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Analytical issue related to fumonisins: A matter of sample comminution?

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ABSTRACT

The aim of the present work was to evaluate the effect of sample particle size on fumonisins recovery during the extraction step. Four maize samples were ground and the resulted flours were separated in six different fractions according to their particle size (1000-250 μm). Fumonisin B1 and B2 were quantified on each fraction, as well as on the unfractionated sample by HPLC-MS/MS. Furthermore, the proximate analysis was carried out to exclude the influence of macro-constituent on the mycotoxins distribution. Although the six maize fractions were found to be characterized by the same macro-constituent composition, a significant increase ($p < 0.000$) of fumonisins content was observed in all the analysed samples. Extraction yields up to five times higher were found in the finer flours. From the above, it is clear that the sample particle size has a significant impact on the fumonisins recovery. The outcomes suggest that sample granulometry should be standardized for ensuring accuracy and reliability of the analysis results. Overall, the results of this study, although still preliminary, may offer a reliable analytical solution to finally address the "hidden fumonisin issue".

1. Introduction

Fumonisins are secondary metabolites produced in cereals by pathogen fungi, mainly *F. verticillioides* and *F. proliferatum*. These fungi species are widely spread in temperate climate countries and maize is one of the most damaged crops (Dall'Asta & Battilani, 2016).

Currently, more than 15 fumonisin homologues have been identified (Braun & Wink, 2018). Among them, fumonisin B1 (FB1) is the most toxic form and, in many cases, co-occurs with other congeners such as fumonisin B2 (FB2) and B3 (FB3) (EFSA CONTAM Panel, 2014). The relevance of these mycotoxins on human and animal health prompted the European Community to introduce maximum permissible limits in food and feed. Indeed, fumonisins content of corn and corn-based products are regulated by EC Regulation n°1126/2007. Furthermore, this regulation makes a clear distinction based on the flour granulometry. The reference value of particle size of 500 μm defined two different legislation limits, 1400 μg/kg for the coarser flours, while for the finer is 2000 μg/kg (Commission Regulation No 1126/2007, 2007).

Structurally, B-type fumonisins are formed by a C-20 (or C-19) long-chain amino-polyol backbone carrying two methyl groups. On the backbone, two propane-1,2,3-tricarboxylic acid (also named tricarballylic acid, TCA) side chains are esterified to hydroxyl groups at positions C14 and C15.

From an analytical point of view, fumonisins are commonly analysed through a water-methanol or water-acetonitrile extraction, followed by a liquid-chromatographic separation coupled with a mass spectrometer detector (Dall'Asta et al., 2009; Gazzotti et al., 2009). These methods often allow to reach satisfactory quality parameters and a very good sensitivity. However, fuomonisins analysis is usually more problematic compared to other mycotoxins. Indeed, data variability and reproducibility are critical issues, as reported elsewhere (Kim, Scott, & Lau, 2003; Park, Scott, Lau, & Lewis, 2004; Dall'Asta et al., 2009).

Over the years, authors have demonstrated that fumonisins extraction is strongly affected by analytical conditions. For instance, Pietri et al. (2012) observed unexpected low recoveries in maize flour samples caused by problems during the extraction step (Pietri & Bertuzzi, 2012). Many studies have been carried to enhance the extraction efficiency changing the solvent mixtures or using buffer, QuEChERS, solid phase extraction and immunoaffinity column purification (Marschik et al., 2013; Bertuzzi, Mulazzi, Rastelli, & Pietri, 2016; Solfrizzo et al., 2011). Lawrence, Niedzwiadek, and Scott (2000) have shown how the solvent temperature can deeply influence the fumonisins recovery (Lawrence et al., 2000). Finally, Dall'Asta et al. (2009) compared five different analytical methods and evidenced significance lack of agreement within the results (Dall'Asta et al., 2009).

All this variability can be explained taking into consideration the

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peculiar ability of fumonisins to interact with matrix constituents (Bryła, Roszko, Krystyna, Jedrzejczak, & Mieczysław W., 2016). Therefore, to overcome the analytical issue, those factors affecting the extraction yield should be effectively controlled. Among them, the interaction between the extraction solvent and the matrix should be maximised, in order to allow the disruption of matrix-analyte complexes. In this context, it is well known that a smaller particle size increases the surface available for the solvent in a solid-liquid extraction.

Therefore, the aim of the present work was to evaluate the effect of sample particle size on fumonisins recovery during the extraction step. For this purpose, maize grain samples were ground, and the resulted flours underwent to sieve-shaking fractionation. According to their particle size, six different fractions were obtained and analysed for FB1 and FB2 content.

2. Material and methods

2.1. Chemicals and sampling

FB1 and FB2 standard solutions (50 µg/ml) were purchased from Romer Labs (Tulln, Austria). LC-grade methanol and acetonitrile, formic acid and potassium hydroxide were obtained from Sigma-Aldrich (Stuttgart, Germany), while bi-distilled water was produced in-house by an Alpha-Q system (Millipore, Marlborough, MA). Certified Reference Materials (CRM), purchased from Trilogy Analytical laboratory (Washington, DC, USA), was used for analytical method validation.

