Food Science and Technology
ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)
DOI: https://doi.org/10.1590/fst.33717


# Validation and application of an analytical method for the determination of mycotoxins in crackers by UPLC-MS/MS

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**Abstract**

An analytical method applying ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was validated for the determination of aflatoxins M2, M1, G2, G1, B2, B1, deoxynivalenol, ochratoxin A, fumonisins B1 and B2, hydrolyzed fumonisins B1 and B2, zearalenone and sterigmatocystin in crackers. The obtained recoveries (70 to 110%) and relative standard deviations (< 13%) were considered satisfactory. Method limits of quantification ranged from 0.20 μg $kg^{-1}$ (sterigmatocystin) to 12.27 μg $kg^{-1}$ (deoxynivalenol). The validated method was then applied for mycotoxin determination in 60 cracker samples (*cream crackers* and *water and salt crackers*) obtained from the metropolitan area of the state of Rio de Janeiro, RJ. Deoxynivalenol, zearalenone and fumonisin B1 were found, respectively, in 100, 50 and 28% of the analyzed samples. The maximum permissible limits established by Brazilian legislation for deoxynivalenol and zearalenone were not exceeded in the analyzed samples.

**Keywords:** mycotoxins; UPLC-MS/MS; cracker; wheat-based biscuits.

**Practical Applications:** A suitable method for routine analysis of mycotoxins in crackers by UPLC-MS/MS was validated. This study reports the results of the first survey on the contamination of crackers in Brazil by aflatoxins M2, M1, G2, G1, B2, B1, deoxynivalenol, ochratoxin A, fumonisins B1 and B2, hydrolyzed fumonisins B1 and B2, zearalenone and sterigmatocystin. The results herein reported suggests that the cracker contamination by deoxynivalenol, zearalenone and fumonisin B1 is an important issue and should be monitored by the Brazilian public health authorities.

## 1 Introduction

Mycotoxins are toxic substances produced naturally as secondary metabolites by several filamentous fungi. These compounds are considered food contaminants, responsible by agriculture and public health problems (International Agency for Research on Cancer, 1993; Peraica et al., 1999; International Agency for Research on Cancer, 2002; Murphy et al., 2006; Zain, 2011; Rocha et al., 2014).

Aflatoxins B1, B2, G1 and G2, deoxynivalenol, fumonisins B1 and B2, ochratoxin A and zearalenone have been established as the main mycotoxins detected in cereals and cereal-based products and their contamination levels have been regulated in food worldwide (Lee & Ryu, 2017; Food and Agriculture Organization of the United Nations, 2004; European Commission, 2006a; Brasil, 2011; Brasil, 2017). Other mycotoxins, such as sterigmatocystin (Mol et al., 2016) and hydrolyzed fumonisins (Dombrink-Kurtzman & Dvorak, 1999), have also been found in these foods. Aflatoxins M1 and M2 can also be produced by fungi, albeit in minor amounts (Bräse et al., 2009; Filazi & Sireli, 2013) and have been reported in both milk and other foodstuffs, such as cereals (Shotwell et al., 1976; Vesonder et al., 1991; Ren et al., 2007; Huang et al., 2010; Ezekiel et al., 2012; Sartori et al., 2015).

Several mycotoxins, mainly deoxynivalenol and zearalenone, have been reported in wheat and wheat products in several countries (Pussemier et al., 2006; Tanaka et al., 2010; González-Osnaya & Farrés, 2011; Mishra et al., 2013). In Brazil, a high occurrence of deoxynivalenol in wheat has been described (Lamardo et al., 2006; Santos et al., 2011). However, scarce studies directed to the determination of mycotoxins in wheat products in the country are available (Oliveira et al., 2000; Almeida et al., 2016).

In 2015, Brazil produced 5 to 6 million tons of wheat (Instituto Brasileiro de Geografia e Estatística, 2015) and domestic consumption was of approximately 11 million tons (Empresa Brasileira de Pesquisa Agropecuária, 2015). Wheat is the raw material for the manufacture of many products consumed daily by the population (Almeida et al., 2016). Brazil is the second largest biscuit producer, producing over one million tons in 2013. The per capita consumption of this product in Brazil in 2015 was of 8.47 kg, and crackers were responsible for 21.4% of this consumption. In 2014, 354 thousand tons of cracker and water and salt crackers were sold, the second most commercialized type of biscuit in Brazil (Associação Brasileira das Indústrias de Biscoitos, Massas Alimentícias e Pães & Bolos Industrializados, 2015). Crackers are an industrialized product containing 90% wheat in their formulation (Scudamore et al., 2009). Despite

*Received 10 Oct., 2017*
*Accepted 03 Jan., 2019*
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the relative importance to consumer health due to the high consumption of this product, only one study has reported contamination by deoxynivalenol in crackers in the country (Souza et al., 2015).

Diverse analytical approaches have been developed aiming at the determination of mycotoxins in food, highlighting the growing application of the liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique in the last years (Krska et al., 2008; Cigić & Prosen, 2009; Köppen et al., 2010; Ediage et al., 2011; Turner et al., 2009; Turner et al., 2015; Berthiller et al., 2016; Berthiller et al., 2017). The selectivity of this technique has enabled the simultaneous analysis of different mycotoxin classes in different food matrices with only minimum sample treatment (Sulyok et al., 2007; Mol et al., 2016; Chiaradia et al., 2008; Frenich et al., 2009; Lacina et al., 2012).

