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Optimization of Matrix Solid-Phase Dispersion method for simultaneous extraction of aflatoxins and OTA in cereals and its application to commercial samples

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ABSTRACT

A method based on Matrix Solid-Phase Dispersion (MSPD) has been developed for the determination of 5 mycotoxins (ochratoxin A and aflatoxins B and G) in different cereals. Several dispersants, eluents and ratios were tested during the optimization of the process in order to obtain the best results. Finally, samples were blended with C_{18} and the mycotoxins were extracted with acetonitrile. Regarding to matrix effects, the results clearly demonstrated the necessity to use a matrix-matched calibration to validate the method. Analyses were performed by liquid chromatography–triple quadrupole-tandem mass spectrometry (LC–QqQ-MS/MS). The recoveries of the extraction process ranged from 64% to 91% with relative standard deviation lower than 19% in all cases, when samples were fortified at two different concentrations levels. Limits of detection ranged from 0.3 ng g^{-1} for aflatoxins to 0.8 ng g^{-1} for OTA and the limits of quantification ranged from 1 ng g^{-1} for aflatoxins to 2 ng g^{-1} for OTA, which were below the limits of mycotoxins set by European Union in the matrices evaluated. Application of the method to the analysis of several samples purchased in local supermarkets revealed aflatoxins and OTA levels.

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1. Introduction

Mycotoxins are toxic chemical products formed as secondary metabolites by few fungal species that readily colonize crops and contaminate them with toxins in the field or after harvest [1]. Surveillance studies showed that mycotoxin contamination is a world-wide problem [2,3], since it is estimated that 25% of the world's crop production and 20% of crop production within the European Union may be contaminated with these contaminants [4]. Economic losses deriving from that are tremendous, including reduction of livestock production and agricultural production, health care, veterinary and regulatory costs [5].

Because of these effects on humans and animals, measures have been set up by authorities in many countries to monitor and control mycotoxins levels. In this way, aflatoxins and ochratoxin A are subject to European Union legislation for a number of years setting maximum levels for these mycotoxins in different commodities [6].

The requirement to apply these regulatory limits has prompted the development of a vast number of analytical methods for the identification and quantification of these mycotoxins in various complex samples, such as food, feed, and another biological samples, especially for the highly toxic and carcinogenic aflatoxins that maximum tolerable levels have been established at ppb level in some matrices as cereals.

Specifically, the International Agency for Research on Cancer (IARC) has classified aflatoxins; aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) as carcinogenic to humans while ochratoxin A (OTA) has been classified as possibly carcinogenic. Moreover, they are the mycotoxins of major significance and hence there has been significant research on broad range of analytical and detection techniques that could be useful and practical.

Aflatoxins and OTA are to be found in agricultural products that are susceptible to contamination include malt, wheat, coffee, green coffee, barley, oat, chicory, maize, cacao, wine, grape juice, dried fruits, peanuts, cotton seed, corn and rice [7–10]. OTA is principally a storage mycotoxin, but it can be produced during the malting process also [11].

In the past years, a trend towards the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) in mycotoxins analysis to reach the low limits established by the legislation has been observed, since by this technique, in contrast to most screening methods, unambiguous analyte confirmation can be obtained [5,12–20]. This idea, has led some researchers to the misconception that the use of LC–MS/MS effectively eliminates matrix effects. In reality, unpredictable increase/decrease in analytical signal intensities may occur due to the co-elution of matrix

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components that disturb the ionization of the analyte [12], so it can be concluded that one of the most crucial and critical step in the analysis of mycotoxins, with independence of the determination technique, is the sample preparation and clean-up [13].

The mycotoxins are mainly determined by single compound analytical methods based on immunoaffinity column clean-up. These columns are also available for the simultaneous determination of aflatoxins and ochatoxin A [14]. Despite minor problems in cross-reactivities, this method was generally considered to be so specific that confirmation was supposed unnecessary. However, increasing quality demands altered this attitude and methods were developed in which mass spectrometry was applied for confirmation purpose [6], apart from its high cost and its matrix dependence [15,16].

For this reason, other alternative techniques, such solid-phase extraction (SPE) or Matrix Solid-Phase Dispersion (MSPD) methods, help simplify protocols, improve selective and performance characteristic and nowadays are applied to the analysis of several residues [17,18].

The possibility of achieving simultaneous extraction and clean-up has been investigated by various groups using MSPD for analysis of aflatoxins in peanuts and chilli powder, green bean and black sesame [19–21].

However, MSPD has been still scarcely used for analysis of mycotoxins from foods. In this paper an extraction procedure is presented for aflatoxins and OTA in coffee, malt and an instant cereal-breakfast beverage based on MSPD, with C_{18} as a dispersing mean and acetonitrile as an eluant after a carefully optimization of different parameters of the extraction process in order to find method that produces least matrix effect and gives high recoveries for the five mycotoxins. The identification and quantification of the analytes were carried out using LC coupled with triple quadrupole mass spectrometry detector.

