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Occurrence and co-occurrence of *Fusarium* mycotoxins in wheat grains and wheat flour from Romania

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Enniatin B1 (PubChem CID: 11262300)
Neosolaniol (PubChem CID: 13818797)
Zearalenone (PubChem CID: 5281576)

ABSTRACT

In this study, the presence of fourteen *Fusarium* mycotoxins, legislated by the European Union – deoxynivalenol, zearalenone, HT-2 and T-2 toxins (EC/1881/2006; 2013/165/EU), or non-legislated (five trichothecens and five "emerging" mycotoxins), was evaluated in 31 whole unprocessed wheat samples and 35 white wheat flour samples from different areas of Romania. For this purpose, a validated multi-mycotoxins liquid chromatography tandem mass spectrometry method was applied. Seventy three percent of the analyzed samples contained at least one mycotoxin. The highest occurrence was for enniatin B, 71% of the analyzed samples being positive (21–407 μg kg^{-1}). Regarding the legislated mycotoxins, deoxynivalenol was detected in 14% (111–1787 μg kg^{-1}) of the samples, while zearalenone was detected in 9% (51–1135 μg kg^{-1}). Only one sample was positive for neosolaniol. Concerning the co-occurrence, 42% of the samples were contaminated with two to five mycotoxins, the most frequent being the binary or tertiary combinations of enniatins. This is the first study applied to Romanian wheat grains and flour samples using a high sensitive multi-mycotoxins method, and which included also "emerging" mycotoxins.

1. Introduction

Mycotoxins can be present in vegetable foods which can serve as a substrate for the growth of filamentous fungi of the genera *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria*.

There are many factors that predispose to mycotoxin production by fungi including substrate type and availability, climate conditions, storage and processing conditions (Covarelli, Beccari, Antonio et al., 2015, Covarelli, Beccari, Prodi et al., 2015; Logrieco, Bottalico, Mulé, Moretti, & Perrone, 2003). Cereals contamination is the most important for mycotoxins occurrence, particularly for wheat, maize and rice which are of major importance (Pereira, Fernandes, & Cunha, 2014). The Rapid Alert System for Food and Feed of the European Union reports mycotoxins on the third position according to the total number of hazard notifications (RASFF, 2015).

Wheat is considered to be the main strategic crop in the world with a global production of 729 million tonnes in 2014. The European Union (EU) is the world's largest wheat producer. Romania is one of the five biggest wheat producers in the EU with a harvested area of 2,107,813 hectares and an annual production of 7,584,814 tonnes in 2014 (RINS, 2015). More than half of Romanian annual wheat production is exported in different countries around the world (USDA, 2015). On the other hand, for Romanian population a high wheat and wheat products consumption is registered (364.6 g/capita/day), more than the European average (295.4 g/capita/day), and the double of the world average (178.8 g/capita/day) (FAOSTAT, 2015).

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Growing wheat in Romania is a traditional and important part for the national agriculture. Main production area for wheat is Danube plain in the South of the country. Other important wheat growing areas are Transylvania, northern part of Moldova in north-eastern Romania, and Banat region in the South-West (Bălan, 2015).

Presence of various moulds and their specific mycotoxins may be different according to climatic conditions. *Fusarium* spp. are the most prevalent toxin-producing fungi in cereals from temperate regions of America, Europe and Asia (Medina, Rodríguez, & Magan, 2015). Romania has a temperate-continental climate with proper temperatures, rainfall and humidity for wheat growing. In the last twenty years, extreme meteorological events have been registered in this area, such as excessive dryness, substantial rainfall, tropical days, or high humidity (Bojariu et al., 2015; USDA, 2015).

Species of *Fusarium* genera are able to produce in wheat three of the most important classes of mycotoxins: fumonisins (FB1, FB2, FB3); zearalenone (ZEA); trichothecenes: HT-2 toxin (HT-2) and T-2 toxin (T-2), diacetoxyscirpenol (DAS), neosolaniol (NEO), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3AcDON), 15-acetyldeoxynivalenol (15AcDON) and fusarenon-X (FUS-X). They can also produce "emerging" mycotoxins like fusaproliferin (FUS), beauvericin (BEA), enniatins (ENA, ENA1, ENB, ENB1) and moniliformin (MON), or other mycotoxins as fusaric acid, fusarin A-D, gliotoxin, butenolite, which are recently discovered (Stanciu et al., 2015).

In vitro and *in vivo* studies have demonstrated for trichothecens, zearalenone and fumonisins their nephrotoxic, hepatotoxic, carcinogenic, immunosuppressive and mutagenic properties (Escrivá, Font, & Manyes, 2015). Moreover, in the last years, toxicological studies about the co-presence of mycotoxins have been demonstrated their synergistic toxic effects (Juan-García, Juan, König, & Ruiz, 2015). Consequently, the EU established for DON, ZEA, sum of FB1 and FB2, sum of HT-2 and T-2 maximum permitted or recommended levels in foodstuffs (EC, 2006a, 2013); the Joint FAO/WHO Expert Committee in Food Additives (JECFA, 2001) and the Scientific Committee on Food (SCF, 2002) have proposed tolerable daily intakes (TDI) for DON and ZEA, and provisional maximum TDIs (PMTDI) for NIV, the sum of DON, 3AcDON and 15AcDON, and the sum of T-2 and HT-2, respectively.

