, these results indicate that more attention should be paid to storage conditions, in order to minimize the content of mycotoxins.

4. Conclusion

The MSPD method presented is a good starting point for further mycotoxins analysis and it can be regarded as a valuable future alternative in this field. However, this study confirms once again the need to carefully evaluate potential matrix effects.

These matrix effects should be solved by using appropriate calibration method. The problem is the absence of one unified way to

solve these matrix effects since all the analytical published works used different methods demonstrating the validity of all of them. In this paper, authors decided to evaluate matrix-matched calibration and internal standard addition, without to cast doubt upon the efficiency to use isotopically labelled internal standard. Moreover, this work goes deeper into the possibility to use a "model" matrix-matched calibration which was used to validate the methods obtaining reliable results. In light of the results obtained in this work, matrix-matched calibration approach allows correct quantitative analysis.

Although consumption of food with traces of mycotoxins does not produce immediate or dramatic reaction, chronic exposure may have adverse effects on the consumers. For this, usually, mycotoxin analysis in samples is required in the commercial transaction for minimizing the public health risk.

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