



Determination of multi-mycotoxins in cereals and of total fumonisins in maize products using isotope labeled internal standard and liquid chromatography/tandem mass spectrometry with positive ionization



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ABSTRACT

Mycotoxins are secondary fungi metabolites present in foods that cause adverse effects in humans and animals. The aims of this work were to develop and validate a multi-mycotoxin method to determine the presence of aflatoxins, citreoviridin, deoxynivalenol, fumonisins ochratoxin A, zearalenone and some metabolites/derivatives in rice, maize-based products and wheat-based products, and a method to determine total fumonisins (free and bound/hidden forms) in maize-based products. The validated multi-mycotoxin method was based on extraction with acidified acetonitrile and LC-ESI⁺-MS/MS analysis, with LOQs ranging from 0.5 to 121 µg/kg, and proved to be suitable for the multi-mycotoxin analysis in wheat, maize and rice products. Bound/hidden fumonisins were determined after extraction of the free forms using the multi-mycotoxin method, followed by a basic hydrolysis of the unextracted bound/hidden and solid-liquid extraction with low temperature purification (SLE-LTP). The hydrolysis efficiency of the bound/hidden extraction procedure was investigated by analyzing a maize reference material and showed recoveries ranging from 75% (HFB2) to 108% (HFB1). The use of isotope internal standards was crucial for mycotoxins quantification in maize meal and wheat flour, while for rice, external calibration and matrix matched curves gave satisfactory results. All 55 samples of wheat-based products analyzed were contaminated with at least one mycotoxin and 16% of 44 rice samples were also contaminated. The most prevalent mycotoxins were DON and ZON in wheat-based products.

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1. Introduction

Mycotoxins are secondary fungi metabolites that can contaminate a wide range of foods [1], and may lead to the development of adverse effects, both in humans and animals [2,3]. The most common classes of mycotoxins are aflatoxins, trichothecenes, especially deoxynivalenol, fumonisins, zearalenone and ochratoxin A, produced mainly by the genera *Aspergillus*, *Penicillium* and *Fusarium* [4]. Their chemical structures are extremely variable, leading to different toxic effects, including genotoxicity, immunotoxicity and nephrotoxicity [2,5]. Cereals are staple foods in diets around the world, and can be a major source of dietary exposure to mycotoxins [1,6,7]. Small grains, such as rice and wheat, are susceptible

to deoxynivalenol, ochratoxin A and zearalenone contamination, and fumonisins are the main mycotoxins found in maize and maize products [8].

In addition to the parental mycotoxin forms, food can also contain derivative/transformed compounds produced during food processing or as a result of plant/animal metabolism [9]. For instance, fumonisins can covalently bind to matrix macroconstituents during food thermal processing (e.g., linkage between fumonisin tricarboxylic acids – TCA and starch, or proteins) [10–12]. The bound-fumonisins are not detected by common analytical methods, which may underestimate fumonisin levels in food [13]. The bound-fumonisin can be hydrolyzed under alkaline conditions, such as in nixtamalization processes (e.g. production of tortillas), releasing the hydrolyzed forms (HFB1) that can be analyzed by routine methods [11,14]. Fumonisins can also be linked to matrix macroconstituents without heat treatment by an associative mechanism, and the products are known as hidden fumonisins or non-covalently bound fumonisins [13].

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In general, methods used to analyze mycotoxins are based on extraction with organic solvents, clean-up (solid phase extraction, immunoaffinity columns), followed by concentration/dilution steps [15–19]. Determination methods include HPLC-FD or UV, GC-FID, and LC-MS or –MS/MS [15,17,20–22]. Matrix matched calibration, internal calibration and sample dilution are procedures commonly used to compensate matrix effects observed in LC-MS/MS analyses [23]. The use of isotope labeled internal standards seems to be the best tool to cope with matrix effects and ensure reliable results. However, the high costs and limited availability of isotopically labeled internal standards restrict its application [24]. Stable isotope dilution in mycotoxin analyses has been reviewed by Rychlik and Asam [25], and successfully applied by several researchers [16,23,26,27].

The aim of this study was to optimize and perform a complete validation of a method for the simultaneous analysis of aflatoxins (AFB1, AFB2, AFG1 and AFG2), citreoviridin (CTV), deoxynivalenol (DON), 15-acetyldeoxynivalenol (15AcDON), 3-acetyldeoxynivalenol (3AcDON), deoxynivalenol-3-glucoside (D3G), deepoxydeoxynivalenol (DOM), fumonisins (FB1, FB2 and FB3) and their hydrolyzed forms (HFB1, HFB2 and HFB3), ochratoxin A (OTA), zearalenone (ZON) and α -zearalenol (α -ZOL) in maize-derived products, rice and wheat-derived products, using isotope labeled internal standards, and LC-MS/MS. To the best of our knowledge, this is the first study reporting determination of total fumonisin (free and bound forms) together with the determination of other mycotoxins.

2. Materials and method

2.1. Chemicals and reagents

HPLC-grade acetonitrile (ACN), ethyl acetate (AcOEt) and methanol (MeOH) were purchased from Merck (Darmstadt, Germany); HPLC-grade toluene was obtained from Mallinckrodt Baker (Phillipsburg, USA); formic acid from Sigma-Aldrich (St. Louis, MO, USA); acetic acid from J.T Baker (Phillipsburg, USA); ammonium formate and ammonium acetate from Fluka (Buchs, Switzerland); anhydrous sodium sulphate, potassium hydroxide (KOH) and hydrochloric acid (HCl) from Vetec (Rio de Janeiro, Brazil); ultrapure water obtained through a Milli-Q purification system, and the syringe filters used were Millex™, both from Millipore (Millipore, Bedford, MA, USA).

Standards of AFB1 (99.0%), AFB2 (99.0%), AFG1 (99.0%), AFG2 (99.5%) and d1-DON (97.5%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). CTV (97.0%) was acquired from Enzo Life Sciences International Inc. (Farmingdale, NY, USA). 15AcDON (98.8%), 3AcDON (99.4%), D3G (96.0%), DOM (98.0%), DON (99.4%), FB1 (97.6%), FB2 (99.0%), FB3 (98.5%), HFB1 (98.4%), ZON (99.4%), α -ZOL (98.7%), ($^{13}\text{C}_{17}$)-AFB1 (99.0%), ($^{13}\text{C}_{17}$)-AFG1 (99.0%), ($^{13}\text{C}_{18}$)-ZON (99.2%), ($^{13}\text{C}_{20}$)-OTA (98.7%) and ($^{13}\text{C}_{34}$)-FB1 (97.8%) were obtained from Biopure (Tulin, Austria). Stock solutions of aflatoxins were prepared in toluene-ACN (9:1), of CVT in ethyl acetate, of OTA in toluene-acetic acid (99:1), and of fumonisins in ACN-water (50:50). Stock solutions of the remaining compounds were prepared in ACN. Concentrations of aflatoxins, OTA, ZON, DON, 3AcDON, 15AcDON and CTV solutions were checked monthly, using UV spectrophotometry. Wavelength and molar absorptivity used to check mycotoxins concentration are shown in Table S1 (Supplementary data) [28]. Parameters for 3AcDON and 15AcDON were obtained from Krska et al. [29], and for CTV from Rocha, Resck and Caldas [30]. Solutions were considered valid when a maximum of 3% variation was estimated in relation to the first check [28].

Maize multi-mycotoxin reference material TR-MT100 (MTC-9999E) containing aflatoxins, fumonisins, DON, OTA and ZON was

purchased from Trilogy Analytical Laboratory (Washington, MO, USA).

2.2. LC-MS/MS conditions

LC-MS/MS analysis was performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan) coupled to a 4000 Qtrap triple-quadrupole mass spectrometer (SCIEX, Framingham, MA, USA), fitted with a Turbo Ion Spray electrospray ionization (ESI) source. System operation and data acquisition were controlled by Analyst® (V 1.5.2) software (SCIEX).

The analyte-dependent MS/MS parameters were optimized by direct infusion of mycotoxin solutions (200–800 ng/mL; dissolved in MeOH/H₂O, containing the selected additive) into the mass spectrometer, at a flow rate of 10 $\mu\text{L}/\text{min}$. The best mobile phase additive was chosen after testing the effects of acetic acid (0.1%), formic acid (0.1%), ammonium formate (5 mM) or ammonium acetate (5 mM) in the preparation of aflatoxins, fumonisins, OTA, CTV, HFB1, DON, 3AcDON, 15AcDON, DOM, D3G and ZON solutions. The additive that gave the best ionization results was also tested in different concentrations. ESI-MS/MS was performed in the multiple reaction monitoring (MRM) mode and both positive (ESI⁺) and negative (ESI[−]) polarities were evaluated. Declustering potential (DP), collision energy (CE) and collision cell exit potential (CXP) were optimized for the three most abundant transitions for each analyte. In most cases, the two most abundant transitions were used in the method (quantifier and qualifier).

