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'Emerging' mycotoxins in cereals processing chains: Changes of enniatins during beer and bread making

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ABSTRACT

Enniatins represent an emerging food safety issue because of their extensive incidence, documented in recent decades, in various small grain cereals. This study was concerned with the fate of these *Fusarium* mycotoxins within malting, brewing, milling and baking, when employed for the processing of contaminated barley and wheat. Besides enniatins A, A1, B and B1, also deoxynivalenol and its conjugated form (deoxynivalenol-3-glucoside) were determined in almost all tested cereal-based samples. Significant decline of enniatins occurred within all technologies, with the largest drop in their concentrations observed in the brewing process. While enniatins were not detectable in final beers, they were almost quantitatively transferred to spent grains, probably because of their limited water solubility. Regarding bread baking, levels of enniatins decreased down to 30% of their concentration in the initial flour used for baking. In this case, degradation at higher temperatures might be assumed.

1. Introduction

The occurrence of *Fusarium* mycotoxins in cereal-based foods and feeds is a global issue of high concern, due to their potential health risks for humans and/or livestock. While maximum levels have been legislatively laid down for the main representatives of this group, namely deoxynivalenol (DON), zearalenone (ZON) and fumonisins (EU., 2006, 2007), for several other *Fusarium* mycotoxins (e.g. HT-2 and T-2 toxins) scientific opinions on health risks are still being prepared by the CONTAM Panel (The Panel on Contaminants in the Food Chain of the European Food Safety Authority). Based on the requests received from the European Commission, EU member states, research institutions, academia, industry, trade and other stakeholders were in the first phase invited by EFSA (EFSA webside., 2012a) to submit data on: (i) levels of T-2 toxin and HT-2 toxin analysed in food and feed, (ii) levels of nivalenol (NIV) analysed in food and feed, and (iii) levels of zearalenone analysed in food (the deadline was November, 2010). In the follow-up phase, collection of information enabling assessment of the risks to human and animal health related to the presence of several other mycotoxin classes in food and feed was required (EFSA webside., 2012b). Several recently emerged *Fusarium* toxins, represented by enniatins (ENNs), beauvericin (BEA), fusaproliferin (FUSA) and moniliformin (MON) were on the list as well. All of these toxins belong to the group of 'emerging' mycotoxins, which are neither routinely determined, nor legislatively regulated; however, the evidence of their incidence is rapidly increasing (Jestoi, 2008; Malachova et al., 2011).

An extensive overview of the physicochemical and interesting biological properties of these emerging *Fusarium* toxins was published by Jestoi (2008). In this work, the distinct significance of these compounds in the human and animal food chains and a strong recommendation for their better assessment was postulated. In any case, compared to regulated *Fusarium* mycotoxins, the prevalence data on these, until recently considered as less important, fungal secondary metabolites are really scarce, generally limited to Northern Europe and the Mediterranean areas. In a recent review, focused on the occurrence of ENNs, BEA and FUSA in small grains, maize and processed grain-based food, a connection between observed contamination pattern and climate changes was highlighted (Santini, Meca, Uhlig, & Ritieni, 2012). The *Fusarium* species capable of producing ENNs are obviously spread across various geographical regions and the extent of grains contamination is occasionally as high as mg/kg (Logrieco, Rizzo, Ferracane, & Ritieni, 2002). For instance, in samples from the Spanish market the most abundant toxin representing this group was enniatin A1 (ENN A1), its concentration as high as 814 mg/kg was found in corn samples (Meca, Zinedine, Blesa, Font, & Manes, 2010). On the other hand, enniatin B (ENN B) was a dominating toxin in most studies conducted on small grains in Nordic countries; its maximum

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concentration level in wheat from Finland was as high as 18.3 mg/kg (Jestoi et al., 2004). The relative concentration ratio of target mycotoxins reported in another study was as follows: ENN B > ENN B1 > ENN A1 > ENN A > BEA (Uhlig, Jestoi, & Parrika, 2007).

In our previous research (Malachova et al., 2011), altogether 23 mycotoxins representing the following four groups: (i) trichothecenes and zearalenone; (ii) ENNs and BEA; (iii) ergot alkaloids; and (iv) alternaria toxins, were monitored in flours and some other cereal-based products, e.g., bakery products, breakfast cereals, and snacks, collected from the Czech market. Common trichothecenes B and four ENNs (A, A1, B and B1) were present in samples at relatively high levels. While the frequent presence of DON together with its conjugate DON-3-glucoside (DON-3-Glc) was in line with our expectation, high occurrence of at least one of four target ENNs practically in all of the 116 examined samples was rather surprising. The most abundant was ENN A, which was detected in 97% of samples (concentration range 20–2532 μg/kg), and followed by ENN B with an incidence in 91% of samples (concentration range 13–941 μg/kg). To get more information on these major mycotoxins in small grains harvested in the Czech Republic, a wide range of wheat and barley samples were analysed within the follow-up initiated monitoring study. The unpublished results confirmed a ubiquitous occurrence of ENNs in cereals; thus additional questions, e.g., 'What is the impact of agricultural practices on their levels?' or 'What is the fate of ENNs during food processing?', have logically emerged.