In this context, the aim of this study was to validate an analytical method suitable for the routine analysis of mycotoxins in crackers by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Sample treatment method comprises simultaneous extraction and clean-up (deffating) steps, followed by extract concentration. The analytical method was applied to 60 cracker samples obtained in the metropolitan region of Rio de Janeiro, RJ, Brazil. Additionally, the results were used to estimate population exposure to deoxynivalenol by cracker consumption.

## 2 Materials and methods

### *2.1 Reagents and chemicals*

Ammonium formate (>99%) and formic acid (mass spectrometry grade) were acquired from Sigma-Aldrich (St. Louis, MO, USA), acetonitrile and methanol (HPLC-grade) were obtained from J.T. Baker (Phillipsburg, NJ, USA), while n-Hexane (purity > 96%), ethyl acetate (for analysis) and potassium hydroxide (pellets for analysis) were obtained from Merck (Darmstadt, Germany). Ultrapure water from a Milli-Q Gradient water system was used (Millipore, Bedford, MA, USA).

### *2.2 Standard solutions*

Solid aflatoxin standards, namely B1, B2, G1, G2, M1, M2, ochratoxin A and sterigmatocystin, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock ochratoxin A (40 μg $mL^{-1}$) solutions were prepared in toluene/acetic acid (99:1, v/v). Individual sterigmatocystin and aflatoxin stock solutions (10 μg $mL^{-1}$) were prepared in acetonitrile. The standard solution concentrations were determined by UV spectrophotometry (Horwitz & Latimer, 2005), and their stability was verified by UV spectrophotometry at least every twelve months. Stock fumonisin B1 and B2 (50 μg $mL^{-1}$) solutions in acetonitrile/water (1:1, v/v) and deoxynivalenol in acetonitrile (100 μg $mL^{-1}$) were obtained from Fluka/Sigma-Aldrich (St. Louis, MO, USA). Stock zearalenone solutions in acetonitrile (100.7 μg $mL^{-1}$) were purchased from Biopure (Tulln, Austria). Hydrolyzed fumonisins B1 and B2 were prepared by the hydrolysis of fumonisin B1 and B2, according to Dall'Asta et al. (2009). Briefly, 5 mL of a standard solution containing fumonisin B1 and B2 (50 μg $mL^{-1}$) in acetonitrile/water (1:1, v/v) was evaporated to dryness under a nitrogen flow at 40 °C using a controlled water bath. Residues were dissolved in 5 mL of a 2 mol $L^{-1}$ KOH solution and left at room temperature for 12 h. The hydrolyzed fumonisins were then extracted thrice with 10 mL of ethyl acetate, combined and then again evaporated in the same conditions. The residues were finally dissolved in 5 mL methanol. No native fumonisins were detected in this final solution by UPLC-MS/MS, indicating that total conversion to the hydrolyzed forms was achieved. Thus, hydrolyzed fumonisin B1 and B2 concentrations in methanol were calculated as 28.1 and 27.6 μg $mL^{-1}$, respectively. All standard solutions were stored at -18 °C.

### *2.3 UPLC-MS/MS analysis*

Liquid chromatography was carried out using an ACQUITY UPLC™ system (Waters). A BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm particle size) maintained at 35 °C was used as the stationary phase. The mobile phase flow rate was set at 0.3 mL $min^{-1}$. Two elution gradients were used in this study to avoid the presence of fumonisin carryover (Sartori et al., 2017). The aqueous mobile phase of the elution gradient used to determined fumonisins, hydrolyzed fumonisins and sterigmatocystin was 0.3% acid formic solution, with the gradient beginning at 60% methanol, increasing to 80% for 3 min, held at 80% for 1 min. Following these steps, the system was re-equilibrated with 60% methanol for 2 min. The aqueous mobile phase of the elution gradient used to determine M2, M1, B2, B1, G2 and G1, ochratoxin A, deoxynivalenol and zearalenon was a 5 mmol $L^{-1}$ formate ammonium solution, beginning at 10% methanol, increasing to 100% during 4 min and held at 100% for 1.5 min. The system was then re-equilibrated for 2 min with 10% methanol. A 5 μL injection volume was used for both gradients.

A tandem quadrupole mass spectrometer (Waters, Quattro Premier™ XE) equipped with an electrospray ionization (ESI) source operated in the positive and negative ionization modes was used for analyte detection. The following parameters were set: capillary voltage at 3.5 kV, extractor voltage at 3 V, rf lens at 0.1 V, multiplier at 750 V, desolvation temperature at 350 °C and source temperature at 120 °C. Nitrogen was used both as cone and desolvation gas, at 50 L $h^{-1}$ and 750 L $h^{-1}$, respectively. Argon was applied as the collision gas at $4 \times 10^{-3}$ mbar. The two selected ion transitions (*m/z*) for each mycotoxin and their acquisition conditions are presented in Table 1 and 2.

