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Analytical Methods

Simultaneous determination of fumonisins B1 and B2 in different types of maize by matrix solid phase dispersion and HPLC-MS/MS

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ABSTRACT

This work involved the optimization and validation of a method, according to Directive 2002/657/EC and the Analytical Quality Assurance Manual of Ministério da Agricultura, Pecuária e Abastecimento, Brazil, for simultaneous extraction and determination of fumonisins B1 and B2 in maize. The extraction procedure was based on a matrix solid phase dispersion approach, the optimization of which employed a sequence of different factorial designs. A liquid chromatography-tandem mass spectrometry method was developed for determining these analytes using the selected reaction monitoring mode. The optimized method employed only 1 g of silica gel for dispersion and elution with 70% ammonium formate aqueous buffer (50 mmol L^{-1}, pH 9), representing a simple, cheap and chemically friendly sample preparation method. Trueness (recoveries: 86–106%), precision (RSD ≤19%), decision limits, detection capabilities and measurement uncertainties were calculated for the validated method. The method scope was expanded to popcorn kernels, white maize kernels and yellow maize grits.

1. Introduction

Maize (*Zea mays*) is a foodstuff that participates in a wide agro-industrial chain of production and processing of food and feed. One of the major problems of the maize chain is contamination by mycotoxin-producing fungi. This contamination can occur in the crop and worsen during harvesting, transportation, drying, processing and/ or develop during storage of maize and its derived products. (Galvão, Miranda, Trogello, & Fritsche-Neto, 2014).

Fumonisins are toxic mycotoxins produced by *Fusarium* fungi (Bezuidenhout et al., 1988). Among all identified fumonisins, B1 and B2 are the most toxic and could cause esophageal cancer in humans (Bordin, Rosim, Neeff, Rottinghaus, & Oliveira, 2014). The toxicity and occurrence of fumonisins in various foods has led the regulatory authorities, such as the Commission of the European Community, Food and Drug Administration of the United States and Agência Nacional de Vigilância Sanitária in Brazil, to establish maximum limits allowed in different foods. (Regulation EC/1881/2006; FDA Regulatory Guidance for Mycotoxins; RDC-ANVISA N° 7/2011).

In order to determine mycotoxin residues in various food matrices, several analytical methods have been proposed. The main sample preparation techniques that have been employed for analysis of mycotoxins, including fumonisins, are: (1) solid-liquid extraction (SLE) followed by clean-up with solid phase extraction (SPE) or by immunoaffinity column or by dispersive solid phase extraction (dSPE) (Beltrán et al., 2013; Abia et al., 2013; Wang et al., 2013; Szekeres et al., 2014; Liao et al., 2015; Petrarca, Rodrigues, Rossi, & De Sylos, 2014; García-Moraleja, Font, Manes, & Ferrer, 2015b; Jung et al., 2015; Ediage, Poucke, & De Saeger, 2015; Bryła, Szymczyk, Jedrzejczak, & Obiedzinski, 2015; Bryła, Roszko, Szymczyk, Jedrzejczak, & Obiedzinski, 2016; Petrarca, Rossi, & De Sylos, 2016), (2) liquid-liquid extraction (LLE) followed by clean-up with immunoaffinity column (Beltrán et al., 2013; Abia et al., 2013; García-Moraleja, Font, Mañes, & Ferrer, 2015a), (3) matrix solid phase dispersion (MSPD) (Rubert, Soler, & Manes, 2011; Rubert, Dzuman et al., 2012; Rubert, Soler, & Mañes, 2012; Serrano, Font, Ruiz, & Ferrer, 2012;Ye, Lai, & Liu, 2013; Blesa, Moltó, Akhdari, Mañes, & Zinedine, 2014), (4) dispersive liquid-liquid microextraction (DLLME) (Arroyo-Manzanares, Huertas-Pérez, Gámiz-Gracia, & García-Campaña, 2013) and (5) Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) (Arroyo-Manzanares et al., 2013; Azaiez, Giusti, Sagratini, Mañes, & Fernández-Franzón, 2014; Arroyo-Manzanares, Huertas-Pérez, García-Campaña, & Gámiz-Gracia, 2014; Pizzutti et al., 2014; Bolechová et al., 2015; Nielsen, Ngemela, Jensen, De Medeiros, & Rasmussen, 2015; Arroyo-Manzanares, Huertas-Pérez,

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Gámiz-Gracia, & García-Campaña, 2015). High performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC) coupled to detectors such as UV–Vis spectrophotometric (Ye et al., 2013), fluorescence (Petrarca et al., 2014; Petrarca et al., 2016) and mass spectrometry (Rubert et al., 2011; Beltrán et al., 2013; Abia et al., 2013; Zhang et al., 2013; Arroyo-Manzanares et al., 2013; Wang et al., 2013; Azaiez et al., 2014; Arroyo-Manzanares et al., 2014; Pizzutti et al., 2014; Liao et al., 2015; Bolechová et al., 2015; Nielsen et al., 2015; García-Moraleja et al., 2015a; García-Moraleja et al., 2015b; Jung et al., 2015; Ediage et al.,2015; Bryła et al., 2015; Bryła et al., 2016), have been the most widely used techniques for fumonisin quantification.

Among the extraction and clean-up techniques abovementioned, MSPD has two important characteristics: extraction and clean-up in a single step and there is no need for solubilizing the solid and semisolid samples. These characteristics make MSPD advantageous for analyzing the mycotoxins in food samples, especially the solid ones (Barker, 2007). MSPD is especially advantageous for fumonisin determination in maize, once the distribution of these mycotoxins in the grain is not homogeneous and supramolecular structures with other maize components need to be broken to ensure efficient extraction (WHO/IARC, 2002). Thus, MSPD has been widely used for the extraction and clean-up of mycotoxins in food, employing chemically modified silica as dispersant and elution with either 100% or at high proportion organic solvents (Rubert et al., 2011; Rubert, Dzuman et al., 2012; Rubert, Soler et al., 2012; Serrano et al., 2012; Ye et al., 2013; Blesa et al., 2014).

In this work, an MSPD method was developed for the extraction and clean-up of fumonisins B1 and B2 in maize using silica gel as dispersant and elution with 70% ammonium formate aqueous buffer (50 mmol L^{-1}, pH 9) . The method was validated according to Directive 2002/657/EC and Manual of Analytical Quality Assurance of the Ministério da Agricultura, Pecuária e Abastecimento, Brazil (BRAZIL, Pecuária e Abastecimento, & Coordenação-Geral de Apoio Laboratorial, 2014). The following validation parameters: matrix effect, linearity, precision, trueness, decision limit, detection capability and measurement uncertainty, were evaluated. The scope of the validated method was expanded to popcorn kernels, white maize kernels and yellow maize grits.