These mycotoxins were selected for this study owing to the hazard they pose to human health and their high incidence in different crops. The selection of the studied matrices were according to the 2002 report on the assessment of dietary intake of OTA by the population of the EU member states [22] in which the contributions of various food commodities were estimated (just to mention the most significant) as 50% for cereals and 10% for coffee. Then cereals and coffee consumption could contribute significantly to human intake of these mycotoxins. Coffee substitutes (in general made with cereals as barley, malt, rye and chicory) are natural products that do not contain caffeine and are therefore suitable for everyone, adults and children.

The present work includes the application of the developed method in three further matrices as well as an investigation of the variability of the matrix effects between individual samples. Based on these additional data for matrix effects, the applicability of the concept of matrix-matched calibration for the developed method is evaluated. Finally, the optimized method was applied to the control of 22 commercial samples.

2. Materials and methods

2.1. Chemical and reagents

Acetonitrile, methanol, hexane, ethyl acetate and dicloromethane were supplied by Merk (Darmstadt, Germany). Solid-phase used for MSPD were silica, amino, phenile, octysilica (C_8) (50 μm), octadecysilica (C_{18}) (50 μm) bonded silica from Analisis Vinicos (Tomelloso, Spain). Florisil® (60–100 mesh) was obtained from Aldrich (Steinheim, Germany).

The standards of aflatoxins (B_1, B_2, G_1, G_2), ochratoxin A (OTA) were supplied by Sigma–Aldrich (St. Louis, MO, USA).

The individual stock solutions of aflatoxins and ochratoxin with concentration 500 mg ml^{-1} were prepared in acetonitrile, kept in security conditions at −20°. All other working standard solutions were prepared immediately before use by diluting the stock solution with acetonitrile.

Ammonium acetate (MS grade) is bought from Sigma–Aldrich (St. Louis, MO, USA). Water for LC mobile phase was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

2.2. Samples

A total of 22 samples were purchased in commercially available size during July 2009 from supermarkets located in the city of Valencia (Spain). The samples were transported to the laboratory under ambient conditions. Samples were milled using a blender Moulinex and a 200 g subsample was analysed [23].

The milled samples were analysed as quickly as possible after the purchase and they were stored at −20 °C.

2.3. 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, 1 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. The homogeneous mixture after solvent evapored was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 10 ml of acetonitrile by applying a slight vacuum. Then, the extract was transferred to a 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 acetonitrile and filtered through a 13-mm/0.45-μm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before their injection into the LC–MS/MS system.

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 C_{18} (250 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 35% of 5 mM ammonium acetate in water (A) and 65% of 5 mM ammonium acetate in acetonitrile (B) during 3 min. After, it was increased linearly to 95% of B in 4 min, and held constantly for 3 min. Then, the gradient backs to the initial conditions during 10 min. The flow rate was 0.25 ml min^{-1}, and 20 μl of standard solutions or extract were injected.

A QqQ mass spectrometer Quattro LC from Micromass (Manchester, UK); equipped with pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software Ver. 4.1 was used for MS/MS analyses. Parameters were optimized in positive and negative mode by continuous infusion of a standard solution (10 μg ml^{-1}) via syringe pump at a flow rate 20 μl min^{-1}. Analysis was performed in positive ion mode. The ESI source values were capillary voltage, 3.50 kV; 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 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: res-

olution 12.0 (unit resolution) for the first and third quadrupole; ion energies, 0.5; entrance and exit energies, 1 and 3; multiplier, 650; collision gas (argon, 99.99% purity) pressure 3.74×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.2 ms. The mass spectrometer was operated in scan, product ion scan, and multiple reaction monitoring (MRM) modes. All the measurements were carried out in triplicate.

2.5. Method validation

Method accuracy and precision were evaluated by performing recovery studies using "blank" samples. Recovery experiments were conducted at two levels—between 1 and 2 μg kg^{-1} (quantification limits, LOQs) and between 10 and 20 μg kg^{-1} (10 × LOQs). "Blank" samples (1 g) were spiked with 1 ml of a working mixture of the compounds at the appropriate concentration. Then, "blank" samples were left to stand 3 levels before the extraction. Five replicates were prepared for each spiking level after solvent evaporation.

For the estimation of the linearity and matrix effects, raw extracts of samples spots without visible fungal infections were fortified using a multi-mycotoxin standard on a range of studied concentration level, diluted and analyzed and the corresponding peak areas were compared to a standard prepared and diluted in neat solvent.

To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated and the signal suppression/enhancement (SSE) due to matrix effects was determined.

The limit of detection (LOD) was estimated from extracted samples, 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 was defined in this study as the lowest calibrator with an acceptable relative uncertainty (coefficient of variation $\leq$19% and an accuracy $\geq 70 \pm 19$%). The LOQ was preliminarily estimated, in the same way as the LOD, using also the criterion of $S/N \geq 10$ for the qualifier ion.