### *2.4 Sample preparation*

The method reported by Sartori et al. (2017) was used for sample preparation, as follows. About 3 g of each sample was weighed in 50 mL centrifuge tubes, followed by the addition of 5 mL n-hexane, 5 mL of a 3% formic acid solution and 10 mL acetonitrile. Each tube was vortex-shaken (IKA Works) for 2 min, then sonicated for 10 min and then centrifuged at 3,000 rpm for 7 min (Hitach- HIMAC CF 7D2). Subsequently, 5 mL aliquots of the extracts (acetonitrile/water) was concentrated to dryness as previously described, at 50 °C in a controlled water bath (Turbo-Vac LV). Finally, residues were dissolved in 1 mL of methanol/water (1:1, v/v) and the resulting solutions were filtered through 0.22 μm PVDF membrane filters. After filtration, the solutions were transferred to vial sand taken to the equipment.

**Table 1**. UPLC-MS/MS parameters for fumonisins, hydrolyzed fumonisins and sterigmatocystin.

| Mycotoxins | $t_R$ (min)[a] | Quantifier transition ion Q (*m/z*) | Qualifier transition ion q (*m/z*) | Q/q[b] | Energy collision (eV)[c] | Cone voltage (V) | Dwell time (s) |
|---|---|---|---|---|---|---|---|
| Hydrolyzed fumonisin B1 | 1.60 (1) | 406.3 > 388.3 | 406.3 > 370.3 | 1.2 (±0.2) | 20 / 20 | 30 | 0.05 |
| Fumonisin B1 | 1.75 (1) | 722.2 > 334.3 | 722.2 > 352.3 | 1.2 (±0.2) | 40 / 40 | 50 | 0.05 |
| Hydrolyzed fumonisin B2 | 2.47 (2) | 390.3 > 372.3 | 390.3 > 354.3 | 1.3 (±0.3) | 20 / 20 | 30 | 0.02 |
| Fumonisin B2 | 2.72 (2) | 706.2 > 336.3 | 706.2 > 318.3 | 2.0 (±0.4) | 35 / 35 | 50 | 0.02 |
| Sterigmatocystin | 3.00 (2) | 325.2 > 281.2 | 325.2 > 310.2 | 1.1 (±0.2) | 35 / 25 | 45 | 0.02 |

ESI in positive mode for all analytes; interchannel delay and interscan delay were both 0.005 s; [a]acquisition windows given in parentheses; [b]relative ion transition intensities (Q/q) and maximum permitted tolerances given in parentheses (European Commission, 2002); [c]values are given as quantifier transition ion/qualifier transition ion.

**Table 2**. UPLC-MS/MS parameters for deoxynivalenol, aflatoxins M2, M1, B2, B1, G2 and G1, ochratoxin A and zearalenone.

| Mycotoxins | $t_R$ (min)[a] | Quantifier transition ion Q (*m/z*) | Qualifier transition ion q (*m/z*) | Q/q[b] | Energy collision (eV)[c] | Cone voltage (V) | Dwell time (s) |
|---|---|---|---|---|---|---|---|
| Deoxynivalenol | 2.15 (1) | 297.1 > 249.1 | 297.1 > 231.1 | 2.2 (±0.6) | 25 / 25 | 25 | 0.15 |
| Aflatoxin M2 | 2.91 (2) | 331.3 > 273.3 | 331.3 > 285.2 | 1.9 (±0.4) | 25 / 25 | 45 | 0.15 |
| Aflatoxin G2 | 3.04 (3) | 331.3 > 245.3 | 331.3 > 285.3 | 1.5 (±0.3) | 30 / 30 | 40 | 0.015 |
| Aflatoxin M1 | 3.05 (3) | 329.2 > 273.2 | 329.2 > 259.2 | 2.1 (±0.5) | 25 / 25 | 50 | 0.015 |
| Aflatoxin G1 | 3.16 (3) | 329.2 > 243.2 | 329.2 > 283.2 | 1.5 (±0.3) | 25 / 25 | 45 | 0.015 |
| Aflatoxin B2 | 3.28 (3) | 315.2 > 287.0 | 315.2 > 259.2 | 1.0 (±0.2) | 25 / 30 | 50 | 0.015 |
| Aflatoxin B1 | 3.38 (3) | 313.0 > 269.2 | 313.0 > 285.2 | 1.8 (±0.4) | 35 / 25 | 40 | 0.015 |
| Ochratoxin A | 3.75 (4) | 404.2 > 239.2 | 404.2 > 358.2 | 1.6 (±0.3) | 25 / 15 | 25 | 0.15 |
| Zearalenone | 4.23 (5) | 316.9 > 174.8 | 316.9 > 130.8 | 1.3 (±0.3) | 25 / 30 | 50 | 0.15 |

ESI in positive mode for all analytes, except for zearalenone; Interchannel delay and interscan delay were both 0.005 s; [a]acquisition windows given in parentheses; [b]relative ion transition intensities (Q/q) and maximum permitted tolerances given in parentheses (European Commission, 2002); [c]values are given as quantifier transition ion/qualifier transition ion.