2. Materials and methods

2.1. Chemicals

Standards of fumonisins B1 (FB1) and B2 (FB2) (minimum purity 98%, Sigma-Aldrich, St. Louis, USA and minimum purity 90%, Wako Pure Chemical Industries, Osaka, Japan, respectively) were used for stock solution preparations. Acetonitrile (ACN), methanol, acetic acid (99% w/w), formic acid (88% w/w), ammonium hydroxide (29% w/w) (HPLC grade, J. T. Baker, Mexico) and tetrahydrofuran (THF) (analytical grade, Dinâmica Química Contemporânea Ltda, Brazil) were used for mobile phase and/or extraction solution preparation. Silica gel (70–120 mesh, Fluka, USA) and silica chemically bound with octadecyl groups (C18 silica) (50 μm, 65A, Phenomenex, USA) were used for MSPD. Ultrapure water from Millipore Direct-Q3 UV purifier (Millipore, USA) was used for aqueous solution preparations.

2.2. Stock and working solutions

Individual stock solutions were prepared at a concentration of 0.8 μg L^{-1} for FB1 and 0.6 μg L^{-1} for FB2 by dissolving the exact mass of each standard in ACN: ultrapure water (1:1 v/v) and were then stored at -10 °C. Working solutions were prepared by mixing the individual stock solutions and diluting them with ACN: ultrapure water (1:1 v/v) to a final concentration of 100 ng L^{-1} of FB1 and 50 ng L^{-1} of FB2. All solutions were stored at −10 °C.

2.3. Samples

Maize was used in the optimization and validation of the method. Popcorn kernels, white maize kernels and yellow maize grits were used to expand the scope of the method. All samples were ground to appropriate particle size and were provided by Laboratório de Controle de Qualidade e Segurança Alimentar of the Ministério da Agricultura, Pecuária e Abastecimento, Brazil. All samples were stored at −10 °C.

2.4. HPLC-MS/MS instrument

The HPLC-MS/MS analyses were performed in a triple quadrupole mass spectrometer with a turbo ion spray interface (API 5000, Applied Biosystems, USA) coupled to a HP Agilent Technologies 1200 series liquid chromatography system equipped with an autosampler and a quaternary pump (Agilent Technologies, USA). Both systems and data treatment were controlled by Analyst 1.5.1 software (Applied Biosystems, USA).

2.5. HPLC-MS/MS conditions

The optimum condition for the separation of FB1 and FB2 using a C18 column (100 × 3 mm, 2.7 μm, Poroshell, Agilent Technologies, USA) was obtained with the mobile phase: 0.1% v/v formic acid in ultrapure water (solvent A) and 0.1% v/v formic acid in ACN (solvent B). The chromatographic gradient was used as follows: from 0 to 3 min the percentage of solution B linearly increased from 20 to 90% and was maintained constant up to 3.4 min; from 3.4 to 3.5 min the percentage of solution B decreased to 20%, which was maintained up to 6 min. The mobile phase flow rate was 0.500 mL min^{-1}, the injection volume was 10 μL and the column temperature was maintained at 40 °C.

Electrospray ionization (ESI) conditions in positive mode were first optimized with direct infusion into the mass spectrometer to select the precursor and the product ions resulting from fragmentation, the declustering potential (DP) and the collision energy (EC) for each fumonisin (Table 1S). This optimization was conducted by direct infusion of standard solutions at 10 μg L^{-1} of FB1 and 5 μg L^{-1} of FB2 at a flow rate of 10 μL min^{-1}. Flow injection analysis (FIA) was used to optimize capillary voltage, curtain and nebulizer gas flow rates and source temperature. The experiments were conducted at a mobile phase flow rate (solvent A: solvent B, 1:1 v/v) of 0.5 mL min^{-1}. The following settings were also applied to the turbo ion spray source: capillary voltage, 4500 V; temperature, 650 °C; nebulizing gas (N_2), 40 (arbitrary units); curtain gas (N_2), 18 (arbitrary units); CAD gas (N_2), 4 (arbitrary units); entrance potential, 10 V. The fumonisins were evaluated employing the Selected Reaction Monitoring (SRM) mode. The most intense transition was used for quantification (352.4 *m/z* for FB1 and 336.4 *m/z* for FB2) and the other two transitions for confirmation (334.5 and 316.4 *m/z* for FB1; 354.3 and 318.3 *m/z* for FB2).

2.6. Preliminary studies for optimization of extraction of FB1 and FB2 by MSPD in maize

2.6.1. MSPD cartridge preparation

1 g of the maize sample and 1 g of the dispersant were weighed, transferred to a ceramic container and mixed. Polypropylene cartridges (15 mL) were mounted using a Teflon filter (20 μm) at the bottom, followed by glass wool and dispersed sample. The car-

tridges were compressed with a vacuum pump and finalized with a Teflon filter (20 μm) at the top.

2.6.2. Dispersant selection

Silica gel and C18 silica were evaluated as dispersants. MSPD cartridges were prepared (Section 2.6.1) and eluted with 16.00 mL of a mixture of 20 mmol L^{-1} ammonium formate buffer (pH 7) and methanol (9:1 v/v). Fractions of approximately 2 mL of the eluate were collected, centrifuged at 4000 rpm for 10 min, filtered with syringe filter (0.22 μm) and 10 μL were injected in the HPLC-MS/MS system.

2.6.3. Optimization of the extraction solution composition

Silica gel was defined as dispersant and the extraction solution composition was evaluated by a 3^2 factorial design (Table 2S). The following factors were evaluated: organic solvent at (−) methanol, (0) ACN and (+) THF levels; aqueous solvent at (−) 20 mmol L^{-1} ammonium formate buffer (pH 6), (0) ultrapure water and (+) 20 mmol L^{-1} ammonium formate buffer (pH 9) levels. MSPD cartridges were prepared (Section 2.6.1) and eluted with 6.50 mL of extraction solution in the constant ratio of 3:7 v/v for organic and aqueous solvents. The extracts were centrifuged at 4000 rpm for 10 min, filtered with syringe filter (0.22 μm) and 10 μL were injected in the HPLC-MS/MS system.