3. Results and discussion

3.1. Optimization of the LC–MS/MS

3.1.1. Optimization of the triple-quad detection method

First, the experiments to select the optimum multiple reaction monitoring parameters (MRM transitions, interface parameters and MS/MS parameters) were performed by direct injection of individual standards at 10 mg ml^{-1}. ESI in both positive and negative ion mode were evaluated, observing that all mycotoxins exhibited higher precursor ion signal intensities or better fragmentation patterns in positive ion mode. Only OTA was efficiently ionized in the negative mode, but lower signal than positive mode, so ESI in positive mode was selected for all of them. In general, all aflatoxins exhibit good ESI ionisation efficiency in the positive ion mode with abundant protonated molecules $[M+H]^+$ and sodium adduct ions $[M+Na]^+$. To validate the identity of the parent, these ions were fragmented into daughter ions with argon gas in the collision cell of the triple quadrupole, but since the sodium adduct did not exhibit specific fragmentation during the collision induced dissociation process for any compound, the protonated molecule was chosen as the precursor ion for each studied mycotoxin in the product ion scan mode. In this context, and as it has been related in the literature [24], the formation of sodium adduct ions can easily be suppressed by the addition of modifiers (ammonium ions) to the mobile phase

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

Mycotoxin	Retention time (min)	Precursor ion	Product ion	Cone	Collision energy
AFB_1	13.04	313.2	241^Q 269^q	47	30 30
AFB_2	12.83	315.2	243^Q 259^q	50	30 30
AFG_1	12.42	329.2	200^Q 215^q	43	40 30
AFG_2	12.23	331.2	189^Q 217^q	46	45 25
OTA	11.12	404.2	239^Q 358^q	20	20 15

Q, Quantification transition.
q, Confirmation transition.

leading to a better MS sensitivity. The product ion spectra of the protonated aflatoxins species contains a number of abundant product ions reflecting bond cleavages and rearrangement reactions of the polycyclic ring system along with loss of water, carbon monoxide and carbon dioxide.

The pathway fragmentation of OTA has been widely studied. In positive mode ionization, the abundant ion is the protonated molecule. Applying soft energy collision energy, the fragments obtained correspond to the loss of a carboxylic group and radical cleavage [25,26].

This method has been specifically developed for confirmation analysis purposes in compliance with the European Union laws in force [27]. On this basis, a substance can be identified using LC–MS/MS, in MRM mode, by at least two transitions. For this purpose all possible fragments were studied and the two transitions with highest chromatographic signal-to-noise (S/N) ratios and with minimum interference from matrix components were chosen.

Quantification was carried out on the primary transition. Each mycotoxin was confirmed by the second transition and the ratio between primary and secondary daughter ion calculated. These ions were selected according to the highest sensitivity and to optimal selectivity for the target compounds. These product ions with the highest intensity provided by fragmentation of the precursor ion and the optimum collision energy are shown in Table 1.

3.1.2. Optimization of the chromatographic method

The direct combination of LC with MS reduces the stress on chromatographic separations because of the mass selectivity and distinctive fragmentation patterns. However, chromatographic separation can be crucial in some cases. In order to reduce analysis time, increase sensitivity and provide good peak shape, main variables with influence on the chromatographic separation were optimized.

Modification of the mobile phase with volatile acids and salts was also carried out because the mobile phase composition has a significant effect on peak shapes and the retention behaviour of the analyte in the LC column, as well as on the MS response.

Different mixtures of water and acetonitrile modified with ammonium acetate were evaluated. Addition of salts influences markedly the mass spectrometric response since it avoid the sodium adduct formation what improve the detection of molecular precursor ion and the consequent fragmentation. Moreover, the addition of ammonium acetate improves the peak shape and the reproducibility of the retention time for OTA.

Therefore, chromatographic separation of the target analytes was finally performed with a mixture of water–acetonitrile with ammonium acetate (5 mM), using an elution time of 20 min.