### *2.5 Validation*

An analytical method for the determination of aflatoxins M2, M1, G2, G1, B2, B1, deoxynivalenol, ochratoxin A, fumonisins B1 and B2, hydrolyzed fumonisins B1 and B2, zearalenone and sterigmatocystin in crackers (*cream crackers* and *water and salt crackers*) was validated. The following analytical performance parameters were assessed: selectivity, matrix effect, linearity, trueness, precision (repeatability and intermediate precision), limit of detection (LOD) and limit of quantification (LOQ).

The method selectivity was evaluated by analyzing matrix blank samples, to evaluate the presence of interfering signals in all cracker samples.

For the investigation of matrix effects, calibration curves for each compound in each matrix extracts (*cream cracker* and *water and salt cracker*) and in methanol/water (1:1, v/v) were prepared at six concentration levels, ranging from 0.5 to 25 ng $mL^{-1}$ (aflatoxins M2, M1, G2, G1, B2, B1 and ochratoxin A and sterigmatocystin), 1 to 100 ng $mL^{-1}$ (zearalenone), 2.5 to 125 ng $mL^{-1}$ (hydrolyzed fumonisins B1 and B2), 1.5 to 75 ng $mL^{-1}$ (fumonisins B1 and B2) and 5 to 250 ng $mL^{-1}$ (deoxynivalenol). The calibration curve slopes were compared by the t-test (Souza, 2007). The effect of sample dilution on the matrix-effect was also evaluated.

Matrix-matched calibration curves at the same concentration levels used to study the matrix effect were used to assess linearity (Souza & Junqueira, 2005). The homocedasticity, independency and normality of the regression residuals were checked. Outliers were successively investigated by the Jacknife standardised residuals test (Belsley et al., 1980). The homocedasticity of residuals, verified by a modified Levene test (Brown & Forsythe, 1974) for all the calibration curves was confirmed (p-values > 0.05). The independency of residuals, verfiied by the Durbin-Watson statistic (Durbin & Watson, 1951) for all calibration curves was confirmed (p-values > 0.05). The normality of residuals, checked by the Ryan-Joiner test (Ryan & Joiner, 1976) for all the calibration curves was confirmed (p-values > 0.05). The regression significance and the lack-of-fit were performed by an analysis of variance (ANOVA) (Draper & Smith, 1998).

Method trueness and repeatability were investigated by carrying out recovery studies using cracker samples spiked with the evaluated mycotoxins at three concentration levels, using four replicates for each level. Intermediate precision was assessed by analyzing spiked samples at the same concentrations as the first concentration level, all analyzed within four days, by four different analysts. Repeatability and intermediate precision are expressed by the relative standard deviation (RSD %), while trueness is expressed by recovery values.

Cracker samples spiked with each investigated compound at the lowest concentration level applied in the recovery studies were used to determine the method limit of detection (LOD) and limit of quantification (LOQ), considering 3 and 10 signal-to-noise ratios, respectively.

### *2.6 Cracker biscuit samples*

A total of 60 cracker samples (*cream crackers*, n = 30 and *water and salt crackers*, n = 30) were randomly purchased from local supermarkets in the metropolitan region of Rio de Janeiro,

RJ, Brazil between 2015 and 2016, from 13 different companies, representing 16 different brands. The samples were ground and passed through a 0.84 μm sieve and then stored at -20 °C until analysis.

## 3 Results and discussion

### *3.1 Method validation*

No interfering signals were observed eluting at the same time as the analytes for all studied crackers. Mycotoxins in samples were identified by comparing analyte retention times to standard solution retention times. Confirmation in each sample was obtained by comparison of the signal intensity ratios of the quantifier and qualifier ion transitions of each analyte with those in standard solutions, considering the maximum permissible limits according to the European Union (European Commission, 2002). Figure 1 displays chromatograms of a cracker sample fortified with the investigated mycotoxins. Retention times, ion ratios and the maximum permissible limits for the obtained ion ratios for each assessed mycotoxin are also displayed.

Significant differences ($p > 0.01$) were detected between the slopes of the solvent calibration curves and in the matrices for most mycotoxins (except sterigmatocystin in *cream crackers* and aflatoxins M1 and M2, and hydrolyzed fumonisin B1 in *water and salt crackers*), demonstrating a significant matrix effect for most of the assessed compounds. Sample dilutions (final extracts) were investigated aiming at reducing or eliminating matrix effects. Sample dilution effects were assessed using two matrix amounts in the final extract (0.1 and 1 g $mL^{-1}$). Results are displayed in Table 3.

