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Free and hidden fumonisins in various fractions of maize dry milled under model conditions

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ABSTRACT

In the present study the milling of corn was made and then in the milled fractions the content of the fumonisins was analysed. These conditions of milling model have many advantages, e.g. the ease unifies the various milling fractions derived from the entire lot which allows for increased precision results. The concentration of free/hidden fumonisins in fractions from dry milled maize under modelled conditions (i.e., in flour and bran/germs) was compared to the initial fumonisin concentration in the grain. Both free and hydrolysed forms of fumonisin were extracted and determined using LC/MS. The concentration of both free and hidden fumonisins in the milled fractions was statistically significantly ($p \leq 0.05$) diversified: higher in the bran and lower in the flour. The free and hidden fumonisin levels in bran increased by 69% and 46%, respectively, while they decreased in flour by 28% and 20%, respectively, relative to maize. Statistically significant ($p \leq 0.05$) differences in the concentrations were also observed before and after hydrolysis in both the unprocessed grain and milled fractions. These findings indicate the components that interact with fumonisins via supra-molecular bonds are non-uniformly distributed throughout the grain volume.

1. Introduction

Fumonisins are secondary metabolites of the *Fusarium* fungi (primarily *Fusarium verticillioides* and *Fusarium proliferatum*) that typically contaminate maize. Therefore, they are commonly found in milled maize fractions and highly processed products. Fumonisins may adversely influence health in mammals including exhibiting hepato-carcinogenic, hepatotoxic, nephrotoxic, and cytotoxic effects (Bryła, Jędrzejczak, et al., 2013; Bryła, Roszko, et al., 2013; Clements, Kleinschmidt, Maragos, Pataky, &White, 2003; Dilkin et al., 2002; Munkvold & Desjardins, 1997). In humans, significant exposition to fumonisin B_1 (FB_1) is suspected to increase the risk of oesophagus cancer (Pagliuca et al., 2005), while consuming maize-based foods contaminated with fumonisins is associated with neural tube defects (Marasas et al., 2004). For these reasons, FB_1 has been classified by the International Agency for Research on Cancer as a group 2B carcinogen (IARC, 2002).

In Europe, the contamination of maize with fumonisins is mainly an issue in Mediterranean countries where the climate favours the fungal development responsible for the biosynthesis of fumonisins, particularly Italy (Pietri, Bertuzzi, Pallaroni, & Piva, 2004). In Poland, maize is increasingly cultivated (both for food and fodder) because it is easy to produce and adapts very well to various climates (CSO, 2013). The degree to which home-cultivated maize is infested with *Fusarium* fungi depends on the growing season (Waśkiewicz & Goliński, 2013). However, maize imported to Poland for food may be significantly more polluted with fumonisins.

The European Union has set the following maximum permissible levels for the $FB_1 + FB_2$ sum in maize: 4000 μg/kg in unprocessed grain, 2000 μg/kg in ≤500 μm milled fractions, 1400 μg/kg in >500 μm milled fractions, 1000 μg/kg in maize intended for human consumption, 800 μg/kg in corn flakes/snacks, and 200 μg/kg in food intended for children (EC 1126/2007).

Fumonisins are chemically stable at elevated temperatures. A significant heat-induced drop is only observed after processing at temperatures above 150 °C (Bolger et al., 2001; Ren, Zhang, Han,

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Han, & Wu, 2011; Waśkiewicz, Beszterda, & Goliński, 2012). According to Bullerman and Bianchini (2007) thermally degrading at least 90% of the initial fumonisin concentration requires heating them for 60 min at 175 °C regardless of the ambient pH. Other studies suggest thermally induced drops in fumonisins content may result from changes in the mycotoxin structure via interactions with other food constituents to form conjugates (Falavigna, Cirlini, Galaverna, & Dall'Asta, 2012).

The reactions between heated FB_1 and reducing sugars, which produces strong covalent bonds, are well understood. The products of such reactions include *N*-(deoxy-D-fructos-1-yl)-fumonisin B_1 (NDF-FB_1) produced by the Amadori rearrangement of the Schiff's base while the FB_1 primary amine reacts with a D-glucose aldehyde group and *N*-carboxymethyl-fumonisin B_1 (NCM-FB_1) produced from NDF-FB_1 (Bullerman, Bianchini, Jackson, Jablonski, & Ryu, 2008; Poling, Plattner, & Weisleder, 2002). Other forms fumonisins (which include hidden fumonisins) also occur naturally. Such compounds may non-covalently interact with macroconstituents in unprocessed maize (e.g., proteins or starch). During these interactions, mycotoxins are physically trapped within biopolymer structures present in the maize grain. Such phenomena invalidates the results of traditional analytic methods developed to determine fumonisins (both free and hidden) when the latter should be well controlled for food and feed in view of the potential toxicity (Dall'Asta, Falavigna, Galavern, Dossen, & Marchelli, 2010; Dall'Asta, Galaverna, et al., 2009; Dall'Asta, Mangia, et al., 2009; Falavigna et al., 2012).

Food/fodder processing may modify the mycotoxin content in raw produce. The sorting/cleaning of maize grain is one of the most effective ways to decrease the fumonisins concentration in the final product because broken grains may contain up to 10 times the fumonisins of healthy ones (FAO/WHO, 2012; Humpf & Voss, 2004; Pascale, Visconti, Prończuk, Wiśniewska, & Chełkowski, 1997).