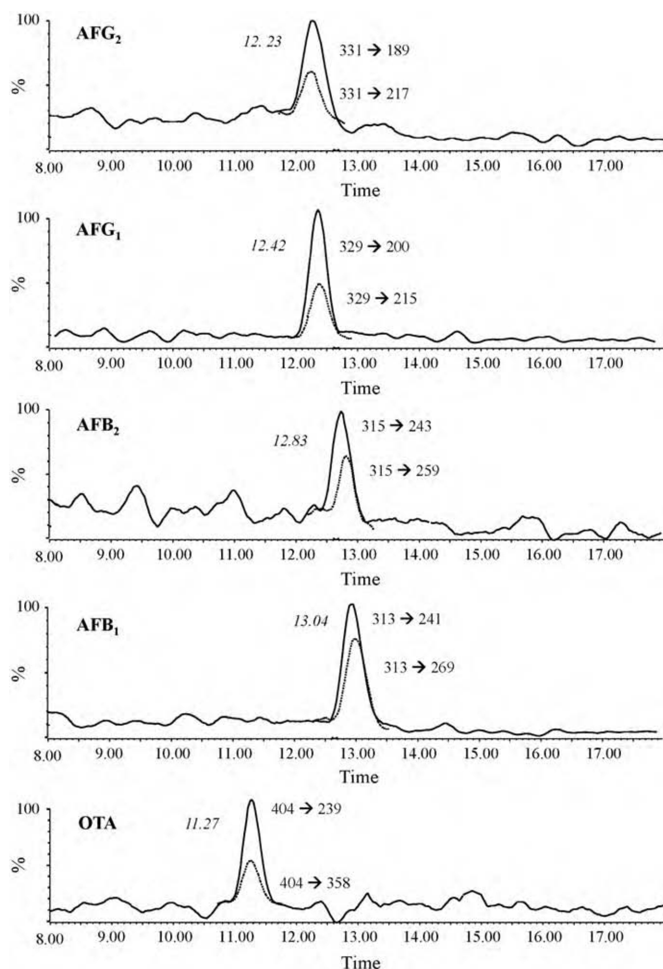

Fig. 1. LC/MS/MS chromatograms of a standard of mycotoxins in acetonitrile at LOD levels with the quantifier and qualifier ions for each compound.

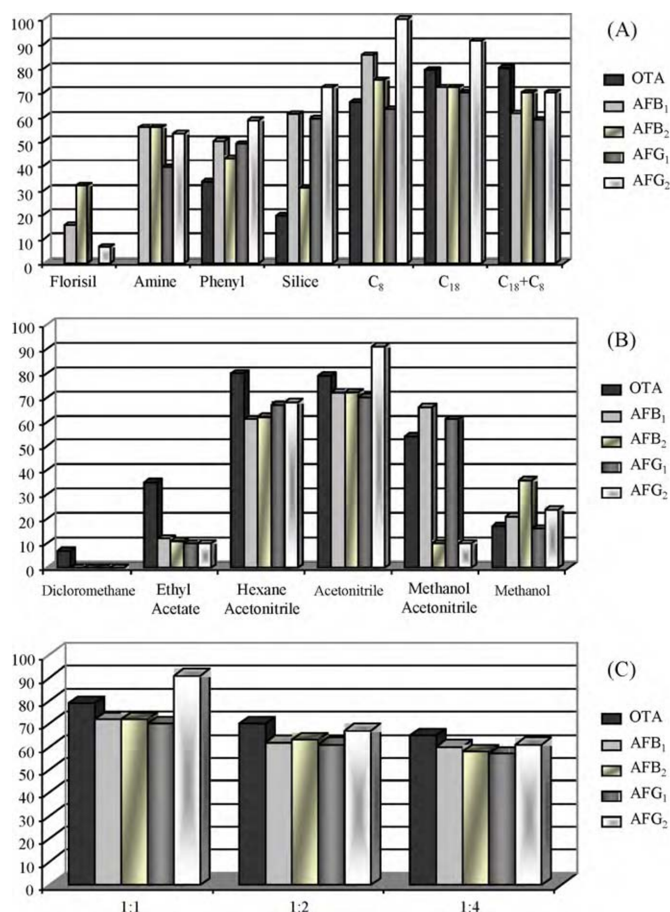

Fig. 2. Studied parameters of extraction optimization method. (A) Recoveries (%) in different solid dispersants, (B) recoveries (%) with different solvents and (C) recoveries (%) employing different ratios of sample.

Fig. 1 shows the chromatogram obtained with a standard solution under the optimum chromatographic conditions commented before at LODs levels. High repeatability and reproducibility of the injections were observed; therefore, the use of an internal standard was unnecessary.

3.2. Optimization of the extraction method

In any multi-mycotoxin method, the critical step is the extraction and clean-up procedure, specially, when the concentration of the analytes is around ppb levels. For this reason, extraction conditions had to be carefully selected to achieve the highest recovery for the mycotoxins contained in the cereals while eliminating most of the interfering matrix components.

Efficiency of MSPD extractions depends on type and quantity of dispersing phase, the amount of sample, and nature and volume of the eluting solvents. For the validation studies of the extraction, the most suitable elution solvents and the polarity of solid-phase were assessed. Malt was selected as model matrix due to its high possibility to present all the studied mycotoxins. The method was applied to the other matrix after its optimization.

3.2.1. Selection of the solid phase

Classic applications of the MSPD technique employ reversed-phase sorbent as dispersants. Octadecyl-silica (C_{18}) and octyl-silica (C_8) are by far the most often used. Theoretically, silica particles disrupt the gross architecture of biological samples whereas the bonded alkyl chains contribute to dissolving their components, providing relatively clean extracts from complex matrices when the polar solvents (acetonitrile, methanol and combinations of these) are used as extractants. In general, species of medium polarity are efficiently extracted under of these conditions.