2.6.4. Selection of significant variables for MSPD

After selecting silica gel as dispersant and pH 9 for extraction solution, the $2^{(4-1)}$ fractional factorial design (Table 3S) was performed to select the variables that significantly influence the MSPD extraction. The following factors were evaluated: ammonium formate buffer concentration at (−1) 20 and (+1) 40 mmol L^{-1} levels; elution volume at (−1) 6 and (+1) 10 mL levels; organic solvent at (-1) ACN and (+1) THF levels; ratio of buffer and organic solvent at (−1) 7:3 and (+1) 9:1 v/v. MSPD cartridges were prepared (Section 2.6.1) and eluted with extraction solution. The extracts were centrifuged at 4000 rpm for 10 min, filtered with syringe filter (0.22 μm) and 10 μL were injected in the HPLC-MS/MS system.

2.7. Optimization of the MSPD procedure for the extraction of FB1 and FB2 in maize

After selecting silica gel as dispersant and pH 9 and THF for extraction solution, the 3^3 Box-Behnken design was performed to select the optimum condition for the extraction of FB1 and FB2 by MSPD (Table 4S). The following factors were evaluated: ammonium formate buffer concentration at (−1) 30, (0) 40 and (+1) 50 mmol L^{-1} levels; elution volume at (−1) 4, (0) 6 and (+1) 8 mL levels; ratio of THF and buffer at (−) 2:8 (0) 3:7 and (+) 4:6 v/v levels. The central point was performed in triplicate. MSPD cartridges were prepared (Section 2.6.1) and eluted with extraction solution. The extracts were centrifuged at 4000 rpm for 10 min, filtered with syringe filter (0.22 μm) and 10 μL were injected in the HPLC-MS/MS system.

2.8. Optimum condition

MSPD cartridges were prepared (Section 2.6.1) and eluted with 8.00 mL of a mixture of 50 mmol L^{-1} ammonium formate buffer (pH 9) and THF (7:3 v/v). The extract was diluted to 10.00 mL with a mixture of ACN and ultrapure water (1:1 v/v), homogenized and centrifuged at 2600 rpm for 10 min. Next, an aliquot of 5.00 mL of this extract was diluted to 25.00 mL with a mixture of ACN and ultrapure water (1:1 v/v), centrifuged at 4000 rpm for 10 min and filtered with syringe filter (0.22 μm).

2.9. Evaluation of the matrix effect

The matrix effect was evaluated by comparing the external calibration curve slope with the standard addition curve slopes for the following samples: maize, popcorn kernels, white maize kernels and yellow maize grits. Three cartridges (Section 2.8) were used to prepare the standard addition curves. Next, 4.00 mL of the extract were transferred to 5.00 mL volumetric flasks, to which working solution aliquots were added so that the final concentrations obtained were: 0, 2.5, 5.0, 10.1, 20.2, 30.3 and 60.5 μg L^{-1} for FB1 and 0, 1.1, 2.3, 4.6, 9.2, 13.7 and 27.5 μg L^{-1} for FB2.

2.10. Validation

2.10.1. Linearity

Linearity was evaluated by the standard addition curve for maize. A MSPD cartridge was prepared and eluted (Section 2.8). Next, 4.00 mL of the extract were transferred to 5.00 mL volumetric flasks, to which working solution aliquots were added so that the final concentrations obtained were: 0, 2.5, 7.5, 15.0, 20.0, 30.0 and 60.0 μg L^{-1} equivalent to 0, 125, 375, 750, 1000, 1500 and 3000 μg kg^{-1} for FB1 and 0, 1.3, 3.8, 7.5, 10.0, 15.0 and 30.0 μg L^{-1} equivalent to 0, 65, 190, 375, 500, 750 and 1500 μg kg^{-1} for FB2. This procedure was performed on three different days. The peak areas of FB1 and FB2 obtained during the three days were used to plot the standard addition curves. Linearity was evaluated by the determination coefficient as well as by the variance analysis.

2.10.2. Trueness and precision

To evaluate trueness, intra-day and inter-day precision, spiked samples were prepared at four concentration levels: 0, 0.5, 1.0 and 1.5 times of the MRL, i.e. 0, 1000, 2000 and 3000 μg kg^{-1} of the sum of FB1 and FB2. Recoveries were calculated by interpolation of each fumonisin peak area on the corresponding standard addition curve. Next, the concentration calculated was decreased of the concentration estimated in the naturally contaminated sample and divided by added standard concentration. These experiments were performed in triplicates at each concentration level for evaluation of the intra-day precision and repeated for two more days for evaluation of the inter-day precision. The precision was calculated by the relative standard deviation (RSD) of the total concentration estimated, i.e., concentration in the naturally contaminated sample with standard addition.

2.10.3. Decision limit, detection capability, detection and quantification limits

The Directive 2002/657/EC defines the decision limit (CCα) as: "means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant" and the detection capability (CCβ) as: "means the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β" (Directive 2002/657/EC). CCα (Eq. (1)) and CCβ (Eq. (2)) were determined considering two contributions: natural contamination of the maize and standard addition curve. CCα and CCβ were calculated at zero concentration level, so their values correspond to the detection limit (LOD) and the quantification limit (LOQ), respectively (Directive 2002/657/EC; BRAZIL, 2014).

$$CC\alpha = [FB]_0 + \frac{t_{(\alpha,\nu)}}{\hat{b}_1}\sqrt{\frac{s^2_{\hat{y}_0}}{K} + s^2_{\hat{b}_0} + (x_0)^2 s^2_{\hat{b}_1} + 2(x_0)\mathrm{cov}_{(\hat{b}_0,\hat{b}_1)}} \quad (1)$$

$$CC\beta = [FB]_0 + 2\frac{t_{(\alpha,\nu)}}{\hat{b}_0}\sqrt{\frac{s^2_{\hat{y}_0}}{K} + s^2_{\hat{b}_0} + (x_0)^2 s^2_{\hat{b}_1} + 2(x_0)\mathrm{cov}_{(\hat{b}_0,\hat{b}_1)}} \quad (2)$$

where: $[FB]_0$ is fumonisin concentration at zero level of the standard addition curve in μg kg^{-1}; $t_{(\alpha,v)}$ is t-value with α significance and v degrees of freedom; $s^2_{y_0}$ is variance of the instrumental response at zero level of the standard addition curve; $s^2_{b_0}$ is variance of the intercept; $s^2_{b_1}$ is variance of the slope; K is the number of replicates of the standard addition curve at zero level; x_0 is fumonisin concentration at zero level of the standard addition curve in μg L^{-1} and $cov_{(b_0,b_1)}$ is covariance between intercept and slope.