The distribution of fumonisins in maize grain/milled fractions was studied by several authors (Brera, Debegnach, Grossi, & Miraglia, 2004; Brera et al., 2006; Broggi et al., 2002; Bullerman & Bianchini, 2007; Vanara, Reyneri, & Blandino, 2009). Most fumonisins were found at the grain periphery in the pericarp, the part of the grain most readily colonised by the fungus. A large concentration is usually also found in the vicinity of the endosperm. Vanara et al. (2009) and Broggi et al. (2002) reported ∑FBs concentrations in bran/germs three times those in whole grain. Pietri, Zanetti, and Bertuzzi (2009) reported FB_1 levels in bran/germs were 50–167% higher than in the whole grain. Katta, Cagampang, Jackson, and Bullerman (1997) and Brera et al. (2004) reported higher levels in bran/germs and lower levels in flour relative to the grain. However, this study is among the first on the distribution of fumonisins in maize grain/maize milling fractions and also investigates hidden fumonisins along with the free forms.

This work aims to compare the concentration of free/hidden fumonisins in fractions produced by dry milling maize under model conditions to initial fumonisin concentration in the maize.

2. Materials and methods

2.1. Chemicals

Only solvents of HPLC grade supplied by Rathburn (Walkerburn, UK) were used in this study. Formic acid, acetic acid, and potassium hydroxide of analytical grade were supplied by POCH (Gliwice, Poland). FB_1, FB_2, FB_3 (50 μg/mL), $^{13}C_{34}$-labelled FB_1 (25 μg/mL), and $^{13}C_{34}$-labelled FB_2, FB_3 (10 μg/mL) certified reference materials were supplied by Biopure (Tulln, Austria). Water was purified using a two-stage system (reverse osmosis – ion-exchange resin) made by Hydrolab (Wiślina, Poland).

2.2. Samples

Naturally contaminated maize from Italy (growing season 2012) was delivered by Silesian Grain (Ziębice, Poland). Approximately 10 kg of grain was milled to the required granulation using an MLU-202 laboratory mill manufactured by Bühler GmbH (Uzwil, Switzerland). The mass was homogenised in an MR-2L grain/flour mixer manufactured by Chopin Technologies (Villeneuve-la-Garenne, France). A 200 g sample was used for the analysis. The remaining portion was sieved through a 1000 μm mesh sieve to separate the bran and germ from the flour. The flour and bran analytical samples were also 200 g. A flowchart of the maize grain processing is shown in Fig. 1. The flour fragmentation was determined using a calibrated sieve as shown in Table 1.

2.3. Analytical procedures for determining fumonisins

The free and total fumonisins were extracted and determined according to the method proposed by Bryła et al. (2014).

2.3.1. Free fumonisins

Samples were ground using a laboratory grinder, and 2.5 g were transferred to a glass beaker. A 10 μL $^{13}C_{34}$-labelled FB_1, FB_2 and FB_3 internal standard solution was added. The sample was homogenised with 10 mL methanol: acetonitrile: water solution 25:25:50 (V/V/V) for 3 min. The solution was centrifuged at 10,730 × g; 5 mL of the supernatant was mixed with 5 mL of water and used for the analysis. The extracts were purified using molecularly imprinted polymer cartridges (FumoZON AFFINIMIP, Polyintell, Val de Reuil, France) conditioned with 2 mL of acetonitrile with a 3–4 droplets per second flow rate, and then washed with 2 mL of water. Four 2 mL portions of the supernatant were loaded onto the cartridge, which was washed with a 6 mL acetonitrile: water mixture 40:60 (V/V). The analytes were eluted with 4 mL of methanol containing 2% formic acid into a 25 mL round bottom flask. The solution was evaporated to dryness at 40 °C using a rotary evaporator, redissolved in a 1 mL methanol: water: acetic acid mixture (10:89.9:0.1), and sonicated in an ultrasound bath. The samples were filtered through a 0.2 μm nylon syringe filter, transferred into glass vials, and loaded using an Accela autosampler (Austin, TX, USA) for analysis in a Thermo-Finnigan LC–MS/MS system (Austin, TX, USA) equipped with an ion trap mass detector.

2.3.2. Total (free + hidden) fumonisins

To determine the hidden fumonisins, the samples were first hydrolysed in an alkaline medium. All fumonisin forms – free, hidden, and covalently bound to other food constituents via carboxyl groups – hydrolyse in alkaline media to produce chains of hydrolysed fumonisins. A well-ground 0.5 g sample was weighed into a PP centrifuge tube, and 10 μL of a $^{13}C_{34}$-labelled FB_1, FB_2 and FB_3 internal standard solution was added. Subsequently, 10 mL of 2 M KOH was added, and the sample was hydrolysed for 24 h at room temperature. The hydrolysed solution was shaken in 12.5 mL of dichloromethane and centrifuged at 10,730 × g. A 5 mL portion of the organic extract was transferred into a round bottom flask and evaporated to dryness using a rotary evaporator operated at 40 °C. The dry residues were dissolved in a 1 mL methanol: water: acetic acid solution (30:69.9:0.1) (V/V/V), filtered through a 0.2 μm nylon syringe filter, transferred into glass vials and analysed using an LC–MS/MS system. The hydrolysed fumonisin concentrations were recalculated as a free form concentration using the molar mass quotient for before and after the hydrolysis as the conversion factor. Both spiked (used to determine the method recovery rate) and real samples were simultaneously hydrolysed with the standard solutions (the same temperature and time).