Normal-phase, non-bonded sorbents (florisil, amino, phenyl and silica) have been proposed as dispersant in many MSPD applications. They interact with sample components solely by adsorption and, obviously, are not able to dissolve the sample matrix. The adsorption properties of these sorbents can be adjusted depending on their water content and acid or basic character.

The effect of these different sorbents on selected mycotoxins recoveries degree was studied, including the most representative as C_{18}, C_8, C_{18}-C_8, silica, florisil, phenyl, and amino. Recoveries obtained by using these solid-phases are analyzed in Fig. 2A. Here, the spiked malt samples at levels of 1 ng g^{-1} for all aflatoxins and 2 ng g^{-1} for OTA (LOQs levels) were used and, since most extraction methods reported for the extraction of mycotoxins in the literature acetonitrile was employed as a solvent, in a preliminary study.

The differences between the mean recoveries obtained with C_{18} and those obtained with phenyl, alumina, silica and florisil were statistical significance, but not those obtained with C_8. This fact was attributed to the preferential adsorption of the four solid phases (phenyl, florisil, silica and amino) by polar components.

These differences were dramatically significant in the case of the recoveries using florisil, amino and phenyl, with which the recovery values did not exceed 58.5% for AFG_2 employing phenyl. In the case of the silica, the recovery values were slightly higher than the other polar solid phases even though only in the case of AFG_1, AFG_2 and AFB_1. The presence of polar groups in the structure of aflatoxins, can

explain the interactions between these compounds and these polar solid phase that not allow their elution at the pass of the medium polar solvent as acetonitrile.

Owing to the good recovery results obtained by the use of C_8 and C_{18}, a mixture of these solid phases (50:50 p/p) was experimented. As it is reflected in the Fig. 2A, C_{18} applied alone provided higher recoveries for all the mycotoxins, maybe for the strong hydrophobic character of the first one, and eliminating the mix of solid-phase steps.

As a conclusion, and according to previous studies [24,25], C_{18} proved to be the best solid support providing high affinity for the studied compounds.

3.2.2. Study of extraction solvent

The nature of the elution solvent is an important matter since the target analytes should be efficiently desorbed while the remaining matrix components should be retained in the column. Solvents are characterized by their polarity and elution strength for a specific sorbent. The extraction solvent is often a compromise between the solvent strength required to efficiently extract mycotoxins from food and the compatibility of solvents with the analytical system.

The optimum extraction solvent was evaluated, checking a variety of solvent with very different polarities such methanol, dicloromethane, acetonitrile, ethyl acetate, hexane and mixtures of them to determine their ability to adequately elute OTA and aflatoxins on C_{18}. The only parameter changed was the type of solvent, maintaining the volume constant (10 ml). Results are presented in Fig. 2B. In this study, aqueous mixtures were discarded owing to the interaction between water and solid phase, leading a doughy consistency that makes the pass of the analytes difficult.

The most apolar organic solvents (ethyl acetate and dicloromethane) gave low mean recoveries for OTA and aflatoxins as the most polar solvent (methanol), for what it was necessary prove solutions of medium polarity as acetonitrile and mixtures of this solvent with apolar solvent (hexane) and with most polar solvent (methanol).

Regarding the acetonitrile solutions (80% of acetonitrile in all proves), adding a hexane part (20%), OTA was determined at similar levels but the aflatoxins recoveries were lower than 100% acetonitrile extraction. However, with methanolic portion (20%), the recoveries were much lower for all compounds than those obtained by acetonitrile extraction, maybe for the high polarity that this solvent has.

Considering these results and to avoid the mix step, the use of only one solvent was selected. Acetonitrile was considered the best organic solvent because of the acceptable recoveries for all the studied mycotoxins and because it gave the cleanest extracts and chromatograms.

3.2.3. Ratio of sample-to-sorbent

In MSPD, a critical parameter is the ratio between matrix and dispersing material. This ratio depends on the sample nature, although ratios of 1:1, 1:2 and 1:4 are frequently applied. For further optimization the sample amount and sorbent mass were varied to assay optimal conditions. The initial study was conducted applying the most usual sample/solid support material ratio.

To verify whether near optimum conditions were used, different amounts of C_{18} (1, 2, and 4 g) were added to the glass mortar and blended with 1 g of sample, and then elution was performed with 10 ml of acetonitrile. Results presented in Fig. 2C, showed that there were no significant differences among the recoveries of the target analytes.

With 1 g of C_{18} and 1 g of sample (ratio 1:1), recoveries were in acceptable range of 77.3–89.7% whereas when 2 and 4 g of C_{18} were used, the recoveries obtained for OTA and aflatoxins were less than 60% so any further increase of C_{18} did not improve the recovery of the studied compounds, maybe because the high dispersion of the sample into the solid-phase dispersant.