However, for "emerging" mycotoxins limited data are available until now, and they are currently under occurrence and toxicity evaluation (EFSA, 2014; Juan-García, Manyes, Ruiz, & Font, 2013).

In the past decade, researchers from different European countries have focused their investigations on wheat and wheat derivatives mycotoxin contamination, especially for legislated mycotoxins like DON, ZEA, HT-2, T-2, aflatoxin and ochratoxin A (Jajić, Jurić, Glamočić, & Abramović, 2008; Juan, Covarelli, Beccari, Colasante, & Mañes, 2016; Lazzaro, Moretti, Giorni, Brera, & Battilani, 2015; Van Der Fels-Klerx et al., 2012; Šliková, Gavurníková, Šudyová, & Gregová, 2013); until now, only few studies were done in Romania, most analyzing DON, ZEA or FB1 by enzyme-linked immunosorbent assay (ELISA), and founding that DON is the most frequent myocotoxin in wheat from eastern and western Romania (Alexa et al., 2013; Banu, Aprodu, & Nicolau, 2011; Stroia, Tabuc, & Neacsu, 2010). Moreover, multi-mycotoxin methods were optimized and validated, with the purpose to analyze more mycotoxins simultaneously, with a high sensibility and sensitivity (Juan et al., 2016). These methods and fungal studies have been demonstrated the co-presence of more than one mycotoxin in the same food matrix, indicating the importance of mycotoxins co-occurrence evaluation (Covarelli, Beccari, Antonio et al., 2015, Covarelli, Beccari, Prodi et al., 2015; Juan, Ritieni, & Mañes, 2013).

The objective of this study was to evaluate the presence and the possible co-occurrence of 14 myotoxins (NIV, DON, 3AcDON, 15AcDON, DAS, NEO, HT-2, T-2, ZEA, BEA, ENA, ENA1, ENB, and ENB1) in 66 wheat and flour samples from Romania. For this, a validated multi-mycotoxins method using liquid chromatography tandem mass spectrometry (LC-MS/MS) was applied. As far as we know, this is the first study which evaluates simultaneously a broad spectrum of *Fusarium* mycotoxins in wheat grains and wheat flour from Romania using a highly sensitive method.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were supplied by PanReac AppliChem (Castellar del Vallés, Spain), whereas methanol LC-MS/MS grade (≥99.9%) was supplied by VWR International Eurolab (Llinars del Valles, Barcelona, Spain).

Ammonium formate (99%) and formic acid (≥98%) for mobile phases were obtained from Sigma Aldrich (St. Louis, USA). Deionized water (<10 MΩcm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Beadford, MA, USA).

Filter papers of Whatman No. 4 type (Maidstone, England) were used to filter the extract samples. Syringe nylon filters (13 mm diameter, 0.22 μm pore size) were purchased from Análisis Vínicos S.L. (Tomelloso, Spain).

The certified standards of ZEA, NIV, DON, 3AcDON, 15AcDON, DAS, NEO, T-2, HT-2, BEA and ENs (A, A1, B, B1) were purchased from Sigma Aldrich (Madrid, Spain).

The individual stock solutions were prepared in acetonitrile at two concentrations, ENs (A, A1, B, B1), and BEA at 500 μg mL^{-1}, and ZEA, NIV, DAS, NEO, DON, 3AcDON, 15AcDON, T-2, and HT-2 at 1000 μg mL^{-1}. Also, a working mixed standard solution in methanol at concentrations between 0.2 and 10 μg mL^{-1} were prepared by diluting the individual stock solutions. This solution was used to prepare the calibration curves, matrix matched calibration curves, recovery and for repeatability studies (intraday and interday). The solutions were kept in glass-stoppered bottles and darkness in safety conditions at −20 °C.

2.2. Sampling

A total of 66 wheat samples, including whole unprocessed wheat (31) and white wheat flour (35), were analyzed in order to investigate the presence of mycotoxins. Whole wheat samples were collected during 2014 harvesting season from four different Romanian areas: Bihor (2) – in the North-West of the country; Braşov (2), Dâmboviţa (7) – in central Romania; Teleorman (20) – in the South of the country. Information about growing area (county and city) was collected. White wheat flour samples were purchased from different markets located in Târgovişte (Dâmboviţa county, Romania), during winter season of 2015, between January and March 2015.

The sampling was performed according to the EU guidelines (EC, 2006b) for the official control of legislated mycotoxins for lots of cereals and cereal products less than 50 tons. Consequently, for both wheat and wheat flour samples, three incremental samples of 1 kg were collected or purchased, obtaining an aggregate sample of 3 kg total weight. After homogenization, samples were packed in plastic bags and kept at −20 °C in a dark and dry place until analysis. Before the analysis, for all the samples, subsamples of 300 g were milled with a blender and divided into three bulks of 100 g each.