2.10.4. Measurement uncertainty

Measurement uncertainty was estimated in accordance with the Bottom-up methodology (BRAZIL, 2014) and was divided into three steps:

(1) To define the measurand (Eq. (3)):

$$[FB] = \frac{([FB]_{HPLC} + [FB]_0).V_{id}.V_{fd}}{V_e.m_s} \quad (3)$$

where: [FB] is fumonisin concentration estimated (μg kg^{-1}) in naturally contaminated maize sample with standard addition; $[FB]_{HPLC}$ is fumonisin concentration estimated by the standard addition curve (μg L^{-1}); $[FB]_0$ is fumonisin concentration at zero level of the standard addition curve (μg L^{-1}); V_{id} is volumetric flask volume (L) used in the initial extract dilution; V_{fd} is volumetric flask volume (L) used in the final extract dilution; V_e is extract volume (L) used in the second dilution; m_s is maize sample weight (kg) used in the cartridge preparation.

(2) To identify the uncertainty sources:

Primary uncertainty sources: fumonisin concentration estimated by the standard addition curve; fumonisin concentration in the naturally contaminated maize sample; volumetric flask volumes used in the initial and final dilution of the extract; extract volume used in the second dilution; maize sample weight used in the cartridge preparation; precision and recovery.

Secondary uncertainty sources: standard addition curve parameters; inter-day precision; resolution of the volume measuring instruments; recipient weight and weight of the recipient containing silica gel and maize sample.

Tertiary uncertainty sources: precision and resolution of the weight measuring instrument.

(3) To estimate the uncertainty of each source and combined standard uncertainty:

Combined standard uncertainty (u_c) was estimated by the Law of Propagation of Uncertainty (Eq. (4)) using the input quantities defined in the measurand (Eq. (3)):

$$\begin{aligned} u_C^2 = {} & (u_{b_0})^2\left(\frac{\partial u_{(FB)}}{\partial u_{b_0}}\right)^2 + (u_{b_1})^2\left(\frac{\partial u_{(FB)}}{\partial u_{b_1}}\right)^2 \\ & + 2cov(b_0,b_1)\left(\frac{\partial u_{(FB)}}{\partial u_{b_0}}\right)\left(\frac{\partial u_{(FB)}}{\partial u_{b_1}}\right) + (u_{V_{id}})^2\left(\frac{\partial u_{(FB)}}{\partial u_{V_{id}}}\right)^2 \\ & + (u_{V_{fd}})^2\left(\frac{\partial u_{(FB)}}{\partial u_{V_{fd}}}\right)^2 + (u_{m_s})^2\left(\frac{\partial u_{(FB)}}{\partial u_{m_s}}\right)^2 + (u_{V_s})^2\left(\frac{\partial u_{(FB)}}{\partial u_{V_s}}\right)^2 \\ & + (u_{[FB]_0})^2\left(\frac{\partial u_{(FB)}}{\partial u_{[FB]_0}}\right)^2 \end{aligned} \quad (4)$$

where: b_0 is intercept and b_1 is slope of the standard addition curve.

According to Regulation EC/401/2006, u_c should not exceed the maximum uncertainty limit (U_{max}) (Eq. (5)):

$$U_{max} = \sqrt{(LOD/2)^2 + (\alpha \times [FB])^2} \quad (5)$$

where LOD is detection limit of the method in μg kg^{-1}; [FB] means fumonisin concentration (μg kg^{-1}) in which u_c was estimated; α is a parameter that depends on the magnitude of fumonisin concentration, α is 0.15 for fumonisin concentration from 501 to 1000 μg kg^{-1} and 0.12 for fumonisin concentration from 1000 to 10,000 μg kg^{-1}.

2.11. Method scope expansion

To expand the validated method scope for popcorn kernels, white maize kernels and yellow maize grits, two cartridges were prepared (Section 2.8) for each sample at the following concentration levels: 0, 0.5, 1 and 1.5 times of the MRL. The extracts without standard addition were used to make the standard addition curves and the extracts with standard addition to estimate recovery and intra-day precision. Linearity, precision, recovery, CCα/LOD and CCβ/LOQ were estimated to evaluate the quality of the method scope expansion.

3. Results and discussion

3.1. HPLC-MS/MS method optimization

The optimum condition selected (Section 2.5) allowed the separation of FB1 and FB2 at 3.5 min with good resolution and signal intensity (Fig. 1S).

3.2. Preliminary studies for optimization of extraction of FB1 and FB2 by MSPD in maize

3.2.1. Dispersant selection

Silica gel and C18 silica were evaluated as dispersants (Section 2.6.2) and the extraction solution volume was fractionated in 2 mL portions in order to check the elution profile of FB1 and FB2 in both dispersants. Extraction of FB1 and FB2 occurred mainly in the first fractions with silica gel and were only eluted in the final fractions with C18 silica (Fig. 2S). Thus, silica gel was selected as the dispersant due to shorter extraction time, lower solvent consumption and lower cost compared to C18 silica.

3.2.2. Optimization of the extraction solution composition

The 3^2 factorial design was performed in order to evaluate the influence of the organic and aqueous solvents in the extraction of FB1 and FB2. FB1 highest area was obtained in the following experiments: (0,+1), i.e., elution with a mixture of ACN and 20 mmol L^{-1} ammonium formate buffer in the ratio 3:7 v/v; and (+1,+1), i.e., elution with a mixture of THF and 20 mmol L^{-1} ammonium formate buffer in the ratio 3:7 v/v (Fig. 3S). FB2 highest area was achieved in experiment (+1,+1) (Fig. 3S). The pK_a for silanol groups is about 6 and the pK_a range for fumonisins is 3.5–9.3, thus, at pH 9 the silanol groups and part of FB1 and FB2 presented negative charge. However, some electrostatic repulsion could have occurred and favored the FB1 and FB2 elution at pH 9. Moreover, the ester groups of fumonisins could have been hydrolyzed in methanol, so lower areas were obtained in this solvent (Visconti, Doko, Bottalico, Schurer, & Boenke, 1994).