Table 2
Evaluation of matrix effects: comparison of the calibration curves slopes and calculation of signal suppression/enhancement (SSE) for selected mycotoxins in malt.

Compound		Slope	*y*-intercept	r^2	SEE
OTA	Solvent	36.421	161.46	0.9973	83
	Matrix-matched	30.255	−9.4998	0.9913	
AFB1	Standard	83.712	265	0.9965	48
	Matrix-matched	39.964	59.943	0.9897	
AFB2	Standard	35.963	84.735	0.9984	56
	Matrix-matched	20.07	30.327	0.9978	
AFG1	Standard	34.864	73.035	0.9974	53
	Matrix-matched	18.492	31.749	0.9952	
AFG2	Standard	63.193	126.22	0.9988	51
	Matrix-matched	32.322	−7.2808	0.9913	

SSE = (slope matrix-matched calibration/slope standard calibration in solvent) × 100.

3.2.4. Study of matrix effects

One of the main problems of LC–MS/MS is that the presence of matrix components can affect the ionisation of the target compounds, reducing or enhancing the response compared with standards in solvents, and the influence of the matrix effect on the response must be studied and, obviously, this affects the quantification, unless matrix effects are removed or compensated.

In order to evaluate matrix effects, the signal suppression–enhancement (SSE) for each analyte in each matrix was calculated, defined as the percentage of the matrix-matched calibration slope divided by the slope of the standard calibration in solvent.

For this objective, triplicate experiments by spiking LOQ level analyte free samples after the extraction and then following the remaining procedure reported in experimental section, were done to obtain a matrix-matched standard calibration for each matrix. The calibration curves showed high linearity ($r^2 > 0.9897$).

The matrix-matched curves slopes were compared with that of the calibration standards in solvent. Results obtained for malt as a representative matrix, are shown in Table 2, where it can be seen that notable signal suppression occurred for aflatoxins. These compounds presented slope ratios that indicate response reduction of 48% for AFB_1 and 56% for AFB_2. Therefore, a reliable quantification of these mycotoxins from food samples using LC–QqQ-MS requires malt-matched standards.

Moreover, it was considered that for accurate quantitative results, the main limitations are different matrices, as well as within given matrix. For this reason, the same experiment was carried out for the other matrices; coffee and instant cereal-breakfast beverage. The results are presented in Table 3. These matrices showed higher suppression matrix effect than those presented for malt. More concretely, SSE due to co-eluting matrix compounds was so pronounced in coffee (reaching a reduction response of 39% for AFG_1). This fact emphasized the necessity of carrying out (sample preparation and chromatography) method validation not only at different concentration but also using different matrices.

According to our results the use of matrix-matched standards calibration as it was reflected in the experimental section was required for the correct quantification of analytes and the data presented so far indicate that the most critical compounds are aflatoxins.

In practice, it is usual to prepare calibration curves for solvent and matrix to calculate the matrix effect by comparing the results.

Table 3
Calculation of signal suppression/enhancement (SSE) for OTA and aflatoxins B and G in coffee and instant breakfast beverage.

Mycotoxin	SSE (%)	
	Instant cereal-breakfast beverage	Coffee
AFB1	43	42
AFB_2	68	57
AFG_1	57	39
AFG_2	66	47
OTA	59	74

SSE = (slope matrix-matched calibration/slope standard calibration in solvent) × 100.

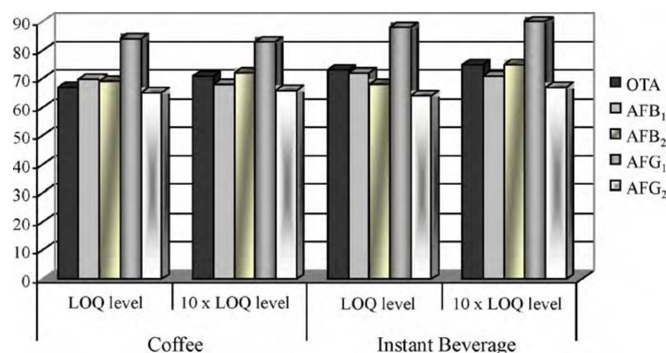

Fig. 3. Recoveries (%) obtained from spiked samples at LOQ levels and 10 times LOQ levels in coffee and instant cereal-breakfast beverage.

3.3. Validation of the method

In order to validate the developed procedure for each cereal sample, recoveries, repeatability as well as limits of detection (LODs) and limits of quantification (LOQs) were determined. The consequence of the latter matrix effects explained is the decrease of sensitivity in terms of LOD and LOQ. To compensate and improve the analytical parameters, validation is performed with a calibration curve for each analyte in real sample matrix and not in standard solution.