Ion source parameters were automatically optimized using flow injection analysis of 80 ng/mL D3G standard solution (0.8 mL/min), which was the less sensitive compound in the preliminary tests. The optimal conditions of the mass spectrometer ion source were: entrance potential at 10 V, ion source at 500 °C, ion source gas 1 and 2 at 50 (GS1) and 40 psi (GS2), ion spray voltage at 5500 V, curtain gas at 12 psi, and collision gas at medium.

Chromatographic separation was performed with a Gemini C18 analytical column (150 \times 4.6 mm, 5 μm) preceded by a C18 security guard cartridge (4.0 \times 3.0 mm, 5 μm), both from Phenomenex® (Torrance, CA, USA). The column was kept at 40 °C and a flow rate of 0.8 mL/min was used. The mobile phase consisted of a gradient of water (A) and methanol (B), both with the additive chosen in the analyte-dependent MS/MS parameters optimization procedure. The gradient started at 40% B; held for 1 min; increased to 86% B in 11 min, held for 2 min; increased to 95% B in 2 min and held for 4 min. The system was equilibrated at the initial condition for 6 min between consecutive runs.

2.3. Multi-mycotoxin extraction optimization

Rice, maize meal and wheat flour were used as model matrix during the development and validation of the analytical procedure. Samples, purchased at local stores, were quartered, ground (blender), homogenized, and sieved (18 mesh) to ensure the uniformity of processing. The homogenized samples were then transferred to polyethylene bags and stored at room temperature for further use. The analytical procedure was based on a solid-liquid extraction (SLE) using an ultrasonic bath, followed by centrifugation and filtration prior to injection, as described below. Five grams of the homogenized samples were weighted into a 50 mL falcon tube, and 20 mL of a solvent mixture were added. The tube was agitated for 20 s (vortex), submitted to the ultrasonic bath for 15 min, and centrifuged at 3500 rpm/10 min/18 °C. 1 mL of the supernatant was evaporated under vacuum (Centrivap Vacuum Concentrator System – LABCONCO/Germany), redissolved in 1 mL methanol:water (40:60), and filtered through a syringe filter (0.45 μm) before injection in the LC-MS/MS.

When isotope labeled internal standards were used, an aliquot of 180 μL of the extract was transferred to an insert and combined with 20 μL of the internal standard working solutions, at the following concentrations: ($^{13}\text{C}_{17}$)-AFB1 = 25.7 ng/mL, ($^{13}\text{C}_{17}$)-AFG1 = 25.9 ng/mL, ($^{13}\text{C}_{18}$)-ZON = 2 $\mu\text{g/mL}$, ($^{13}\text{C}_{20}$)-OTA = 414 ng/mL, ($^{13}\text{C}_{34}$)-FB1 = 753 ng/mL and d1-DON = 5 $\mu\text{g/mL}$. Isotope dilution assay was used for internal calibration for all mycotoxins, except CTV (no isotope available). ($^{13}\text{C}_{17}$)-AFB1 was used for quantification of AFB1 and AFB2; ($^{13}\text{C}_{17}$)-AFG1 for AFG1 and AFG2; ($^{13}\text{C}_{18}$)-ZON for ZON and α -ZOL; ($^{13}\text{C}_{20}$)-OTA for OTA; ($^{13}\text{C}_{34}$)-FB1 for fumonisins and their hydrolyzed forms; and d1-DON for D3G, DON, DOM, 15AcDON and 3AcDON.

In order to select the best solvent mixture for the extraction procedure, three different compositions were tested for each matrix: A = ACN:H₂O (80:20 0.1% formic acid), B = ACN:H₂O (80:20), and C = MeOH:H₂O (80:20). Recovery tests were carried out in triplicate, for each solvent composition, at concentrations ranging from 2.2 $\mu\text{g/kg}$ (AFB1, AFG1) to 224 $\mu\text{g/kg}$ (15AcDON, 3AcDON). Matrix matched standard curves were prepared at concentrations between 0.96 and 1600 $\mu\text{g/kg}$. The optimized method was submitted to validation.

2.4. Multi-mycotoxin method validation

The method was validated according to the parameters established by Brazilian government [28]. For this purpose, the extraction procedure was miniaturized, using 0.5 g of sample and the same sample/solvent ratio (1 g/4 mL). Selectivity was evaluated by analyzing the LC-MS/MS chromatograms of blank and fortified samples, checking for interferences eluting at the same retention time as the mycotoxins of interest. Matrix effect was evaluated for each analyte at five concentration levels, six replicates at each level, using both internal and external calibration; % matrix effect in each case was estimated by the ratio between the average instrument response (areas) of matrix matched standards and neat solution standards. Signal suppression/enhancement above $\pm 20\%$ was considered a significant matrix effect.

Linearity was checked by analyzing the same set of samples used in the matrix effect evaluation. The linear parameters of the regression were estimated by the ordinary least squares method, the presence of outliers verified by the Grubbs test, the homogeneity of variances by Cochran test, and the coefficient of determination (R^2) and significance of the regression obtained using ANOVA. For heteroscedastic data, different weighting factors were tested ($1/x$, $1/x^2$, $1/y$, $1/y^2$, $\ln x$ and $\ln y$) and the one that produced the lowest sum of relative errors was chosen for the regression. Calibration curves (five points) ranged from LOQ to 5xLOQ for D3G and α -ZOL, from LOQ to 10xLOQ for DOM, FB3 and HFB1, and from LOQ to up 100xLOQ for 15AcDON, 3AcDON, AFB1, AFB2, AFG1, AFG2, CTV, DON, FB1, FB2, OTA and ZON. For isotope internal calibration, weighted/ordinary calibration curves were obtained for each analyte by plotting the compound concentrations versus the relative areas, which was the ratio between the analyte peak area and the corresponding isotope internal standard peak area.

Recoveries of the analytes in each matrix were evaluated by fortifying the samples at 5 different levels and comparing the areas of the spiked samples with the area of the matrix matched external standards, expressed as%. The experiment was performed on the same day by the same analyst. For isotope internal calibration, recoveries were obtained by comparing relative areas of the spiked samples with relative areas of the matrix matched set, also expressed as%. Repeatability was expressed as the relative standard deviations (%RSD_r). Intermediate precision was evaluated by the analysis of samples fortified at five concentration levels, by the same analyst, on different days (%RSD_p). Due to the limited amount of standards available, 3 replicates were made with samples forti-

fied with D3G, α -ZOL, HFB1 and DOM, while 6 replicates were done for DON, 3AcDON, 15AcDON, AFs, fumonisins, OTA, CTV and ZON, but some outliers were removed. Hence the number of replicates in each case varied from 3 to 6.

Trueness was evaluated by analyzing five replicates of the TR-MT100 multi-mycotoxin maize reference material. For each mycotoxin, the LOQ was defined as the lowest level for which the method was satisfactorily validated (recovery between 80 and 120% and RSD_r $\leq 20\%$ and RSD_p $\leq 25\%$). All replicates used for validation were prepared from independent working solutions. Spiked samples were left for 2 days at room temperature in the fume hood, protected from light, to allow solvent evaporation and analyte-sample equilibration.

2.5. Preparation of hydrolyzed fumonisin standards

Since standards of hydrolyzed fumonisin B2 and B3 were not commercially available, they were prepared from pure fumonisins standards, based on the procedure of Dall'Asta et al. [31]. Solutions of FB1, FB2 and FB3 were evaporated to dryness under vacuum, redissolved in KOH 2 M (1 mL/50 μg standard) and allowed to react in a thermal bath (60 °C) under constant agitation, during 0, 15, 30, 60, 120 and 180 min. After hydrolysis, the mixture was extracted three times with acetonitrile (1 mL/50 μg standard), agitated (vortex), and centrifuged (1500 rpm, 15 °C, 5 min). Organic phases were pooled, evaporated, redissolved in ACN:H₂O (50:50), filtered through a syringe filter (0.45 μm), and injected in the LC-ESI⁺-MS/MS to determine the yield of the reaction. The optimizing experiments were conducted in triplicate.

2.6. Total fumonisin extraction procedure

Total fumonisins concentrations were obtained by determining both free and bound/hidden fumonisin forms. Free fumonisins were extracted from maize meal using the multi-mycotoxin procedure (Section 2.3). Bound forms are fumonisins covalently bound to matrix constituents (starch or proteins) after a heat treatment and hidden fumonisins are formed by non-covalent interactions with matrix constituents, without heat treatment. Both bound and hidden forms are separated from matrix constituents after the alkaline hydrolysis procedure described below, and determined as hydrolyzed fumonisins (HFB1, HFB2 and HFB3), which were converted to FB1, FB2 and FB3 using molar mass ratios. Results of total fumonisins were expressed as the sum of free and bound/hidden fumonisins.