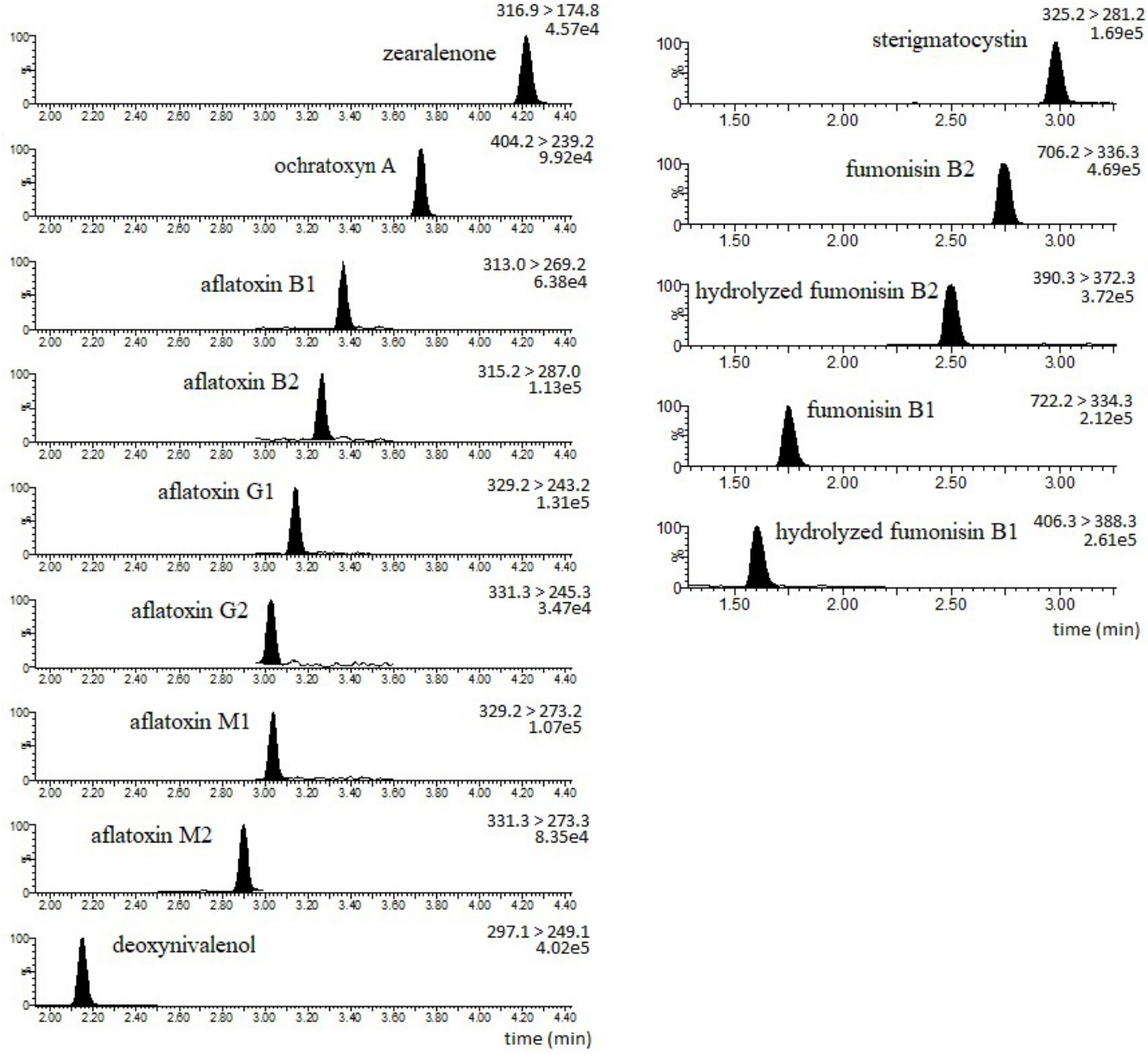


**Figure 1**. Chromatograms of a cracker sample fortified with the mycotoxins investigated herein.

A decreased was observed matrix effect for certain mycotoxins with increasing sample dilution. Despite a still significant matrix effect for most mycotoxins, sample dilution was effective in eliminating the matrix effect for deoxynivalenol. For the analytes displaying matrix effects, matrix-match calibrations were used in routine analyses.

Concerning the linearity studies, the following aspects were confirmed for all the calibration curves: homoscedasticity, independency of the residuals, and normality of the residuals (p values >0.05). In addition, high regression significance (p-values < 0.001) and non-significant lack-of-fit (p-values > 0.05) were also noted, attesting curve linearity. Linearity results are displayed in Table 4.

Recovery values ranged from 70 to 110%, with RSD lower than 14% for all investigated mycotoxins under repeatability conditions. The RSD obtained in the intermediate precision study was always lower than 17%. Thus, results were deemed satisfactory according to Commission Decision 2002/657/EC (European Commission, 2002) and Commission Regulation EC 401/2006 (European Commission, 2006b).

LOD and LOQ values are also displayed in Table 5. Method sensitivity was considered suitable for the routine analysis of the assessed mycotoxins in crackers, in view of the maximum permissible limits for regulated mycotoxins (Brasil, 2011; European Commission, 2006a; Food and Agriculture Organization of the United Nations, 2004).