The LODs and LOQs were based on minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10 times the background chromatographic noise, respectively. Estimated values for all matrices of LODs were in the range of 0.8 ng g^{-1} for OTA and 0.3 ng g^{-1} for all aflatoxins, except for the aflatoxin AFG2 that was 0.4 ng g^{-1}, whereas LOQ values were in the range from 2 ng g^{-1} for OTA to 1 ng g^{-1} for the aflatoxins. The results are summarized in Table 4. The same table contains the maximum levels (MLs) for the studied mycotoxins set by the European Union (EU) considering all tested matrices.

As it can be observed LODs and LOQs were lower than the established ML (or at least similar to them), indicating that the proposed method is suitable for quantification of selected mycotoxins in the studied matrices. The main cereals contained in the composition of the instant breakfast beverage are barley, malt and chicory, for what their values are indicated in the table at individual form.

The recovery of the extraction step of each mycotoxin at two fortification levels (5 and 50 ng g^{-1} for OTA and 2 and 20 ng g^{-1} for aflatoxins) was studied, showing the obtained results in malt in Table 5. Recoveries and repeatability of the developed analytical method were carried out by injection of the same matrix-matched standard five consecutive times within the day (intra-day precision), and for five consecutive days (inter-day precision) for each analyzed compound in each selected matrix.

For all compounds mean recoveries in malt were satisfactory, ranging from 69% to 91%. The precision in the present study, estimated by the relative standard deviation (RSD) of the recovery was in the range of 7–19%. These results were very similar at those obtained in coffee and instant breakfast beverage as it can be observed in Fig. 3. From the results obtained, the developed method was found to be precise (with run-to-run instrumental RSD values between 7 and 19% and day-to-day RSD values between 8 and 17%). The method can thus be qualified as "acceptable" according to the EU criteria [27]; an average recovery (n = 5) between 70 and 120% and a repeatability (RSD) of 20% or less. The results of performance characteristics of the developed method are in good agreement with the performance criteria of the mentioned regulation.

The confirmation of positive samples was carried out by acquiring the full scan product ion spectra of the suspected compounds from a matrix-matched standard. The ion abundances were com-

Table 4
LODs, LOQs, and MLs for the three studied matrices.

			ML (EU)			
Compound	LOD (ng g^{-1})	LOQ (ng g^{-1})	Coffee (ng g^{-1})	Malt (ng g^{-1})	Barley (ng g^{-1})	Chicory (ng g^{-1})
OTA	0.8	2	5	3	3	3
AFB_1	0.3	1	2	2	2	2
AFB_2	0.3	1				
AFG_1	0.3	1	4*	4*	4*	4*
AFG_2	0.4	1				

* Expressed as the sum of the four aflatoxins ($AFB_1 + AFB_2 + AFG_1 + AFG_2$).

Table 5
Recovery values (%) and relative standard deviations (%) given in brackets calculated at two concentration levels (ng g^{-1}).

Compound	Intra-day precision[a]				Inter-day precision[b]			
	Low level		High level		Low level		High level	
	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery
OTA	5	71 (11)	50	75 (9)	5	74 (10)	50	71 (12)
AFB_1	2	72 (13)	20	70 (10)	2	69 (14)	50	74 (12)
AFB_2	2	72 (9)	20	74 (7)	2	73 (9)	50	72 (8)
AFG_1	2	91 (14)	20	88 (12)	2	91 (12)	50	90 (13)
AFG_2	2	69 (18)	20	70 (19)	2	70 (17)	50	71 (17)

[a] Number of replicates:5.
[b] Different days: 5.

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	Precursor ion	Product ion	Ion ratio expected[a,*] (RSD %)	Ion ratio observed[b,*]		
				Malt (RSD %)	Coffee (RSD %)	Instant breakfast beverage (RSD %)
AFB_1	313.2	241[Q] 269[q]	0.77	0.60 (5)	0.55 (6)	0.58 (9)
AFB_2	315.2	243[Q] 259[q]	0.50	0.55 (6)	0.59 (8)	0.57 (7)
AFG_1	329.2	200[Q] 215[q]	0.31	0.45 (11)	0.48 (10)	0.44 (10)
AFG_2	331.2	189[Q] 217[q]	0.37	0.35 (17)	0.40 (15)	0.39 (14)
OTA	404.2	239[Q] 358[q]	0.38	0.35 (7)	0.45 (6)	0.43 (9)

[a] Ratio determined in matrix-matched solution at LOQ level in acetonitrile ($n = 5$).
[b] Ratio determined in fortified sample at LOQ level in malt, coffee and instant cereal-breakfast beverage ($n = 5$).
[*] 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%).

pared with those calculated for fortified malt, coffee, and instant cereal-breakfast beverage samples. Table 6 lists the extracted fragment ions that were monitored to quantify and identify at the same concentration, and the calculated ratio of their abundances. Confirmatory analysis was found to be successful in all the cases. The quantification ion was the most abundant. The ratio of the two major products ions and the retention time deviation were within the interval established by the European Union Guidelines [27].