After the multi-mycotoxin extraction procedure, maize meal sample solid residues were completely dried in the lyophilizer (Lio-bras/K105), and submitted to the procedure by Dall'Asta et al. [31]. In summary, 1 g of lyophilized sample was transferred to a 50 mL falcon tube, 10 mL of KOH (2 M) added under constant stirring (vortex) and the mixture allowed to react in a thermal bath (60 °C) under constant agitation, during 60 min. The mixture was extracted three times with 10 mL of ACN, vortexed, centrifuged (1500 rpm, 15 °C, 5 min), the organic phases pooled and evaporated to dryness under vacuum for LC-MS/MS analysis. However, this procedure required a long time to evaporate the organic phase, which still contained some aqueous basic solution, and yielded low recoveries (<50% for most analytes; data not shown).

In order to improve the extraction procedure, solid-liquid extraction with low temperature purification was used (SLE-LTP). After the hydrolysis step described above, 10 mL of ACN was added to the falcon tube, the pH adjusted to 3.0 ± 0.5 , the tube taken to sonication for 15 min, centrifuged (3500 rpm/5 min/12 °C), and left in the freezer (−18 °C) for 12 h. After this time, the organic phase (ACN) remained liquid, while the aqueous phase froze at the bottom of the falcon tube. The liquid supernatant was removed, filtered in

filter paper containing anhydrous sodium sulfate and evaporated under vacuum. The residues were dissolved in 1 mL MeOH:H₂O (40:60), filtered through a syringe filter (0.45 µm), and injected in the LC–MS/MS.

2.7. Analysis of mycotoxins in cereal products

A total of 99 samples of rice ($n = 44$) and wheat-based food products ($n = 55$) were purchased at retail stores in Brasília (Federal District), from May 2015 to February 2016. At least 500 g/sample was collected, except for crackers (50 g minimum per sample). Cereals samples were quartered, grounded (blender), homogenized, sieved (18 mesh) and stored in polyethylene bags at room temperature until analyzed using the validated multi-mycotoxin method. Three fortified samples (at the intermediate level) were included in each extraction batch for internal quality control. Maize-product samples were not analyzed in the context of this study.

3. Results and discussion

3.1. Optimization of LC–MS/MS

Direct infusion of mycotoxins solutions in positive mode showed sufficient formation of the ammonium adducts $[M+NH_4]^+$ for 15AcDON (5 mM ammonium formate and 5 mM ammonium acetate), DOM (5 mM ammonium formate), D3G (5 mM ammonium formate) and HFB1 (5 mM ammonium formate). However, except for D3G, intensities were lower than those obtained for the protonated adducts $[M+H]^+$.

In the negative mode, only 3AcDON, DOM, DON and D3G yielded good responses when using 5 mM ammonium acetate, while ZON was successfully ionized using all four additives tested. The formation of the acetate adduct was only observed for 3AcDON, DOM and DON. Responses of all product ions formed in negative mode were significantly lower than in positive mode using the same additives. These results agree with Boevre et al. [32], who also observed better ionization results for DON, D3G, 3AcDON, 15AcDON, ZON, α -ZOL and DOM in positive mode. However, other studies reported higher ionization in negative mode, mainly for ZON [18,23,33,34]. Considering the overall intensities obtained in both polarities for all analytes, positive mode was chosen for the LC–MS/MS multi-mycotoxin method.

Fig. 1 shows the responses obtained in positive mode for the two most intense transitions for each analyte, and the additive used in the mobile phase. Higher intensities were observed for all compounds using ammonium acetate, except for fumonisins, the major mycotoxin in maize products. Overall, the use of ammonium formate as additive produced reasonable responses for all analytes, and was selected in this study. However, during the optimization of the chromatographic conditions, it was found that the addition of 0.1% formic acid to the mobile phase was needed to improve the chromatography of fumonisins [35–37].

The effect of different concentrations of ammonium formate in the analyte response was also evaluated. Mycotoxins solutions (AFB1, CTV, OTA, DON, FB1 and ZON) containing 0.1% formic acid and 0.1, 0.25, 0.5, 1 and 5 mM ammonium formate were infused into the mass spectrometer and ionization enhancement or suppression observed. Results showed that 5 mM ammonium formate caused ionization suppression for all analytes, except AFB1, and 1 mM ammonium formate gave the best overall results (data not shown).

Table 1 summarizes the optimized ESI⁺ parameters for the mycotoxins and isotope internal standards obtained by direct infusion of the analyte solutions diluted in MeOH:H₂O (50:50),

containing 0.1% formic acid and 1 mM ammonium formate, which was the final conditions of the mobile phase. For all analytes, the protonated forms $[M+H]^+$ were monitored, except for D3G, for which the ammonium adduct $[M+NH_4]^+$ was selected. The DON acetylated isomers (15AcDON and 3AcDON) coeluted under the chromatographic conditions used. Acetonitrile and methanol, both containing 0.1% formic acid and 1 mM ammonium formate, were also tested as the organic component of the mobile phase. Although acetonitrile increased the sensitivity for most compounds (aflatoxins, OTA, fumonisins), the presence of methanol was essential to improve the peak shapes of DON and its derivatives. Tests with different injection volumes (10–100 µL, $n = 5$ in each case) showed that peak areas increased proportionally with injection volumes up to 50 µL and, from this point on, peak width began to increase. Thus, as a compromise between sensitivity and peak integration quality, 25 µL was selected as injection volume.

3.2. Multi-mycotoxin analysis – optimization and method validation

In the optimization of the extraction procedure, as in most studies, the best results were obtained using acidified ACN as the extraction solvent, and was chosen for the validation procedures (data not shown). Except for fumonisins in maize, for which a blank sample was not available, the chromatograms of blanks did not show any interfering peaks eluting in the same retention times of the analytes under evaluation, indicating satisfactory selectivity of the method. Validation results are shown in Tables 2–4. For maize meal (Table 2), matrix effects using external calibration showed ion suppression of over 30% for eight analytes, including aflatoxins (–54.9 to –72.2%), and a large ion enhancement for HFB1 (70%) (Table 2). When using isotope internal calibration, matrix effects were in the range of –23.3 to 21.7% for all analytes, except for HFB1 (41.4%). The behavior of the residues of the analytical curves obtained by the least squares method showed heteroscedasticity ($C_{\text{calculated}} < C_{\text{critical};5;6}$) for all mycotoxins, except D3G and α -ZOL (homoscedastic, ordinary least squares adjustment). For the heteroscedastic compounds, the best weighting factors found were $1/y$ (15AcDON and ZON), $1/x$ (DON, 3AcDON, DOM, FB1 and FB2) and $1/x^2$ (AFB1, AFB2, AFG1, AFG2, CTV, FB2, HFB1 and OTA) (Table 2). Coefficients of determination (R^2) were higher than 0.98, regressions were significant ($p < 0.05$), and there was no lack-of-fit for the regressions used in calibration procedures (data not shown).

LOQs ranged from 0.5 to 1.2 µg/kg for aflatoxins in maize meal, being higher for DON and its derivatives (up to 121 µg/kg for 15AcDON). Recoveries ranged from 91.4% (α -ZOL) to 116.1% (FB2), considering all levels of fortification and using matrix matched curves and isotope internal standard, except for CTV for which no isotope standard was available. Precision was evaluated both as repeatability (r) and intermediate precision (p). RSD_r ranged from 7.5% (FB2) to 15.6% (CTV), and RSD_p from 10.9% (DON) to 27.8% (α -ZOL), all within the acceptable range. Recoveries and precision obtained at each level of fortification are shown in Table S2 (Supplementary data).