Statistical models were fitted by the least squares method with the areas of FB1 and FB2 from 3^2 factorial design (Eqs. (3) and (4)). The explained percentages of the models were 80 and 92% for FB1 and FB2, respectively, and did not show lack of fit at 95% confidence level ($F_{FB1} = 3.06$, $F_{FB2} = 2.70$ and $F_{critical\ (0.05;3;9)} = 3.86$). The quadratic term of the organic solvent was not significant at 95% confidence level for FB1 nor was the quadratic term for FB2. Thus, the organic and aqueous solvent factors were significant for the

extraction of FB1 and FB2 in maize by MSPD. Since higher areas were obtained in the experiments (0,+1) and (+1,+1), both at pH 9, this pH was selected and new experiments were performed with ACN and THF solvents.

$$\hat{y}_{FB1} = (60.9 \pm 9.3) \times 10^3 + (18.3 \pm 5.2) \times 10^3 OS + (22.7 \pm 5.2) \times 10^3 AS - (2.4 \pm 9.0) \times 10^3 OS^2 + (9.1 \pm 9.0) \times 10^3 AS^2 + (9.9 \pm 6.4) \times 10^3 OS \cdot AS \quad (6)$$

$$\hat{y}_{FB2} = (30.5 \pm 4.9) \times 10^3 + (21.3 \pm 2.7) \times 10^3 OS + (12.8 \pm 2.7) \times 10^3 AS + (4.8 \pm 4.7) \times 10^3 OS^2 - (4.5 \pm 4.7) \times 10^3 AS^2 + (10.0 \pm 3.3) \times 10^3 OS \cdot AS \quad (7)$$

Eqs. (3) and (4) describe model coefficient ± confidence interval, where OS is organic solvent and AS is aqueous solvent.

3.2.3. Selection of significant variables for MSPD

The 2^{4-1} fractional design was performed to verify whether the variables: buffer concentration, elution volume, organic solvent and ratio of buffer and organic solvent, were significant for the extraction of FB1 and FB2 in maize by MSPD. The highest areas for FB1 and FB2 were obtained in experiment (+1, −1, +1, −1), which was eluted with 6.00 mL of a mixture of 40 mmol L^{-1} ammonium formate buffer and THF (3:7 v/v) (Fig. 4S). The effects were calculated and plotted at a normal probability plot of the effects to determine which factors affected significantly the extraction of FB1 and FB2 (Fig. 5S). Since 2^{4-1} fractional design is resolution four, the main effects are mixed with third-order interactions and second order interactions are mixed among themselves. However, interaction of the three factors could be considered negligible if compared to the main effects. Analyzing the normal probability plot (Fig. 5S), it could be observed that all the main effects were significant, since they were far from of the origin. Although the factors were significant, THF was selected because it provided the highest area in experiment (+1, −1, +1, −1) of the 2^{4-1} fractional design and had already provided higher areas, especially for FB2, in all experiments performed previously.

3.3. Optimization of the MSPD procedure for the extraction of FB1 and FB2 in maize

After verifying that the factors:ratio of buffer and THF, buffer concentration and elution volume, were relevant for the extraction of FB1and FB2 in maize by MSPD, the 3^3Box-Behnken design was carried out to optimize the extraction procedure. By analyzing the results, it was verified that most extraction efficiency of FB1 and FB2 was obtained at higher ammonium formate buffer concentration (experiment: +1, +1, 0), suggesting predominance of electrostatic interactions between fumonisins and silica gel and/or maize matrix (Fig. 6S). The increase of the THF content did not influence significantly the extraction, showing that the hydrophobic interactions present in the dispersed material were sufficiently broken with 20% of organic solvent. The increase in the elution volume provided an increase in the extraction efficiency of FB1 and FB2, within the measured range, which was in accordance with the chromatographic elution principles.

Statistical models were fitted by the least squares method to evaluate the influence of buffer concentration, elution volume, ratio of THF and buffer. For both fumonisins, buffer concentration, elution volume and their interaction were significant at 95% confidence level. However, ratio of THF and buffer was not significant for MSPD procedure. The explained percentages of the models were 74 and 78% for FB1 and FB2, respectively, and did not show lack of fit at 95% confidence level (F_{FB1} = 12.01, F_{FB2} = 13.58 and $F_{critical\ (0.05;3;2)}$ = 19.16).

Response surfaces were constructed to evaluate graphically the influence of the factors studied on the extraction of FB1 and FB2 (the latter is not shown because it presented a similar profile to FB1) (Fig. 1). By analyzing the response surfaces, it could be observed that the ratio of THF and buffer did not influence significantly the extraction. On the other hand, the increase of the ammonium formate buffer concentration as well as the elution volume favored the extraction, however, the latter in a less extend. Thus, the optimum extraction condition was elution with 8.00 mL of a mixture of THF and 50 mmol L^{-1} ammonium formate buffer at pH 9 (3:7 v/v).

Some MSPD methods for mycotoxin extraction in different samples are available in the literature. Rubert et al. proposed the dispersion of 1 g of cereal flours with 1 g of C18 silica followed by packing into a glass column and eluting with 20 mL of a mixture of ACN and methanol (1:1 v/v) containing 1 mmol L^{-1} ammonium formate (Rubert et al., 2011). Next, this method was applied by Rubert et al., Serrano et al. and Blesa et al. in different analyses (Rubert, Dzuman et al., 2012; Rubert, Soler et al., 2012; Serrano et al., 2012; Blesa et al., 2014). In another work, Ye et al. proposed a similar method in which the elution was performed with 10 mL of 10 mmol L^{-1} formic acid in methanol (Ye et al., 2013).

Thus, the advantages of the optimized method in this work are: the use of silica gel as a dispersant, taking into account that it costs about five times less than C18 silica; the use of a smaller organic solvent volume, the extraction solution contained 30% of organic solvent while the method by Rubert et al. and Ye et al. used 100%. In addition, a lower elution volume was used, which further reduced the organic solvent consumption making the elution process faster and thus generating less residue amount.

3.4. Evaluation of the matrix effect

The matrix effect was evaluated by comparing the external calibration curve slope with the standard addition curve slopes for the following samples: maize, popcorn kernels, white maize kernels and yellow maize grits. All curves were performed with seven concentration levels and the weighted least squares regression was used to fit the models, since only white maize kernels provided homogeneous response variances. Next, the F-test was applied to evaluate the variance homoscedasticity between the external calibration curve slope and the standard addition curve slope. The pooled variance *t*-test was applied when the slope variances were homogeneous ($F_{calculated} < F_{critical}$) and the unpooled variance *t*-test when the slope variances were heterogeneous ($F_{calculated} > F_{critical}$). The $t_{calculated}$-values were higher than the $t_{critical}$ one, showing the influence of the matrix on the FB1 and FB2 signals (Table 1). Thus, standard addition curves should be used to determine FB1 and FB2 in the matrices evaluated by the optimized method. After checking the matrix effect, the maize sample was used for method validation and the other matrices for method scope expansion.