Until now, practically no information is available on the structural/concentration changes of ENNs and BEA during food processing. The aim of the current study is to contribute to filling in this gap, thus enable refining consumers' dietary exposure estimate and, if possible, to generate data, on the basis of which respective stakeholders might take up preventive measures, ensuring minimisation of risks associated with ENNs intake. Four important cereals processing technologies, represented by milling, baking, malting and brewing, were used to learn more about ENNs transfer from raw materials, barley and wheat, into the final products. To obtain comprehensive information on the fate of other mycotoxins contained in experimental grains grown under various agricultural conditions, DON and masked DON-3-Glc, co-occurring in cereals with ENNs, were also monitored.

2. Materials and methods

2.1. Reagents and chemicals

The analytical standards of ENN A, ENN A1, ENN B, ENN B1 and BEA were purchased from Alexis Biochemicals (New York, NY). Trichothecenes standards of DON, DON-3-Glc, and isotopically labelled $^{13}C_{15}$-deoxynivalenol (used as internal standard) were obtained from Biopure (Tulln, Austria). Stock standard solution, 0.1 mg/mL for enniatins and 1 mg/mL, were prepared in acetonitrile and stored at −20 °C. A composite working standard solution in acetonitrile (5000 ng/mL) used for spiking experiments and for calibration purposes was prepared by mixing of suitable aliquots of each individual standard stock solution. Organic solvents used for extraction and LC–MS analysis (HPLC grade acetonitrile and methanol) as well as ammonium formate and sodium chloride were obtained from Sigma–Aldrich (Taufkirchen, Germany). Ultra-pure water was produced by Milli-Q system (Millipore Corporation, Bedford, MA). Anhydrous magnesium sulphate was obtained from Penta (Prague, Czech Republic).

2.2. Samples

2.2.1. Malting and brewing

For the malting and brewing experiments, spring barley of variety Radegast (recommended by Czech Beer and Malt Association for production of typical Czech beer) was used. It was grown at the experimental station of Agrotest Fyto Ltd. (Kromeriz, Czech Republic) under two agricultural conditions: (i) low-input system without any chemical treatment (NON) and (ii) conventional system with the application of fungicides (FUN). Both samples of barley, NON as well as FUN-treated, were micro-malted at the Research Institute of Brewing and Malting (Brno, Czech Republic). Finally, two series of malts and malting samples were collected during the malting process. Each series contained seven intermediate samples: input barley; barley after one, two and three days of steeping; green malt; final malt; and rootlets. Altogether, 14 samples of both NON and FUN series were analysed, covering the whole malting process. Identical conditions as those used in our previous study concerned with the fate of trichothecenes during brewing were employed for beer production (Lancova, Hajslova, Poustka, et al., 2008). Two types of malts NON and FUN, obtained as a final product of our above mentioned micromalting processes, were used as raw materials for beer production. For both of these series, the following samples were collected during brewing: first wort, sweet wort, young wort, green beer, final beer and waste product (spent grains).

2.2.2. Milling and baking

For milling and baking experiments, winter wheat cultivar Eurofit, classified as A bread supplementary (quality bakery wheat) was grown under conventional (CON) and organic (ORG) farming conditions. Both types of wheat were milled under the laboratory-scale conditions using a Bühler laboratory mill (MLU-202 type, Bühler, Switzerland), when milling fractions white flours, shorts and bran were obtained for CON as well as ORG series. Both samples of flour were further used for baking experiments. For preparation of one batch of pilot bread-baking, 300 g flour, 12 g leavening agent, 3 g fat, 5.1 g salt, 4.5 g sucrose and approximately 160 mL of distilled water were used. The conditions of milling and baking technological parameters are described in detail in our earlier paper (Lancova, Hajslova, Kostenlanska, et al., 2008). All milling fractions, three dough intermediates (kneaded, fermented, proofed dough) and final bread of both CON and ORG series were used for LC–MS analysis. All of the above described brewing and baking samples were analysed in duplicate and presented concentration levels were obtained as a mean value of these two analysis.

2.3. Sample preparation procedures

Two sample preparation procedures were implemented for this purpose. Based on water content, respective sample was processed either as a "solid" or "liquid" as follows.

2.3.1. Solid samples

Modified QuEChERS extraction/clean-up procedure described earlier (Zachariasova, Lacina, et al., 2010) was implemented and validated for solid cereal matrices, dough and bread (samples of dough were lyophilised prior to homogenisation and extraction; bread samples were dried down). Briefly, to 2 g of homogenous representative sample weighed into a PTFE cuvette, 7.5 mL of deionised water and 10 mL of acetonitrile were added. The suspension was shaken vigorously by hand for 3 min. After addition of 1 g of NaCl and 4 g of $MgSO_4$, the mixture was shaken again. The separation of aqueous phase from organic one was achieved using centrifugation (5 min, 5000 rpm). To control potential mycotoxins losses, e.g., due to the partition between an organic and an aqueous phase, an aliquot of $^{13}C_{15}$-labelled DON standard solution corresponding to a contamination level of 100 μg/kg was added into each sample prior to sample processing.