Validation results obtained for rice are shown in Table 3. Matrix effects were less pronounced for rice compared to maize meal, and although the use of isotope internal standard compensated the effect for most analytes, external calibration was considered satisfactory for all analytes ($\pm 20\%$), except AFB2 (–21.9%, Table 3). Analytical curves showed heteroscedastic behavior for all mycotoxins ($C_{\text{calculated}} < C_{\text{critical};5;6}$), with $1/x^2$ the best weighting factor found for seven of the analytes (Table 3); R^2 were higher than 0.98, regressions were significant ($p < 0.05$), and there was no lack-of-fit. The lowest LOQs were also found for aflatoxins (0.5–1.6 µg/kg) and the highest LOQ for D3G (84 µg/kg). Recoveries were considered acceptable for all analytes except HFB1 (55.2%; $n = 3$), which

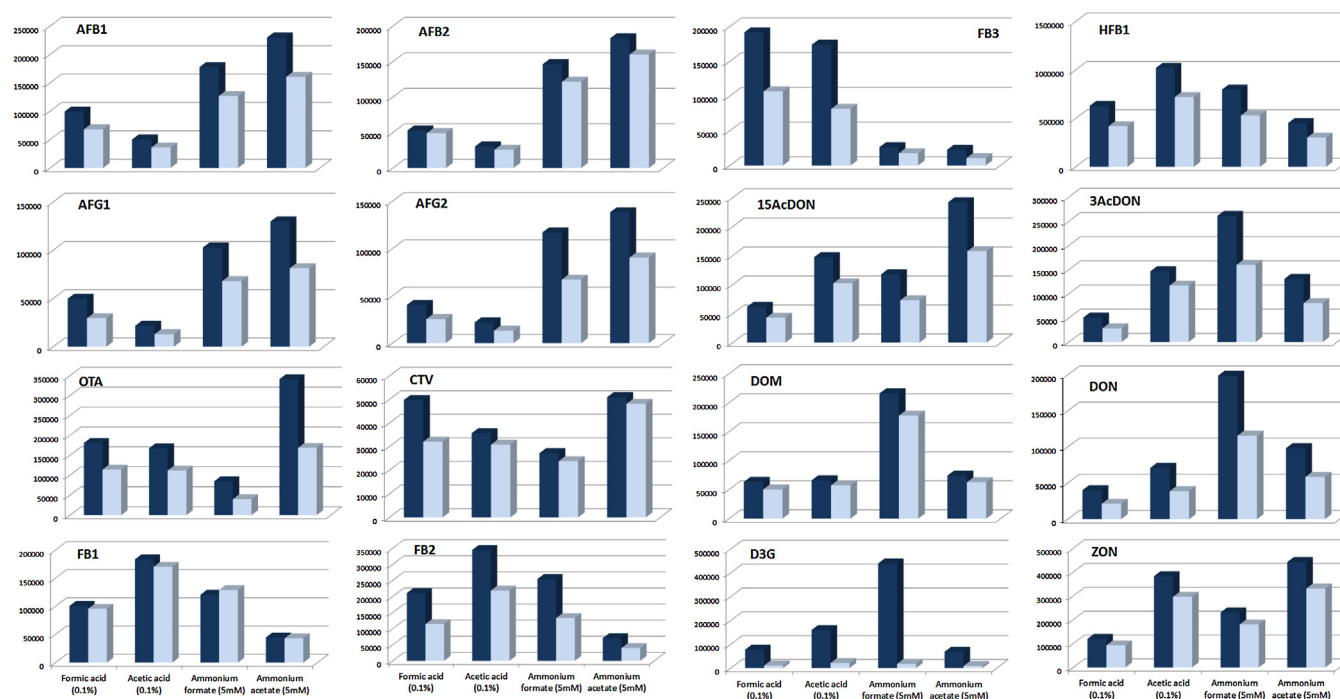


Fig. 1. Intensity of selected transitions (quantifier, darker bar, and qualifier) after direct infusion into the mass spectrometer in positive ionization mode using different additives (0.1% Formic acid; 0.1% Acetic acid; 5 mM Ammonium formate; 5 mM Ammonium acetate).

is not relevant for rice. Repeatability (RSD_r) exceeded 20% for three analytes (up to 25.6% for HFB1), and RSD_p were acceptable for all mycotoxins ($\leq 25\%$; $n = 3$ –6 each level; 2 days), except HFB1 (29.9%). Complete recoveries and precision data obtained for rice are shown in Table S3 (Supplementary data).

Table 4 shows the validation parameters obtained for wheat flour. When external calibration was used, signal suppression was observed for most compounds (up to 53% for AFB2), and signal enhancement of almost 40% was observed for DOM. The use of isotope internal standard compensated the matrix effects in all cases, but was still important for AFB2 (–32.3%) and DOM (53.3%). The analytical curves were homoscedastic for only D3G and α -ZOL; for the heteroscedastic compounds the best weighting factors were either $1/x^2$ or $1/x$ (Table 4). R^2 were higher than 0.98, regressions were significant, with no lack-of-fit. The lowest LOQs were found for aflatoxins (0.6–1.6 $\mu\text{g/kg}$), and the highest for the acetylated forms of DON (72 and 80 $\mu\text{g/kg}$). Recoveries ranged from 80.1% (3AcDON) to 117.1% (α -ZOL). Repeatability was higher than 20% for only 15AcDON (21.1%) and intermediate precision was below 25% in all cases. Recoveries and precision obtained for each level of fortification in wheat flour are shown in Table S4 (Supplementary data).

Trueness of the validated multi-mycotoxin method was evaluated through the analysis of maize multi-mycotoxin reference material, naturally contaminated with aflatoxins (total aflatoxin level reported, AFs), fumonisins (total fumonisin level reported, FBs), DON, OTA and ZON (Table 5). The results were within the reported uncertainty range for all mycotoxins, except for zearalenone, for which the level found (238.1 $\mu\text{g/kg}$) was slightly below the lower bound of the uncertainty range (239.4 $\mu\text{g/kg}$). Considering the standard deviation ranges also reported for the reference material, the results found were in the first range for fumonisins, in the second range for aflatoxins, deoxynivalenol and ochratoxin A, and in the third range for zearalenone (Table 5).

Heteroscedastic behavior was found in this study for all compounds for which the range of the calibration curves was wider (10 or 100xLOQ), but not for those with a narrow working range





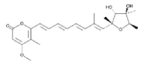
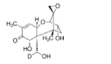
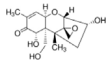
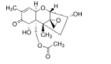
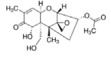
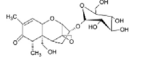
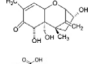
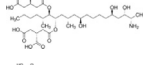
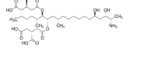
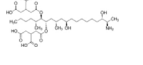
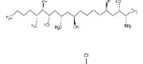
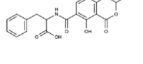
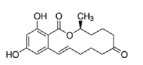
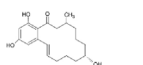
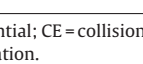
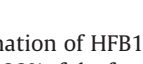
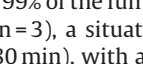
(5xLOQ; D3G and α -ZOL), except rice. The matrix effect found for almost all analytes was expected, since the extraction procedure did not include any clean-up or dilution of the extracts. The use of stable isotope internal standard was essential to compensate these effects for maize meal and wheat flour, but may be less important when determining mycotoxins in rice. Matrix effect comparisons using stable isotope internal standard or external calibration have not been presented in most studies [16,26,27], except for Varga et al. [23], who confirmed our findings for aflatoxins, fumonisins, DON, OTA and ZON in maize, in addition to the trichothecenes T2 and HT-2.

Overall, the LOQs found in this study were similar to those reported by Sulyok et al. [17], Malachová et al. [24], Liao et al. [16], Varga et al. [23] and Frenich et al. [19] for cereals and derived products using LC-ESI⁺-MS/MS. The limits obtained for DON, 3AcDON, 15AcDON and ZON were higher than those reported by some authors for maize or wheat (0.7–12 $\mu\text{g/kg}$) [19,23,24,32], although, with exception of the work by Malachová et al. [24], the LC-MS/MS methods were not validated (precision and recovery) at the reported limits. Furthermore, lower LOQs for ZON and DON were obtained in a separated chromatographic run using negative ionization mode [24], or the method included a fewer groups of mycotoxins such as zearalenone, trichothecenes and their metabolites [32]. Liao et al. [16] validated a LC-MS/MS method for 26 mycotoxins in rice, maize and wheat, including aflatoxins and OTA at 10 $\mu\text{g/kg}$, and DON, ZON and fumonisins at 100 $\mu\text{g/kg}$. However, the authors reported much lower LOQs (0.3–0.9 for aflatoxins and OTA, and 8.3–10.4 for DON and ZON). Hence, comparison of LOQs among studies should consider the different ways in which this parameter is defined.

3.3. Preparation of hydrolyzed fumonisins standards

In this study, the reaction time for hydrolysis of fumonisin standards under 60 °C and basic conditions (2 M KOH) was investigated. Hydrolysis efficiency was determined using quantification of both the remaining parental fumonisin standards (FB1, FB2 and FB3) and

Table 1Optimized ESI⁺- MS/MS parameters and chromatographic retention times used for the multi-mycotoxin LC-MS/MS analysis of cereals and derived products.