**Table 3**. Matrix effects (%) for the investigated mycotoxins at different extract dilutions.

| Mycotoxins | Matrix-effect (%)[a] | | | |
|---|---|---|---|---|
| | *Cream cracker*[b] | *Cream cracker (diluted)*[c] | *Water and salt cracker*[b] | *Water and salt cracker (diluted)*[c] |
| Deoxynivalenol | -29.0 | -0.4 | -42.3 | 4.8 |
| Aflatoxin M2 | 4.9 | 0.4 | -4.5 | 3.4 |
| Aflatoxin M1 | 8.1 | -5.3 | -2.4 | 3.3 |
| Aflatoxin G2 | -52.0 | -14.0 | -61.3 | -9.3 |
| Aflatoxin G1 | -57.0 | -12.0 | -66.6 | -15.2 |
| Aflatoxin B2 | -58.2 | -19.2 | -68.8 | -21.7 |
| Aflatoxin B1 | -57.1 | -18.6 | -66.3 | -21.2 |
| Fumonisin B1 | -27.4 | -5.7 | -19.8 | 2.0 |
| Fumonisin B2 | -19.7 | -0.95 | -16.1 | 4.2 |
| Hydrolyzed fumonisin B1 | -26.4 | -5.5 | -13.9 | 6.6 |
| Hydrolyzed fumonisin B2 | -16.4 | 9.5 | -0.2 | 23.9 |
| Ochratoxin A | -24.0 | -1.5 | -43.0 | -0.1 |
| Zearalenone | -42.6 | -9.2 | -58.6 | -7.3 |
| Sterigmatocystin | -4.2 | -4.1 | -7.1 | 9.3 |

**Table 4**. Linearity results for the matrix-matched calibrations.

| Mycotoxins | *Cream cracker* | | | *Water and salt cracker* | | |
|---|---|---|---|---|---|---|
| | Linear range (ng mL$^{-1}$) | Equation | $r^2$ | Linear range (ng mL$^{-1}$) | Equation | $r^2$ |
| Deoxynivalenol | 5 - 250 | $y = 32.5x + 59.9$ | 0.99 | 5 - 250 | $y = 26.5x + 116.6$ | 0.99 |
| Aflatoxin M2 | 0.5 - 20 | $y = 946.6x + 307.2$ | 0.99 | 0.5 - 25 | $y = 861.2x - 59.0$ | 0.99 |
| Aflatoxin M1 | 0.5 - 25 | $y = 999.8x - 19.2$ | 0.99 | 0.5 - 25 | $y = 902.6x - 219.0$ | 0.98 |
| Aflatoxin G2 | 0.5 - 25 | $y = 391.3x + 79.8$ | 0.99 | 0.5 - 25 | $y = 316.9x + 4.6$ | 0.98 |
| Aflatoxin G1 | 0.5 - 25 | $y = 1219.3x + 416.9$ | 0.99 | 0.5 - 25 | $y = 946.3x + 64.6$ | 0.99 |
| Aflatoxin B2 | 0.5 - 25 | $y = 1061.1x + 71.3$ | 0.99 | 0.5 - 25 | $y = 791.3x + 125.5$ | 0.99 |
| Aflatoxin B1 | 0.5 - 15 | $y = 1448.3x + 745.0$ | 0.99 | 0.5 - 25 | $y = 1136.9x + 88.9$ | 0.99 |
| Fumonisin B1 | 1.5 - 45 | $y = 438.5x + 1214.2$ | 0.99 | 1.5 - 45 | $y = 484.4x + 532.9$ | 0.99 |
| Fumonisin B2 | 1.5 - 45 | $y = 1084.5x + 2414.6$ | 0.99 | 1.5 - 45 | $y = 1133.6x + 1081.0$ | 0.99 |
| Hydrolyzed fumonisin B1 | 2.5 - 125 | $y = 548.0x + 631.1$ | 0.99 | 2.5 - 125 | $y = 640.8x - 1403.3$ | 0.99 |
| Hydrolyzed fumonisin B2 | 2.5 - 75.0 | $y = 750.2x + 1867.3$ | 0.99 | 2.5 - 125 | $y = 895.5x - 1536.6$ | 0.99 |
| Ochratoxin A | 0.5 - 25 | $y = 598.0x + 189.8$ | 0.99 | 0.5 - 25 | $y = 449.1x + 120.8$ | 0.99 |
| Zearalenone | 1.0 - 50 | $y = 1009.1x + 961.6$ | 0.99 | 1.0 - 50 | $y = 726.9x + 385.9$ | 0.99 |
| Sterigmatocystin | 0.5 - 25 | $y = 1995.1x - 282.3$ | 0.99 | 0.5 - 25 | $y = 1933.2x - 174.0$ | 0.99 |

$r^2$: determination coefficient.