3.4. Application to differents samples

To evaluate the applicability of the method proposed, 22 samples were obtained from a local supermarket. 10 samples of malt, 7 samples of coffee and 5 samples of instant-based cereal-breakfast beverage. The results are statistically represented in Fig. 4.

Four samples of the total malt samples were positive for AFG_2 and AFG_1, and traces of AFB_1 and AFB_2 were detected. Although these levels were below the maximum level established by EU, they can indicate that more attention should be paid to storage conditions, in order to minimize the content of these analytes.

On the other hand, seven different coffee samples were collected and after the analysis, only two samples were positive for OTA, although the concentrations levels were lower than those established by the legislation.

Finally, none of the instant breakfast beverage analyzed presented traces of the mycotoxins studied.

Fig. 5 shows the chromatogram of a malt positive real sample, which maintains the good characteristics of those obtained from spiked samples.

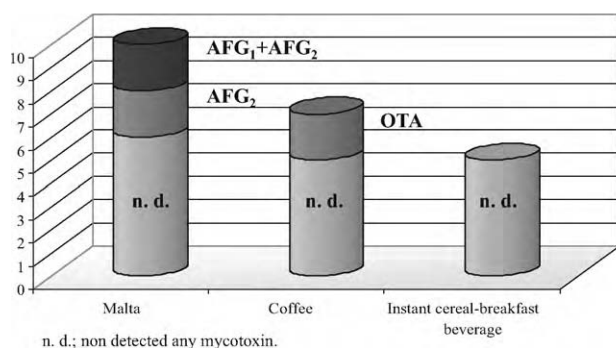

Fig. 4. Results of the analysis of 22 different real samples and the incidence of mycotoxins.

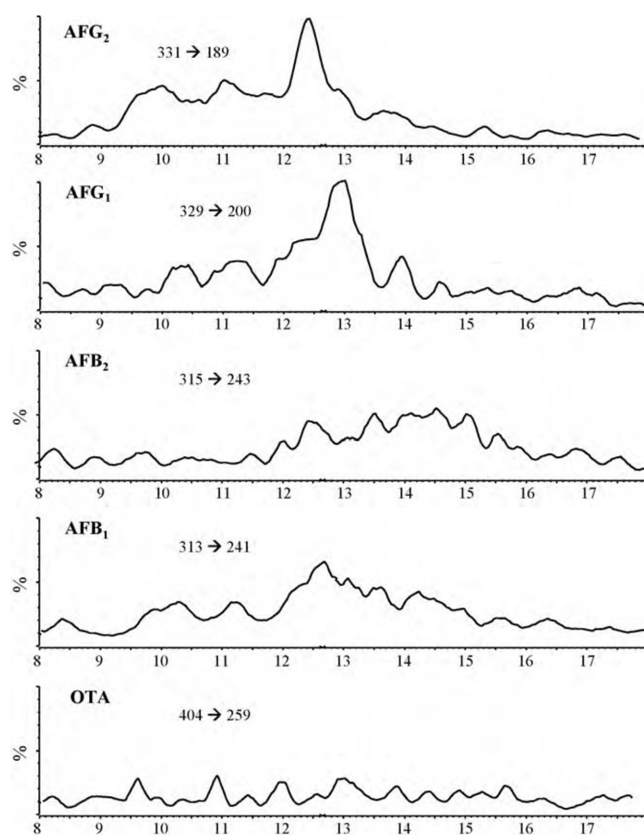

Fig. 5. Chromatogram of real malt sample that contains AFG1 and AFG2.

Although 6 samples gave evidence of contamination, this level did not exceed the ML fixed by the EU.

4. Conclusions

The MSPD method presented is a good starting point for further development of sample analysis in a single run and it can be regarded as a valuable alternative to the more classical sample preparation methods because it allows a significant reduction in both the sample size and solvent consumption needed for multi-residue analysis. Moreover, it offers a valid clean-up alternative to immunoaffinity columns, which are expensive and cannot be used to perform a multi-mycotoxin extraction, being also suitable for

routine analysis. It should be emphasize the necessity to optimize the solid-phase dispersant, the elution solvent and the ratio of the extraction method.

The results obtained in the extraction optimization confirm once again the need to carefully evaluate potential matrix effects. Only appropriate sample extraction, clean-up and good chromatographic separations allow us to considerably reduce matrix effects and to obtain the best method performances in terms of repeatability and accuracy of quantitative measurements.

This study showed that matrix effects vary from sample and from analyte, and it can considerably affect quantification accuracy. Therefore, for a full method validation, the matrix effects should be carefully evaluated on all analytes in each specific matrix under investigation.

In an application of the methodology, six out of 22 radom samples gave evidence of contamination, however these levels did not exceed the MRL fixed by the EU. These results suggest that it is important to monitor malt for the presence of aflatoxins and OTA, especially when it may be possible the store in dubious conditions. These amounts of OTA and AFs detected may be attributed to improper packaging and long storage time.

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

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