Analyte	Structure	DP (V)	Transition (<i>m/z</i>)		CE (V)	CXP (V)	Retention time (min)	Ion ratio (RSD; %)
U-(¹³ C ₁₇)-AFB1		86	330	→	301	35	8.0	1.6 (5.2)
AFB1			330	→	251	55		
AFB2			313	→	285	33		
AFB2		111	313	→	241	53	7.5	1.3 (11.2)
			315	→	287	37		
			315	→	259	41		
U-(¹³ C ₁₇)-AFG1		81	346	→	257	39	6.9	1.7 (8.8)
AFG1			346	→	212	57		
AFG2			329	→	243	39		
AFG2		91	329	→	311	31	6.3	1.3 (10.5)
			331	→	313	35		
			331	→	245	43		
CTV		71	403	→	315	13	12.4	1.4 (18.7)
			403	→	139	33		
						10		
d1-DON		56	298	→	249	15	3.4	2.0 (9.7)
			298	→	203	23		
						14		
DON		76	297	→	249	17	3.4	2.0 (10.4)
			297	→	203	23		
						16		
15AcDON		81	339	→	321	13	5.6	1.9 (6.8)
			339	→	137	17		
						10		
3AcDON		71	339	→	231	17	5.7	1.8 (5.8)
			339	→	203	23		
						16		
D3G		41	476	→	297	19	3.0	1.8 (12.2)
			476	→	459	11		
						16		
DOM		66	281	→	233	17	4.5	1.2 (11.8)
			281	→	215	19		
						14		
U-(¹³ C ₃₄)-FB1		106	756	→	374	53	8.0	1.0 (9.4)
			756	→	356	59		
						18		
FB1		106	722	→	334	57	8.1	1.1 (8.6)
			722	→	352	53		
						10		
FB2		96	706	→	336	51	10.0	1.8 (8.7)
			706	→	318	55		
						22		
FB3		116	706	→	336	53	9.2	1.6 (12.2)
			706	→	668	41		
						24		
HFB1		66	406	→	388	25	6.6	1.4 (8.3)
			406	→	370	29		
						12		
U-(¹³ C ₂₀)-OTA		41	424	→	250	35	12.7	1.1 (7.3)
			424	→	377	21		
						10		
OTA		61	404	→	239	35	12.7	1.2 (16.0)
			404	→	358	21		
						10		
U-(¹³ C ₁₈)-ZON		41	337	→	319	13	12.3	1.3 (5.9)
			337	→	301	17		
						24		
ZON		66	319	→	301	15	12.3	3.2 (26.6)
			319	→	283	19		
						8		
α-ZOL		56	321	→	303	11	12.1	1.2 (13.0)
			321	→	285	17		
						24		

DP = declustering potential; CE = collision energy; CXP = collision cell exit potential; Ion ratio: quantifier/qualifier obtained through the validation experiments (N = 270); RSD: relative standard deviation.

the expected formation of HFB1 (the only commercially available standard). At least 99% of the fumonisins were hydrolyzed at 0 time (RSD up to 1.3%; n = 3), a situation that remained over time (15, 30, 60, 120 and 180 min), with a single exception (98.6% of FB3 at 30 min). Hence, the conditions chosen were 60 min/60 °C in order to ensure a complete hydrolysis in other maize products, including breakfast cereals and snacks. The quantification of HFB1 also showed that all FB1 was hydrolyzed to HFB1.

The produced HFB2 and HFB3 were infused into the mass spectrometer and the analyte-dependent MS/MS parameters optimized. Transitions monitored for HFB2 were: 390/372 (DP: 61 V; CE: 27 V; CXP: 12 V) and 390/336 (DP: 61 V; CE: 33 V; CXP: 20 V);

and for HFB3: 390/354 (DP: 51 V; CE: 27 V; CXP: 24 V) and 390/336 (DP: 51 V; CE: 31 V; CXP: 22 V).

3.4. Total fumonisins extraction procedure

The hydrolysis efficiency of fumonisins (2M KOH at 60 °C/60 min) in maize meal was confirmed by analyzing six replicates of the maize reference material, and comparing with samples where the KOH was replaced by water (non-hydrolyzed maize meal). In the non-hydrolyzed maize meal, mainly the parental fumonisins were found (FB1, FB2 and FB3), while in the hydrolyzed maize meal just the hydrolyzed forms were found

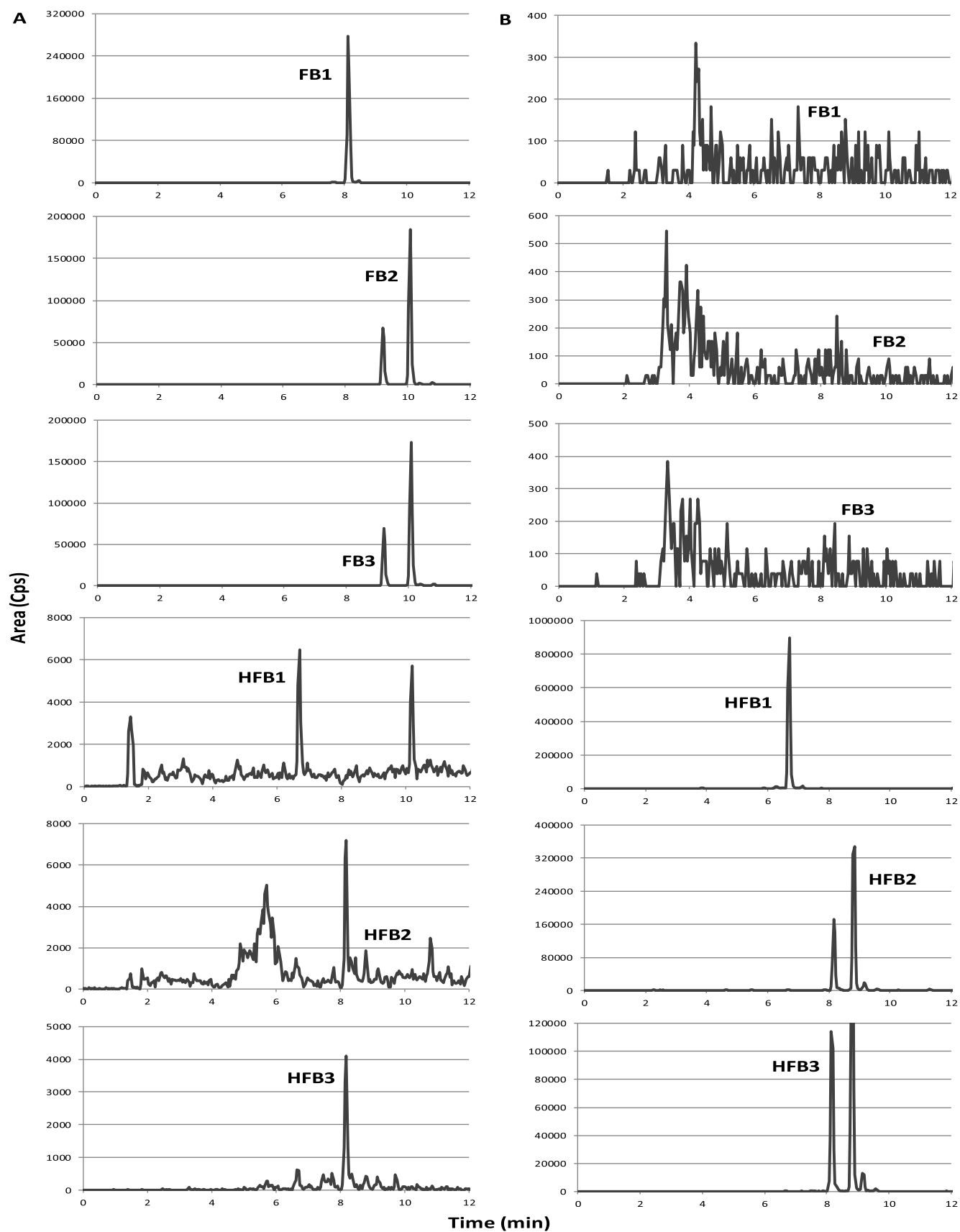


Fig. 2. LC-ESI+–MS/MS chromatograms of maize reference material submitted to the total fumonisin extraction procedure. (A) non-hydrolyzed maize flour; (B) hydrolyzed maize flour.

Table 2

Validation parameters obtained at five different concentration levels for maize meal.