**Table 5**. Validation parameters of the analytical method.

| Mycotoxins | LOD | LOQ | 1 μg kg$^{-1}$ [a] | | | 5 μg kg$^{-1}$[a] | | 15 μg kg$^{-1}$[a] | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Rec | $RSD_r$ | $RSD_R$ | Rec | $RSD_r$ | Rec | $RSD_r$ |
| Deoxynivalenol | 3.68 | 12.27 | 77.9 | 6.2 | 5.0 | 91.2 | 8.3 | 82.2 | 1.5 |
| Aflatoxin M2 | 0.23 | 0.78 | 76.2 | 3.0 | 7.4 | 109.6 | 9.9 | 84.7 | 4.3 |
| Aflatoxin M1 | 0.40 | 1.33 | 85.3 | 12.2 | 13.3 | 107.6 | 9.0 | 79.0 | 10.2 |
| Aflatoxin G2 | 0.88 | 2.93 | 104.4 | 7.2 | 16.7 | 109.8 | 5.9 | 86.5 | 4.8 |
| Aflatoxin G1 | 0.43 | 1.44 | 70.0 | 3.3 | 3.8 | 91.2 | 8.5 | 78.6 | 2.4 |
| Aflatoxin B2 | 0.41 | 1.38 | 70.3 | 12.3 | 4.0 | 106.7 | 9.1 | 86.3 | 1.8 |
| Aflatoxin B1 | 0.33 | 1.11 | 72.3 | 13.4 | 5.4 | 91.4 | 8.1 | 75.9 | 2.1 |
| Fumonisin B1 | 0.51 | 1.71 | 83.6 | 8.0 | 4.0 | 96.2 | 3.9 | 85.0 | 3.6 |
| Fumonisin B2 | 0.44 | 1.47 | 80.2 | 10.1 | 6.4 | 93.8 | 2.9 | 87.0 | 5.3 |
| Hydrolyzed fumonisin B1 | 2.22 | 7.41 | 71.5 | 11.0 | 8.8 | 91.6 | 2.7 | 77.9 | 5.7 |
| Hydrolyzed Fumonisin B2 | 1.26 | 4.20 | 70.5 | 9.9 | 6.6 | 89.4 | 0.9 | 73.0 | 3.4 |
| Ochratoxin A | 0.09 | 0.31 | 88.0 | 7.6 | 5.1 | 99.5 | 7.3 | 79.3 | 7.9 |
| Zearalenone | 0.68 | 2.25 | 75.6 | 1.9 | 4.6 | 101.0 | 6.2 | 88.7 | 1.3 |
| Sterigmatocystin | 0.06 | 0.20 | 70.9 | 4.9 | 6.3 | 87.0 | 4.1 | 73.9 | 5.5 |

[a]Spiked concentration levels for aflatoxins M2, M1 G2, G1, B2 and B1, ochratoxin A and sterigmatocystin (one hundred times higher for deoxynivalenol and ten times higher for fumonisins and hydrolyzed fumonisins); LOD: Limit of Detection (μg kg$^{-1}$); LOQ: Limit of Quantification (μg kg$^{-1}$); Rec: Recovery (%); $RSD_r$ (%): Relative Standard Deviation (intra-day, n = 4); $RSD_R$ (%): Relative Standard Deviation (inter-ddayay, n = 3).

### *3.2 Sample analysis*

The validated analytical method was applied for the determination of the 14 target mycotoxins (aflatoxins M2, M1, G1, B2, B1, deoxynivalenol, ochratoxin A, fumonisins B2 and B1, fumonisins B2 and B1 hydrolysates, zearalenone and esterigmatocistin) in 60 cracker samples.

Deoxynivalenol was found in 60 (100%) of the analyzed cracker samples. The mean concentration for the cracker samples was 481.14 μg kg$^{-1}$. In *cream cracker* samples, concentrations ranging from 66.4 to 1507.4 μg $kg_{-1}$ were found, with a means of 437.4 μg kg$^{-1}$. Similar levels (between 73.4 and 1444.8 μg kg$^{-1}$, with a mean of 524.9 μg kg$^{-1}$) were found in the *water and salt cracker* samples. The maximum concentration found for deoxynivalenol (1507.4 μg kg$^{-1}$) was lower than the maximum level (5295 μg kg$^{-1}$) found in the study conducted by Souza et al. (2015) with 23 cracker samples from Rio Grande do Sul, Brazil. Compared to studies conducted in other countries evaluating deoxynivalenol contamination, the maximum level found in the present study was higher than the values found by Savi et al. (2016) in crackers (1159 μg kg$^{-1}$) and Tanaka et al. (2010) in wheat-based biscuits (791 μg kg$^{-1}$), and similar to the value found by Almeida et al. (2016) in crackers (1720 μg kg$^{-1}$). The incidence of deoxynivalenol in the present study was higher than the 78% and 30% reported by Souza et al. (2015) and Savi et al. (2016) and similar to the 98% found by Tanaka et al. (2010).

Zearalenone was found in 30 (50%) of the 60 analyzed samples, in concentrations ranging from the LOD tp 14.83 μg kg$^{-1}$, with means of 6.22 μg kg$^{-1}$. Similar levels were found for this mycotoxin in the cream cracker and water and salt crakers, in 13 cream cracker samples (LOD to 13.58 μg kg$^{-1}$) and in 17 water and salt crackers samples (LOD to 14.83 μg kg$^{-1}$).

In a study carried out by Andrade (2016) in Brasilia, all 14 (100%) analyzed cracker samples were contaminated with zearalenone and the means found was of 560.0 μg kg$^{-1}$, above that found in the present study. In a study carried out in Japan by Tanaka et al. (2010), the levels found are in agreement with those found in the present study, although the incidence of zearalenone-contaminated samples was lower (2%).

Fumonisin B1 was detected (> LOD) in 17 (28%) of the analyzed samples, 8 cream cracker and 9 water and salt cracker samples. No studies were found on the presence of fumonisins in crackers, but their presence has been reported in wheat. In the south of Brazil, Mallmann et al. (2001) found fumonisin B1 in one wheat sample. Stankovic et al. (2012) analyzed Serbia wheat samples and found fumonisin B1 in 92 of the 103 analyzed samples, in concentrations ranging from 750 to 5400 μg kg$^{-1}$. Li et al. (2015) found fumonisin B1 in 22 (6%) out of 369 wheat flour samples in China, at concentrations ranging between 0.3 and 34.6 μg kg$^{-1}$. The fumonisin contamination found in the samples analyzed herein may be due to the possible presence of corn starch in the samples.