3.5. Validation

3.5.1. Linearity

Initially, the F-test was performed to assess the area variance homoscedasticity obtained for FB1 and FB2 along the study range. The F-value was calculated by the ratio between the greatest and the smallest variance obtained for the seven concentration levels evaluated. Since the calculated F-values were higher than the critical F-value ($F_{(0.95,\ 2,\ 2)}$ = 19.00), the area variances of FB1 and FB2 were heterogeneous (Table 2). Therefore, the weighted least squares regression was applied using the ratio between the sum of the area variances and the area variance at each concentration level as weight (Table 2). Linear fit quality was evaluated by the determination coefficient (R^2) and the variance analysis.

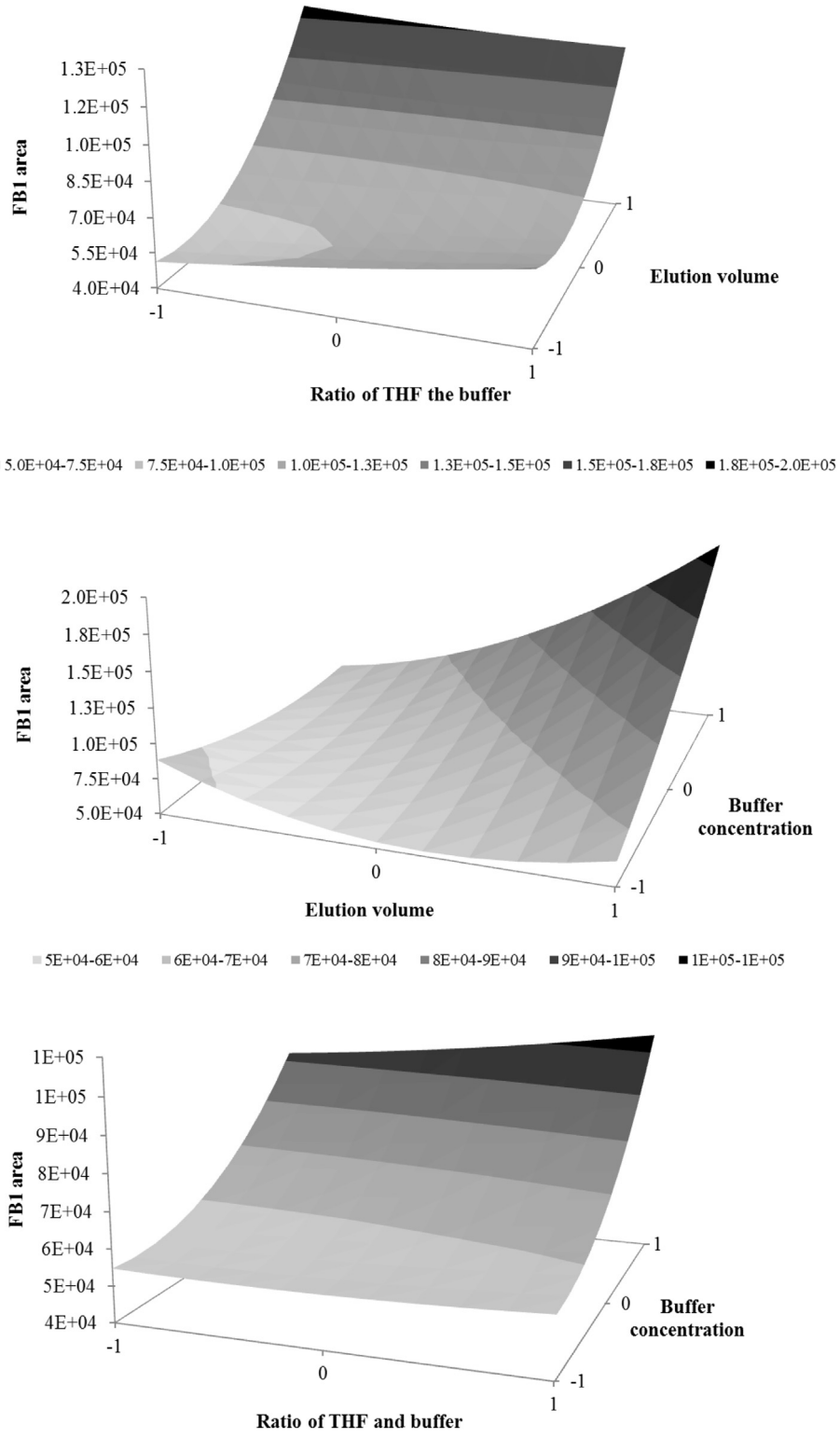

Fig. 1. Response surfaces for FB1 obtained from the 3^3 Box-Behnken design.

R^2-values were higher than 0.999, $F_{lack\ of\ fit}$-values were lower than the critical value ($F_{(0.95,\ 5,\ 14)} = 2.96$) and the $F_{significance}$-values were over a thousand times greater than the critical F-value ($F_{(0.95,\ 1,\ 19)} = 4.38$) (Table 2). Analyzing these three parameters, it was verified that the linear fit was appropriate for the concentration range studied. In addition, the R^2-values were similar to those described in the literature (Table 5S), where linearity was evaluated only by R^2-values.

3.5.2. Trueness and precision

Intra-day precision was evaluated by RSD for estimated total concentration, i.e., concentration in the naturally contaminated sample with standard addition at the following levels: 0.5, 1.0 and 1.5 times the MRL. To evaluate inter-day precision, the same procedure was performed for two more days. Since concentration values at the levels evaluated were higher than 500 $\mu g\ kg^{-1}$, according to Regulation EC/401/2006, the RSD-values should be

Table 1
Statistical comparison between external calibration curve and standard addition curves in different matrices.