Mycotoxin	Matrix effect (RSD), %		Internal calibration			
	External calibration	Internal calibration	Weighting factor	LOQ (μg/kg)	Recoveries (RSD _r), %	Intermediate precision (RSD _p), %
AFB1	−72.2 (16.7)	4.5 (15.7)	1/x ²	1.2	109.3 (11.9)	93.0 (23.6)
AFB2	−64.9 (14.1)	21.7 (14.2)	1/x ²	0.7	104.8 (12.0)	100.9 (22.4)
AFG1	−59.7 (23.4)	−9.3 (13.1)	1/x ²	0.7	106.8 (9.2)	96.2 (22.3)
AFG2	−54.9 (12.7)	7.5 (10.6)	1/x ²	0.5	104.4 (11.4)	96.1 (22.8)
CTV	−39.0 (19.6)	NA	1/x ²	16	111.3 (15.6)	96.9 (23.7)
DON	9.0 (11.0)	−11.9 (14.2)	1/x	39	103.7 (9.7)	99.9 (10.9)
15AcDON	−11.2 (10.5)	−13.0 (11.8)	1/y	121	110.9 (10.9)	98.9 (16.5)
3AcDON	−11.5 (9.8)	−11.5 (9.8)	1/x	77	109.6 (11.0)	99.5 (15.8)
D3G ^a	−10.3 (7.6)	−12.0 (8.4)	Ordinary	84	98.4 (15.1)	85.8 (20.9)
DOM	1.8 (13.3)	3.0 (15.3)	1/x	40	98.0 (12.3)	91.6 (15.9)
FB1	14.6 (14.2)	−13.6 (13.8)	1/x	19	93.7 (8.3)	82.4 (21.2)
FB2	−19.6 (17.3)	−23.3 (27.1)	1/x ²	8	116.1 (7.5)	99.3 (20.6)
FB3	17.6 (20.4)	−13.1 (22.0)	1/x	32	105.6 (12.0)	90.7 (24.5)
HFB1	70.8 (16.0)	41.4 (17.5)	1/x ²	6	98.1 (12.1)	87.7 (20.1)
OTA	−41.6 (16.8)	−17.6 (12.1)	1/x ²	4	106.4 (9.8)	92.6 (21.9)
ZON	−54.5 (12.0)	−10.0 (9.7)	1/y	24	104.3 (9.9)	96.4 (14.5)
α-ZOL ^a	−46.1 (10.8)	0.7 (10.0)	Ordinary	39	91.4 (7.8)	85.8 (27.8)

LOQ: limit of quantification; RSD: relative standard deviation; NA: isotope labeled internal standard not available; Matrix effect: six replicates; Recoveries: 3–6 replicates; Intermediate precision: triplicates, two different days.

^a Recoveries and intermediate precision: only two different fortification levels (LOQ and medium).

Table 3

Validation parameters obtained at five different concentration levels for rice.

Mycotoxin	Matrix effect (RSD), %		Internal calibration			
	External calibration	Internal calibration	Weighting factor	LOQ (μg/kg)	Recoveries (RSD _r), %	Intermediate precision (RSD _p), %
AFB1	−10.8 (14.3)	−2.7 (13.3)	1/y	0.5	101.5 (14.6)	91.5 (20.5)
AFB2	−21.9 (10.0)	−13.3 (12.0)	1/x ²	1.2	102.2 (13.7)	92.4 (17.4)
AFG1	−15.5 (12.2)	−8.1 (11.6)	1/y ²	1.0	104.3 (14.6)	93.3 (19.8)
AFG2	−9.6 (17.1)	−4.8 (12.9)	1/x ²	1.6	108.5 (16.5)	92.7 (19.3)
CTV	−2.4 m (13.5)	NA	1/x ²	16	90.0 (15.3)	86.1 (19.7)
DON	−8.2 (12.4)	−4.5 (16.7)	1/x	40	100.3 (18.9)	98.5 (20.1)
15AcDON	−3.5 (10.4)	−4.8 (14.0)	1/x ²	72	100.2 (19.0)	94.9 (19.2)
3AcDON	−5.1 (9.7)	−1.5 (15.2)	1/x ²	48	100.7 (19.1)	94.5 (18.9)
D3G ^a	−0.4 (7.1)	6.1 (11.6)	1/y ²	84	83.0 (17.0)	84.6 (23.3)
DOM	4.1 (9.7)	7.9 (16.3)	1/x	24	102.8 (17.7)	96.4 (18.4)
FB1	0.8 (13.9)	−3.5 (16.0)	1/x ²	21	86.6 (20.3)	85.9 (20.6)
FB2	−2.8 (8.9)	−6.6 (18.5)	1/x ²	12	85.7 (13.6)	83.8 (16.7)
FB3	−1.6 (14.3)	−7.5 (15.5)	1/x	24	92.2 (18.6)	87.8 (21.5)
HFB1	1.7 (16.0)	−6.2 (17.2)	1/x	8	55.2 (25.6)	66.0 (29.9)
OTA	−8.6 (14.1)	−1.5 (14.2)	1/x	3	88.1 (17.3)	87.3 (19.0)
ZON	−10.3 (16.0)	1.6 (12.7)	1/y ²	16	101.0 (12.2)	94.5 (15.0)
α-ZOL ^a	−11.3 (11.3)	−2.5 (13.4)	1/y	39	93.3 (22.4)	82.6 (20.4)

LOQ: limit of quantification; RSD: relative standard deviation; NA: isotope labeled internal standard not available; Matrix effect: six replicates; Recoveries: 3–6 replicates; Intermediate precision: triplicates, two different days.

^a Recoveries and intermediate precision: only two different fortification levels (LOQ and medium).

(HFB1, HFB2 and HFB3), proving the efficiency of the procedure (Fig. 2).

The LC–MS/MS was more sensitive to the hydrolyzed forms than the parental compounds (FB1, FB2 and FB3). Since there was no maize meal free of fumonisins available, when the contaminated sample was submitted to hydrolysis, the free forms found in the sample were also hydrolyzed and the levels of HFB1, HFB2 and HFB3 were higher than the initial matched matrix calibration point. Therefore, quantification was conducted using calibration curve made in the solvent (MeOH:H₂O; 40:60) and isotope internal calibration (¹³C₃₄–FB1). Recoveries for HFB1, HFB2 and HFB3 were evaluated in six replicates fortified with the prepared standards (Section 2.5) at level of 1.2, 1.8 and 2.5 μg/kg, respectively. Recoveries were 75.6% (RSD of 6.6%) for HFB1, 108.0 (RSD of 10.6%) for HFB2 and 74.9% (RSD of 12.2%) for HFB3.

3.5. Mycotoxins in cereal products samples

As shown previously, matrix effects in rice were compensated by using *in matrix* external calibration for the quantification of myco-

toxins in this cereal. For wheat-based products, the use of isotope labeled internal standards was necessary. Internal quality controls (n = 3) included in each batch of extraction were within the acceptable range for recoveries (70–120%) and repeatability (RSD_r < 20%), confirming that the method performed well during routine analysis.

Mycotoxin occurrences in rice and wheat-based samples are summarized in Table 6. DON was found in all 55 wheat-based products analyzed, with the highest mean and maximum levels found in crackers (560.7 and 916.1 μg/kg, respectively). D3G was found in 18 wheat-based products, mainly crackers, and ZON in 46 samples, including all 14 cracker samples (mean of 60.7 μg/kg). α-ZOL was found in only one sample (wheat snack) and OTA in two wheat pasta samples. CTV was found in 5 rice grain samples (1 parboiled and 4 polished) and in one cracker sample at very high level (8640 μg/kg). AFB1 was found in only one sample (rice pasta). Levels of AFs, fumonisins, DON, OTA and ZON found in samples analyzed did not exceed the established maximum limits (ML) set by

Table 4

Validation parameters obtained at five different concentration levels for wheat flour.

Mycotoxin	Matrix effects (RSD), %		Internal calibration			
	External calibration	Internal calibration	Weighting factor	LOQ ($\mu\text{g/kg}$)	Recoveries (RSD _r), %	Intermediate precision (RSD _p), %
AFB1	−38.8 (18.7)	−11.0 (11.5)	1/x ²	0.6	103.9 (9.4)	95.7 (16.4)
AFB2	−53.0 (18.7)	−32.3 (12.1)	1/x	1.2	104.3 (12.0)	97.3 (18.0)
AFG1	−37.7 (19.6)	−13.3 (11.3)	1/x	1.2	109.7 (10.7)	100.7 (11.8)
AFG2	−39.1 (16.1)	−15.2 (11.4)	1/x ²	1.6	104.4 (9.8)	95.4 (15.8)
CTV	−26.4 (14.2)	NA	1/x ²	12	110.9 (12.1)	100.0 (18.1)
DON	8.6 (26.3)	−8.3 (13.2)	1/x	40	114.0 (9.5)	112.5 (13.5)
15AcDON	−23.5 (12.5)	−14.3 (14.0)	1/x ²	80	92.6 (21.1)	84.2 (20.7)
3AcDON	−23.0 (14.7)	−15.9 (14.6)	1/x ²	72	80.1 (19.4)	77.5 (16.2)
D3G ^a	21.5 (15.5)	14.3 (18.3)	ordinary	61	102.5 (15.6)	95.9 (14.5)
DOM	39.4 (13.5)	53.3 (14.8)	1/x ²	40	104.7 (11.9)	96.6 (16.3)
FB1	−26.5 (17.8)	2.8 (13.3)	1/x ²	19	104.3 (14.3)	98.8 (19.0)
FB2	−48.5 (20.2)	−28.7 (12.9)	1/x	8	113.3 (13.4)	100.9 (21.8)
FB3	−32.3 (19.7)	2.2 (14.7)	1/x	24	98.9 (16.7)	92.6 (18.9)
HFB1	10.0 (19.1)	21.5 (20.3)	1/x ²	8	113.0 (9.8)	99.9 (21.0)
OTA	−33.5 (19.4)	−1.6 (13.2)	1/x	3	111.2 (7.5)	103.8 (15.5)
ZON	−46.9 (15.7)	−6.5 (9.3)	1/x	16	107.5 (9.5)	97.8 (15.5)
α -ZOL ^a	−40.0 (13.3)	11.4 (14.4)	ordinary	39	117.1 (16.0)	110.7 (20.9)