Four naturally contaminated maize samples were included in the study. Whole maize kernels were finely ground with a laboratory mill (A11 Basic Analytical Mill, IKA, Stauffen, Germany) and 100 g of the obtained flour underwent to fractioning process using five certified ISO sieves and shaker Octagon 200CL (Endecotts, London, UK). The obtained fractions and the mass balance (calculated as average of the four samples), are reported in Table 1.

2.2. Fumonisins determination

Two grams of each fraction were extracted with 8 ml of CH_3OH/H_20 solutions (75:25, v/v). After homogenisation step (2 min × 14000 rpm) with Ultra turrax T18 (IKA, Stauffen, Germany) and centrifugation (10 min × 4000 rpm), supernatant was recovered, transferred into a vial and directly injected in the LC-MS/MS analysis. Each analysis was carried in triplicate.

The LC-MS/MS analysis was performed with 2695 Alliance (Waters, Milford, MA, USA) coupled with a QuattroTM triple quadrupole mass spectrometer using an ESI source (Micromass, Waters, Manchester, UK). Chromatographic conditions were as follow: column, C18 Synergi Fusion – RP (Phenomenex, 50 mm × 2.0 mm, 2,5 µm) equipped with a C18 precolumn cartridge; column temperature, 30 °C; flow rate, 0.2 ml/min; injection volume, 10 µL. Chromatographic separation was achieved using water (eluent A) and methanol (eluent B), both acidified with formic acid 0.2%. The gradient is described as follow: start with 30% B; 0–8 min to 65% B; 8–20 min to 90% B; 20–23 min isocratic step (column washing); 23–30 min to 30% B (initial conditions) as a re-equilibration step.

Table 1
Particle size range and mass balance of the ground maize fractions considered within this study.

Fraction	Particle Size (µm)	Percentage (%)[a]
F6	Ø > 1000	21.2
F5	710 ≤ Ø ≤ 1000	15.8
F4	500 ≤ Ø ≤ 710	13
F3	355 ≤ Ø ≤ 500	7.4
F2	250 ≤ Ø ≤ 355	8.3
F1	Ø ≤ 250	34.3

[a] The percentage is calculated as average of the four samples.

Table 2
Main in-house validation parameters.

	FB1	FB2
Recovery (%)	91.5 ± 3.2	79.7 ± 4.2
Calibration Range (µg Kg-1)[a]	25–1000	25–1000
LOD (µg Kg-1)[b]	25	25
LOQ (µg Kg-1)[b]	25	25

[a] High linearity (R2 > 0.99) has been observed in the used calibration.
[b] Since no blank samples were available, LOD and LOQ were set to the lower point of the calibration range providing sufficiently high S/N ratio.

MS parameters: the ESI source was operated in positive ionization mode (ESI+); capillary voltage 4,0 kV; extractor voltage 2,0 V; cone voltage 50 V; source block temperature 120 °C; desolvation temperature 350 °C; desolvation and cone gas (N_2) 700 and 50 L/h, respectively. Detector was used in the multiple reaction monitoring mode (MRM) and two transitions were monitored for each analyte (see Table 1 Supplementary Material). The main transition was used for quantification (quantifier ion), while the second as qualifiers transition. Matrix-matched calibration curves (calibration range 1000–4000 µg $kg-1$) were used for target analyte quantification. A good linearity was obtained for all the considered mycotoxins ($R^2 > 0.99$).

The present analytical method was in-house validated following the EURACHEM guidelines (Magnusson & Ornemark, 2014). The main validation parameters are briefly summarized in Table 2.

2.3. Proximate analysis

Proximate analysis was carried on each fraction to verify eventual differences in macro-constituent composition. An equal amount (100 g) of each sample were mixed before the fractionation step. Thus, the obtained fractions were representative of the entire sample set.

Macro-constituent characterization of each fraction was carried in term of lipid, protein and starch. Protein and lipid amounts were determined using Kjeldhal and Soxhlet apparatus, respectively (both from Velp Scientifica, Usmate Velate, Italia). The procedures were in accordance with AACCI Method 46–12.01 and AACCI Method 30–25.01 (International AACCI, Method 30-25.01) (International AACCI, Method 46-11.02). Starch content was determined by polarimetric method performed with Model 341 Polarimeter (Perkin Elmer, Waltham, MA, USA) (Mitchell, 1990).

2.4. Scanning electron microscopy

To visualize differences depending on the granulometry, qualitative images of the six fractions were acquired using a scanning electron microscope Phenom ProX (Phenom-World, Eindhoven, The Netherlands).

0.5 g of sample were transferred to the appropriate adhesive holder with the aid of a polypropylene tip. The powder particles not fixed on the adhesive were eliminated by air spraying. The samples were presented to the instrument and the images were obtained in different scales.

3. Results

3.1. Fumonisins extraction yield

To understand the relevance of the comminution step on the extraction of fumonisins, four maize samples were fractionated using certified ISO sieves. Samples were then analysed using LC-MS/MS to determine the fumonisins content, using a classical water/methanol extraction. The results, expressed as sum of FB1 + FB2 (µg/kg), are summarized in Fig. 1.