Solvent or matrix	$^{*}F_{calculated}$	Intercept ± s	Slope ± s	R^2	$^{**}F_{calculated}$	$t_{calculated}$	$t_{critical}$
FB1							
Solvent	1319	$(1.5 \pm 0.9) \times 10^3$	$(10.1 \pm 0.1) \times 10^3$	0.9992	–	–	–
Maize	137	$(5.4 \pm 0.3) \times 10^4$	$(12.1 \pm 0.3) \times 10^3$	0.9995	3.15	27	0.05
Popcorn kernels	12	$(4 \pm 1) \times 10^4$	$(11.7 \pm 0.7) \times 10^3$	0.9881	16.83	19	0.05
White maize kernels	11	$(1.3 \pm 0.3) \times 10^4$	$(11.4 \pm 0.1) \times 10^3$	0.9980	2.01	31	0.02
Yellow maize grits	123	$(2.7 \pm 0.2) \times 10^4$	$(11.9 \pm 0.2) \times 10^3$	0.9999	1.52	32	2.02
Solvent or matrix	$^{*}F_{calculated}$	Intercept ± s	Slope ± s	R^2	$^{**}F_{calculated}$	$t_{calculated}$	$t_{critical}$
FB2							
Solvent	3151	$(4.3 \pm 0.7) \times 10^3$	$(20.9 \pm 0.3) \times 10^3$	0.9986	–	–	–
Maize	62	$(3.8 \pm 0.4) \times 10^4$	$(25.6 \pm 0.6) \times 10^3$	0.9984	4.43	33	2.05
Popcorn kernels	26	$(2.3 \pm 0.5) \times 10^4$	$(25.2 \pm 0.8) \times 10^3$	0.9998	1.14	22	2.02
White maize kernels	14	$(3 \pm 1) \times 10^3$	$(24.9 \pm 0.2) \times 10^3$	0.9999	1.62	52	2.02
Yellow maize grits	184	$(1.5 \pm 0.1) \times 10^4$	$(25.4 \pm 0.3) \times 10^3$	0.9999	1.06	52	2.02

s: standard deviation.
* $F_{calculated}$: F-value obtained by comparing response variances for each curve; $^{*}F_{(0.95,\ 2,\ 2)} = 19.00$.
** $F_{calculated}$: F-value obtained by comparing the external calibration curve slope and the standard addition curve slope; $^{**}F_{(0.95,\ 20,\ 20)} = 2.12$.

Table 2
Statistic results for linearity, CCα/LOD, CCβ/LOQ, precision and recovery.

Fumonisin	FB1	FB2	FB1 + FB2
Slope ± s	$(7.0 \pm 0.4) \times 10^3$	$(1.46 \pm 0.07) \times 10^4$	–
Intercept ± s	$(6.1 \pm 0.3) \times 10^4$	$(4.2 \pm 0.3) \times 10^4$	–
R^2	0.9983	0.9991	–
$F_{lack\ of\ fit}$	0.05335	0.04819	–
$F_{significance}$	4610	4443	–
CCα/LOD (μg kg^{-1})	514	176	–
CCβ/LOQ (μg kg^{-1})	594	210	–
$RSD_{intra\text{-}day}$ (%)	2–11	2–19	2–13
$RSD_{inter\text{-}day}$ (%)	8–10	2–20	6–13
Recovery $_{inter\text{-}day}$ (%)	89–102	86–106	88–103

$F_{(0.95,\ 2,\ 2)} = 19.00$; $F_{(0.95,\ 5,\ 14)} = 2.96$; $F_{(0.95,\ 1,\ 19)} = 4.38$; s: standard deviation.

at most 20% for intra-day and 30% for inter-day precision. All RSD-values obtained (Table 2) were lower than this recommendation and were in accordance with MSPD methods described in the literature (Table 5S).

Trueness was evaluated by recovery at the following concentration levels: 0.5, 1.0 and 1.5 times the MRL, for three days. Since concentration values at the levels evaluated were higher than 500 μg kg^{-1}, according to Regulation EC/401/2006, the recovery percentage should be between 70–110%. All recoveries obtained (Table 2) were within this recommended range and were in accordance with MSPD methods described in the literature (Table 5S).

3.5.3. Decision limit, detection capability, detection and quantification limits

CCα/LOD and CCβ/LOQ were determined considering the contributions of the natural contamination of the maize and standard addition curve. Since the maize sample analyzed presented high contamination of FB1 and FB2, the CCα/LOD and CCβ/LOQ estimated (Table 2) were much higher than the LOQ reported in the literature (Table 5S). However, if a sample with a low natural contamination or blank were used, such values would decrease considerably. This was confirmed in the method scope expansion (Table 4).

3.5.4. Measurement uncertainty

Measurement uncertainty was estimated as described in Section 2.10.4. The concentrations of FB1 and FB2 determined by standard addition curves in a naturally contaminated maize sample

Table 3
Estimated concentrations of FB1 and FB2 in corn, their respective combined uncertainty and acceptability criteria for uncertainty.

MRL level added	*Estimated concentration (μg kg^{-1})			u_c (μg kg^{-1})			U_{max} (μg kg^{-1})		
	FB1	FB2	FB1 + FB2	FB1	FB2	FB1 + FB2	FB1	FB2	FB1 + FB2
0	546	178	724	108	68	128	270	94	362
0.5	1253	506	1759	186	142	234	298	116	405
1.0	1726	717	2443	262	192	324	330	139	453
1.5	2361	982	3344	375	282	469	383	172	530

* Estimated concentration of the naturally contaminated corn with different standard addition levels.

Table 4
Results obtained in the method scope expansion for popcorn kernels, white maize kernels and yellow maize grits.

Sample	Fumonisin	Slope ± s	Intercept ± s	R^2	$^{*}F_{lack\ of\ fit}$	$^{**}F_{significance}$	CCα/LOD (μg kg^{-1})	CCβ/LOQ (μg kg^{-1})	RSD (%)	Recovery (%)
Popcorn kernels	FB1	$(1.30 \pm 0.01) \times 10^4$	$(2.46 \pm 0.04) \times 10^4$	1.000	1.25	714,962	101	107	7–11	113
	FB2	$(2.89 \pm 0.06) \times 10^4$	$(1.76 \pm 0.06) \times 10^4$	0.9999	1.63	718,967	34	38	6–12	98
	FB1 + FB2	–	–	–	–	–	–	–	7–10	109
White maize kernels	FB1	$(12.72 \pm 0.06) \times 10^3$	$(6.6 \pm 0.5) \times 10^3$	0.9999	3.42	480,498	33	39	7–15	92
	FB2	$(2.90 \pm 0.04) \times 10^4$	$(4.6 \pm 0.2) \times 10^3$	0.9999	6.17	736,049	12	16	9–16	70
	FB1 + FB2	–	–	–	–	–	–	–	8–16	85
Yellow maize grits	FB1	$(12.1 \pm 0.8) \times 10^3$	$(23.9 \pm 0.1) \times 10^3$	1.000	0.07	36,887,030	100	102	3–8	101
	FB2	$(27.0 \pm 0.5) \times 10^3$	$(1.3 \pm 0.1) \times 10^4$	0.9999	1.97	243,845	40	32	1–8	87
	FB1 + FB2	–	–	–	–	–	–	–	2–8	96

* $F_{(0.05,\ 5,\ 7)} = 3.97$; $^{*}F_{(0.01,\ 5,\ 7)} = 7.46$.
** $F_{(0.05,\ 1,12)} = 4.75$.