LOQ: limit of quantification; RSD: relative standard deviation; NA: isotope labeled internal standard not available; Matrix effect: six replicates; Recoveries: 3-replicates; Intermediate precision: triplicates, two different days.

^a Recoveries and intermediate precision: only two different fortification levels (LOQ and medium).

Table 5

Analysis of maize reference material for aflatoxins, fumonisins, deoxynivalenol, ochratoxin A e zearalenone.

Mycotoxin	Reported value range ^a	SD range ^b	Measured value (RSD _r), %
AFs	22.1 $\mu\text{g/kg}$ 14.4–29.8 $\mu\text{g/kg}$	18.4–25.8/14.7–29.5/11.0–33.2	15.0 $\mu\text{g/kg}$ (14.0)
FBs	37.1 mg/kg 27.2–47.0 mg/kg	32.9–41.3/28.7–45.5/24.5–49.7	39.7 mg/kg (6.8)
DON	2.6 mg/kg 2.2–3.0 mg/kg	2.4–2.8/2.2–3.0/2.0–3.2	2.2 mg/kg (3.6)
OTA	4.0 $\mu\text{g/kg}$ 0.5–7.5 $\mu\text{g/kg}$	2.3–5.7/0.6–7.4/0–9.1	6.1 $\mu\text{g/kg}$ (10.4)
ZON	352.0 $\mu\text{g/kg}$ 239.4–464.6 $\mu\text{g/kg}$	306–398/260–444/214–490	238.1 $\mu\text{g/kg}$ (10.0)

AFs: AFB1 + AFB2 + AFG1 + AFG2; FBs: FB1 + FB2 + FB3; RSD: relative standard deviation.

^a Including uncertainty.

^b 1st range/2nd range/3rd range.

Table 6

Mycotoxins occurrence in rice and wheat product samples.

Mycotoxin	Wheat-based products		Rice and rice products	
	Number of positive samples/analyzed	Median (range) $\mu\text{g/kg}$	Number of positive samples/analyzed	Median (range) $\mu\text{g/kg}$
DON	55/55	385.6 (79.7–916.1)	0/44	–
D3G	18/55	93.2 (54.8–335.2)	0/44	–
ZON	46/55	48.4 (17.8–205.6)	0/44	–
α -ZOL	1/55	149.2	0/44	–
CTV	1/55	8640.0	5/44	27.0 (24.3–3472.0)
FB1 + FB2 + FB3	4/55	48.4 (22.8–130.0)	0/44	–
OTA	2/55	5.3 (5.3–5.3)	0/44	–
AFB1	0/55	–	1/44	0.6

Brazilian authorities (BRASIL, 2011), except for one whole-wheat pasta sample contaminating ZON at 205.6 $\mu\text{g/kg}$ (ML = 200 $\mu\text{g/kg}$).

4. Conclusions

Cereals and derived products are highly consumed worldwide and since the complete elimination of mycotoxins from these products is not feasible, it is critical that their occurrence be constantly monitored, so dietary risk assessment can be performed to ensure the safety of consumers. This requires the availability of simple, costly effective, sensitive and validated analytical methods.

In this study, the use of acidified ACN as the extraction solvent followed by LC–MS/MS analysis proved to be suitable, rapid and cost effective method for the multi-mycotoxin determination in wheat, maize and rice products. The method was satisfactorily validated for the simultaneous analysis of AFB1, AFB2, AFG1, AFG2,

CTV, DON, 15AcDON, 3AcDON, D3G, DOM, FB1, FB2, FB3, HFB1, OTA, ZON and α -ZOL using LC–ESI⁺–MS/MS. Matrix effects were compensated using external calibration and matrix matched standard curves for rice, but to accurately determine mycotoxins in maize meal and wheat flour, the use of isotope internal standard was important. Hydrolyzed fumonisin standards were successfully prepared and total fumonisin content was obtained through an optimized procedure. To the best of our knowledge, this is the first study reporting the determination of total fumonisin (free and bound forms) together with the determination of other mycotoxins.

The multi-mycotoxin method was applied to the analysis of mycotoxins in rice (polished, parboiled and bran), rice products (pasta and rice flour) and wheat products (pasta and crackers). A high prevalence of DON and ZON was found in wheat-based product samples analyzed. The prevalence of CTV was low, although high levels were found in samples of crackers and rice.

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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.chroma.2017.02.027>.