In the present study, four samples showed simultaneous contamination by deoxynivalenol, zearalenone and fumonisin B1. Stankovic et al. (2012) analyzed 103 wheat samples and reported that 47.8% of the samples contained deoxynivalenol, zearalenone and fumonisin B1, also indicating simultaneous contamination by these compounds.

The concentrations found herein were evaluated according to RDC Resolution No. 07/2011, in force during the sample collection period, that establish the maximum permissible level of 200 and 1750 μg kg$^{-1}$ for zearalenone and deoxynivalenol in crackers, respectively (Brasil, 2011). The results were also evaluated according to RDC No. 138/2017, in effect from January 2017, establishing maximum permissible level of 100 and 1000 μg kg$^{-1}$ for zearalenone and deoxynivalenol in crackers, respectively (Brasil, 2017). No samples exceeded the maximum permissible level according to RDC No. 07/2011 for deoxynivalenol and zearalenone. However, if we consider RDC No. 138/2017, a total of 7 (11.7%) samples would exceed the maximum permissible level for deoxynivalenol. A summary of the results found for the analyzed samples is exhibited in Table 6.

**Table 6**. Results found for the samples analyzed in the present study.

| Mycotoxins | Positive samples (%) | Cream crackers (μg kg$^{-1}$) | | | Water and salt crackers (μg kg$^{-1}$) | | |
|---|---|---|---|---|---|---|---|
| | | minimum | maximum | average | minimum | maximum | average |
| Deoxynivalenol | 60 (100%) | 66.4 | 1507.4 | 437.4 | 73.4 | 1444.8 | 524.9 |
| Zearalenone | 30 (50%) | >LOD | 13.58 | 6.64 | >LOD | 14.83 | 6.38 |
| Fumonisin B1 | 17 (28%) | >LOD | < LOQ | - | >LOD | <LOQ | - |

LOD: limit of detection; LOQ: limit of quantification.

### *3.3 Estimation of deoxynivalenol exposure in cream crackers and water and salt crackers*

The maximum tolerable intake represents the maximum human exposure allowed as a result of the natural occurrence of a certain substance in food, without damaging the health of the individual. To calculate this, the levels established by government agencies should be considered (Souza et al., 2015).

The Brazilian Association of Biscuit Industries estimated that per capita Brazilian consumption of biscuits was of 8.47 kg in 2015, with the consumption of crackers corresponding to 21% of this total (Associação Brasileira das Indústrias de Biscoitos, Massas Alimentícias e Pães & Bolos Industrializados, 2015). Based on these data, cracker consumption was estimated at 4.8 g per capita per day. Exposure to deoxynivalenol through cracker consumption was then estimated according to previously published studies (Ibáñez-Vea et al., 2011; Pacin et al., 2010). Exposure to deoxynivalenol through crackers was estimated considering that this mycotoxin was present in all samples. The mean amount of deoxynivalenol found in biscuits, of 481 μg kg$^{-1}$, would result in a daily intake of 0.04 μg kg$^{-1}$ of body weight per day considering a 60 kg adult. This value would not exceed the limit proposed by the JECFA safety authorities (Joint FAO/WHO Expert Committee on Food Additives, 2001), which is of 1.0 μg kg$^{-1}$ body weight per day. Considering the highest concentration found in the analyzed samples (1507.4 μg kg$^{-1}$), 0.12 μg kg$^{-1}$ of body weight per day would be ingested by a 60 kg person, which would also not exceed the maximum limit allowed. Thus, cracker and salt and water crackers can contribute to 3.8% of total deoxynivalenol consumption recommended by JECFA.

Souza et al. (2015) and Savi et al. (2016) also estimated deoxynivalenol intake in crackers, which did not exceed the limit recommended by JECFA. In the study carried out by Savi et al., cracker consumption contributed to 3% of deoxynivalenol ingestion, in agreement with the values observed in the present study. The deoxynivalenol concentrations present in the biscuit samples of this study did not exceed the acceptable value defined by JECFA, but it is worth noting that cracker and salt and water crackers are not the only dietary source of deoxynivalenol, since wheat is present in many other products consumed daily. In addition, the estimated consumption data did not consider a higher daily intake or intake by different age groups.

## 4 Conclusions

An analytical method for the determination of fourteen mycotoxins in crackers by UPLC-MS/MS was validated. The method includes major regulated mycotoxins in wheat-based products (deoxynivalenol and zearalenone). The sample treatment method developed herein is useful for routine analyses, since it involves a simple simultaneous extraction/clean-up step followed by concentration of the obtained extracts. The validated method was applied to the determination of target mycotoxins in 60 cracker samples (cream cracker and water and salt crackers). Deoxynivalenol, zearalenone and fumonisin B1 were found, respectively, in 100, 50 and 28% of the analyzed samples.

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