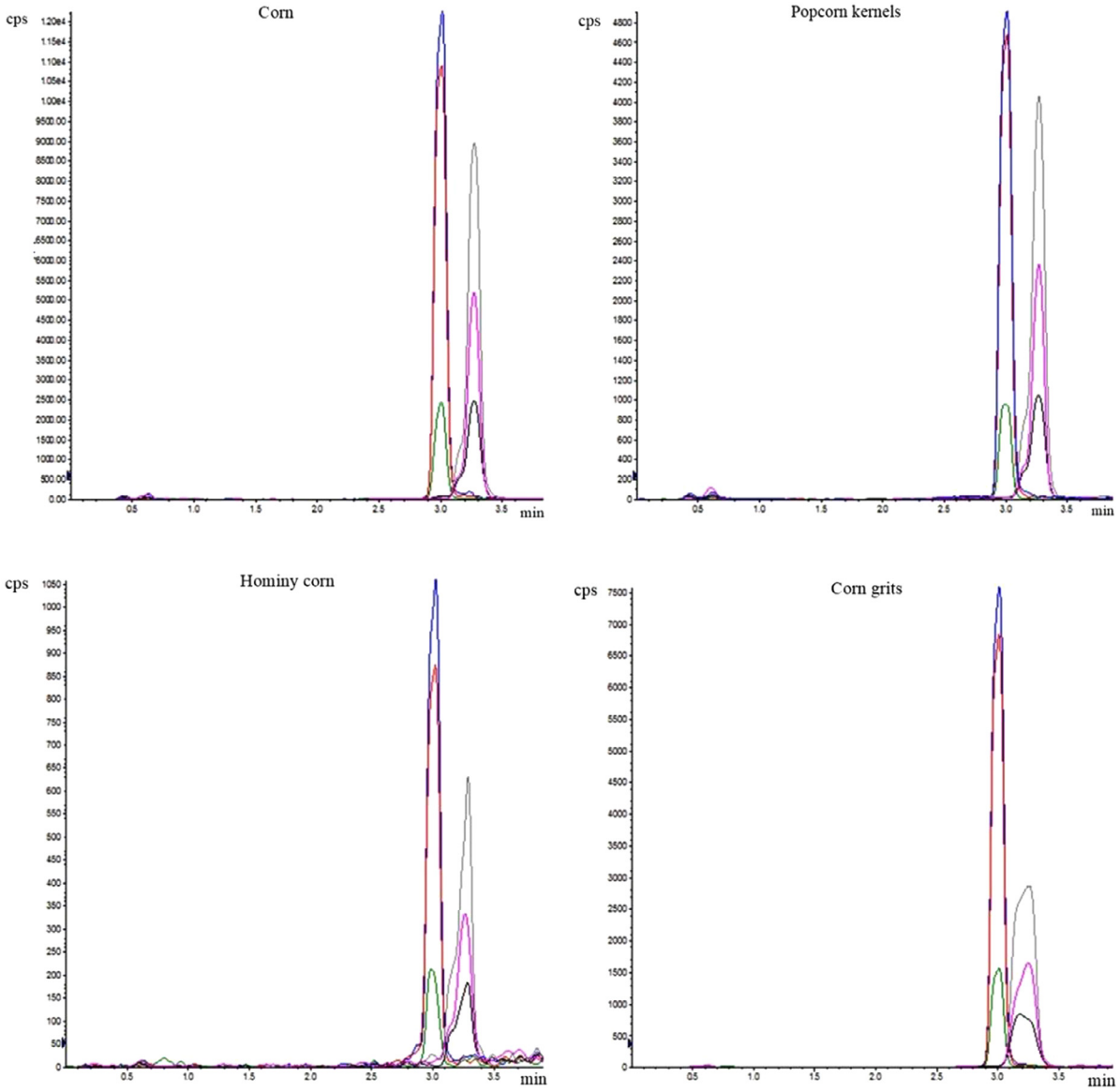

Fig. 2. Total ion chromatograms of FB1 (3 min) and FB2 (3.3 min) extracted from different types of maize in optimum conditions (Sections 2.5 and 2.8). FB1 transitions (from the highest peaks to the lowest): 722.5 → 352.4, 722.5 → 334.5 and 722.5 m/*z* → 318.3. FB2 transitions (from the highest peaks to the lowest): 706.5 → 336.4, 706.5 → 354.4 and 706.5 → 318.3 m/*z*.

with standard addition at levels 0, 0.5, 1.0 and 1.5 times the MRL as well as their respective u_c and acceptability criteria for uncertainty, i.e., U_{max} (Table 3). U_{max} was only not satisfactory for FB2 with standard addition, however, the legislation refers to the concentration sum of FB1 and FB2 and these were satisfactory for all concentration levels, therefore, the method is in accordance with legislation. It is noteworthy that the measurement uncertainty is one of the most important metrological parameters to assess the quality of an analytical result, because it allows comparison with other results and legal limits. None of the methods described in Table 5S have evaluated this variable.

3.6. Method scope expansion

After MSPD method validation, it was expanded to popcorn kernels, white maize kernels and yellow maize grits (Section 2.11). To evaluate the method performance for these matrices, linearity, intra-day precision, recovery, CCα/LOD and CCβ/LOQ were estimated. The results (Table 4) show excellent performance of the method for all matrices, so it can be applied to simultaneously determine FB1 and FB2 in the different types of maize. Martins et al. (2012) reported the contamination of these types of maize with fumonisins, however, to our knowledge, this work is the first study to develop and validate a method for the extraction and quantification of FB1 and FB2 in these matrices. Fig. 2 shows the total ion chromatograms for the samples analyzed.

4. Conclusion

The present work describes the optimization of a MSPD method for the extraction and clean-up of FB1 and FB2 in maize by multivariate approach and subsequent quantification by HPLC-MS/MS. This procedure allowed fumonisin extraction and clean-up in one step, using silica gel as dispersant and elution with 70% ammonium formate aqueous buffer (50 mmol L^{-1}, pH 9). This method was validated and met the international and national legislation prerequisites, and validation parameter values obtained were in accordance with other works described in the literature. After evaluation of

linearity, precision, trueness, CCα/LOD and CCβ/LOQ, it was verified that the method can also be applied in the determination of FB1 and FB2 in popcorn kernels, white maize kernels and yellow maize grits .

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.04.091.

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