References

- [1] J.C. Frisvad, U. Thrane, R.A. Samson, *Mycotoxin producers*, in: R.A. Dijksterhuis, J. Samson (Eds.), *Food Mycology: A Multifaceted Approach to Fungi Food*, CRC Press, New York, 2007, pp. 135–160.
- [2] P. Nicholson, Rapid detection of mycotoxigenic fungi in plants, in: N. Magan, M. Olsen (Eds.), *Mycotoxins in Food: Detection and Control*, Woodhead Publishing Limited, Cambridge, England, 2004, pp. 111–136.
- [3] J.I. Pitt, What are mycotoxins? *Aust. Mycotoxin Newsl.* 7 (1996).
- [4] Council for Agricultural Science and Technology (CAST), *Mycotoxins: Risks in Plant, Animal, and Human Systems*, Task Force Report n° 139, Council for Agricultural Science and Technology, Ames, Iowa – USA, 2003.
- [5] C. Brera, B. Santis, F. Debegnach, M. Miraglia, *Mycotoxins*, in: Y. Picó (Ed.), *Comprehensive Analytical Chemistry*, 1st ed., Elsevier, Amsterdam, The Netherlands, 2008, pp. 363–427.
- [6] Food and Agriculture Organization of the United Nations (FAO), *FAOSTAT – Statistics Division of the Food and Agriculture Organization of the United Nations*, 2014 (accessed 15.02.17) <http://faostat.fao.org/site/291/default.aspx>.
- [7] P.D. Andrade, E.D. Caldas, Aflatoxins in cereals: worldwide occurrence and dietary risk assessment, *World Mycotoxin J.* 8 (2015) 415–431, <http://dx.doi.org/10.3920/WMJ2014.1847>.
- [8] J.I. Pitt, C.P. Wild, R.A. Baan, W.C.A. Gelderblom, J.D. Miller, R.T. Riley, F. Wu, Improving Public Health Through Mycotoxin Control, International Agency for Research on Cancer Lyon, France, 2012.
- [9] M. Rychlik, H.U. Humpf, D. Marko, S. Dänicke, A. Mally, F. Berthiller, H. Klaffke, N. Lorenz, Proposal of a comprehensive definition of modified and other forms of mycotoxins including masked mycotoxins, *Mycotoxin Res.* 30 (2014) 197–205, <http://dx.doi.org/10.1007/s12550-014-0203-5>.
- [10] H.U. Humpf, K.A. Voss, Effects of thermal food processing on the chemical structure and toxicity of fumonisin mycotoxins, *Mol. Nutr. Food Res.* 48 (2004) 255–269, <http://dx.doi.org/10.1002/mnfr.200400033>.
- [11] W. Seefelder, A. Knecht, H.U. Humpf, Bound fumonisin B1: analysis of fumonisin-B1 glyco and amino acid conjugates by liquid chromatography-electrospray ionization-tandem mass spectrometry, *J. Agric. Food Chem.* 51 (2003) 5567–5573, <http://dx.doi.org/10.1021/jf0344338>.
- [12] W.T. Shier, P. Resch, F. Badria, H.K. Abbas, Biological consequences of fumonisins, *Bull. Inst. Compr. Agric. Sci.* 8 (2000) 67–74.
- [13] C. Dall'Asta, M. Mangia, F. Berthiller, A. Molinelli, M. Sulyok, R. Schuhmacher, R. Krska, G. Galaverna, A. Dossena, R. Marchelli, Difficulties in fumonisin determination: the issue of hidden fumonisins, *Anal. Bioanal. Chem.* 395 (2009) 1335–1345, <http://dx.doi.org/10.1007/s00216-009-2933-3>.
- [14] C. Falavigna, M. Cirlini, G. Galaverna, C. Dall'Asta, Masked fumonisins in processed food: co-occurrence of hidden and bound forms and their stability under digestive conditions, *World Mycotoxin J.* 5 (2012) 325–334, <http://dx.doi.org/10.3920/WMJ2012.1403>.
- [15] M.I. Almeida, N.G. Almeida, K.L. Carvalho, G.A.A. Gonçalves, C.N. Silva, E.A. Santos, J.C. Garcia, E.A. Vargas, Co-occurrence of aflatoxins B₁, B₂, G₁ and G₂, ochratoxin A, zearalenone, deoxynivalenol, and citreoviridin in rice in Brazil, *Food Addit. Contam. A Chem. Anal. Control Expo. Risk Assess.* 29 (2012) 694–703, <http://dx.doi.org/10.1080/19440049.2011.651750>.
- [16] C. Liao, J.W. Wong, K. Zhang, D.G. Hayward, N.S. Lee, M.W. Trucksess, Multi-mycotoxin analysis of finished grain and nut products using high-performance liquid chromatography-triple quadrupole mass spectrometry, *J. Agric. Food Chem.* 61 (2013) 4771–4772.
- [17] M. Sulyok, R. Krska, R. Schuhmacher, A liquid chromatography/tandem mass spectrometric multi-mycotoxin method for the quantification of 87 analytes and its application to semi-quantitative screening of moldy food samples, *Anal. Bioanal. Chem.* (2007) 1505–1523, <http://dx.doi.org/10.1007/s00216-007-1542-2>.
- [18] A.L. Capriotti, P. Foglia, R. Gubbiotti, C. Rocchia, R. Samperi, A. Laganà, Development and validation of a liquid chromatography/atmospheric pressure photoionization-tandem mass spectrometric method for the analysis of mycotoxins subjected to commission regulation (EC) No. 1881/2006 in cereals, *J. Chromatogr. A* 1217 (2010) 6044–6051, <http://dx.doi.org/10.1016/j.chroma.2010.07.018>.
- [19] A.G. Frenich, J. Luis, M. Vidal, R. Romero-gonzález, M.M. Aguilera-luiz, Simple and high-throughput method for the multimycotoxin analysis in cereals and related foods by ultra-high performance liquid chromatography/tandem mass spectrometry, *Food Chem.* 117 (2009) 705–712, <http://dx.doi.org/10.1016/j.foodchem.2009.04.045>.
- [20] G.C. Dors, V. da S. Bierhals, E. Badiale-Furlong, Parboiled rice: chemical composition and the occurrence of mycotoxins, *Ciência E Tecnol Aliment* 31 (2011) 172–177, <http://dx.doi.org/10.1590/S0101-20612011000100025>.
- [21] S.T. Tran, T.K. Smith, G.N. Girgis, A survey of free and conjugated deoxynivalenol in the 2008 corn crop in Ontario, Canada, *J. Sci. Food Agric.* 92 (2012) 37–41, <http://dx.doi.org/10.1002/jsfa.4674>.
- [22] L.M. Kawashima, L.M. Valente Soares, Incidência de fumonisina B₁, aflatoxinas B₁, B₂, G₁ e G₂, ocratoxina A e zearalenona em produtos de milho, *Ciência E Tecnol. Aliment* 26 (2006) 516–521, <http://dx.doi.org/10.1590/S0101-20612006000300005>.
- [23] E. Varga, T. Glauner, R. Köppen, K. Mayer, M. Sulyok, R. Schuhmacher, R. Krska, F. Berthiller, Stable isotope dilution assay for the accurate determination of mycotoxins in maize by UHPLC-MS/MS, *Anal. Bioanal. Chem.* 402 (2012) 2675–2686, <http://dx.doi.org/10.1007/s00216-012-5757-5>.
- [24] A. Malachová, M. Sulyok, E. Beltrán, F. Berthiller, R. Krska, Optimization and validation of a quantitative liquid chromatography-tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices, *J. Chromatogr. A* 1362 (2014) 145–156, <http://dx.doi.org/10.1016/j.chroma.2014.08.037>.
- [25] M. Rychlik, S. Asam, Stable isotope dilution assays in mycotoxin analysis, *Anal. Bioanal. Chem.* 390 (2008) 617–628, <http://dx.doi.org/10.1007/s00216-007-1717-x>.
- [26] S. Asam, M. Rychlik, Studies on accuracy of trichothecene multitoxin analysis using stable isotope dilution assays, *Mycotoxin Res.* 23 (2007) 191–198, <http://dx.doi.org/10.1007/BF02946047>.
- [27] G. Häubl, F. Berthiller, J. Rechthaler, G. Jaunecker, E.M. Binder, R. Krska, R. Schuhmacher, Characterization and application of isotope-substituted (13C15)-deoxynivalenol (DON) as an internal standard for the determination of DON, *Food Addit. Contam.* 23 (2006) 1187–1193, <http://dx.doi.org/10.1080/02652030600654390>.
- [28] MAPA, Manual of Analytical Quality Assurance, Ministry of Agriculture, Livestock and Food Supply, Brasília, (2011).
- [29] R. Krska, P. Schubert-Ullrich, R.D. Josephs, H. Emteborg, G. Buttinger, H. Pettersson, H.P. Van Egmond, R.C. Schothorst, S. MacDonald, D. Chan, Determination of molar absorptivity coefficients for major type-B trichothecenes and certification of calibrators for deoxynivalenol and nivalenol, *Anal. Bioanal. Chem.* 388 (2007) 1215–1226, <http://dx.doi.org/10.1007/s00216-007-1369-x>.
- [30] M.W. da Rocha, I.S. Resck, E.D. Caldas, Purification and full characterisation of citreoviridin produced by *Penicillium citreonigrum* in yeast extract sucrose (YES) medium, *Food Addit. Contam. A* 32 (2015) 584–595, <http://dx.doi.org/10.1080/19440049.2014.961177>.
- [31] C. Dall'Asta, G. Galaverna, M. Mangia, S. Sforza, A. Dossena, R. Marchelli, Free and bound fumonisins in gluten-free food products, *Mol. Nutr. Food Res.* 53 (2009) 492–499, <http://dx.doi.org/10.1002/mnfr.200800088>.
- [32] M. Boevre, J.D. Di Mavungu, P. Maene, K. Audenaert, D. Deforce, G. Haesaert, M. Eeckhout, A. Callebaut, F. Berthiller, C. Van Peteghem, S. De Saeger, Development and validation of an LC-MS/MS method for the simultaneous determination of deoxynivalenol, zearalenone, T-2-toxin and some masked metabolites in different cereals and cereal-derived food, *Food Addit. Contam. A* 29 (2012) 819–835, <http://dx.doi.org/10.1080/19440049.2012.656707>.
- [33] F. Berthiller, C. Dall'Asta, R. Schuhmacher, M. Lemmens, G. Adam, A.R. Krska, Masked mycotoxins: determination of a deoxynivalenol glucoside in artificially and naturally contaminated wheat by liquid chromatography-tandem mass spectrometry, *J. Agric. Food Chem.* 53 (2005) 3421–3425, <http://dx.doi.org/10.1021/jf047798g>.
- [34] E. Beltrán, M. Ibáñez, T. Portolés, C. Ripollés, J.V. Sancho, V. Yusà, S. Marín, F. Hernández, Development of sensitive and rapid analytical methodology for food analysis of 18 mycotoxins included in a total diet study, *Anal. Chim. Acta* 783 (2013) 39–48, <http://dx.doi.org/10.1016/j.aca.2013.04.043>.
- [35] L. Sorensen, T. Elbak, Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry, *J. Chromatogr. B* 820 (2005) 183–196, <http://dx.doi.org/10.1016/j.jchromb.2005.03.020>.
- [36] C. Cavaliere, P. Foglia, E. Pastorini, R. Samperi, A. Lagan, Development of a multiresidue method for analysis of major Fusarium mycotoxins in corn meal using liquid chromatography/tandem mass spectrometry, *Rapid Commun. Mass Spectrom.* 19 (2005) 2085–2093, <http://dx.doi.org/10.1002/rcm.2030>.
- [37] M. Sulyok, F. Berthiller, R. Krska, R. Schuhmacher, Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize, *Rapid Commun. Mass Spectrom.* 20 (2006) 2649–2659, <http://dx.doi.org/10.1002/rcm.2640>.