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Natural co-occurrence of mycotoxins in wheat grains from Italy and Syria

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ABSTRACT

This article describes the application of an analytical method for the detection of 25 mycotoxins in wheat grain based on simultaneous extraction using matrix solid-phase dispersion (MSPD) followed by liquid chromatography coupled to tandem mass spectrometry, a hybrid triple quadrupole-linear ion trap mass spectrometer (QTrap®). Information Dependent Acquisition (IDA), an extra confirmation tool for samples that contain the target mycotoxins, was used. The analysis of 40 Syrian and 46 Italian wheat grain samples interestingly showed that Syrian samples were mainly contaminated with ochratoxin A and aflatoxins, whereas Italian samples with deoxynivalenol and 15-acetyldeoxynivalenol. Emerging *Fusarium* mycotoxins were predominant in Italian samples compared to the Syrian. Among the analysed samples, only one was found containing zeralenone with level above the maximum European recommended concentration (100 ppb). These results confirm that climatic differences between Syria and Italy, both in Mediterranean basin, play a key role in the diversity of fungal genera and mycotoxins in wheat grains.

1. Introduction

FAO estimation for global wheat production in 2011 was at a record 676 million tonnes, while for the European Union (EU), the world's largest producer, was 142 million tonnes (FAO, 2011). In Syria, wheat is considered to be the main strategic crop with an annual production that ranged from 4 to 5 million tonnes in the last years (FAO, 2011; NAPC, 2009; Sadiddin & Atiya, 2009). In Syria wheat two main products, bread and bulgur, are common food commodities (Haydar, Benelli, & Brera, 1990) with an average consumption of bread of 12.9 kg per capita per month (FAO, 2003). The importance of wheat has been mainly attributed to its ability to be ground into flour and semolina, which form the basic ingredients for bread and other bakery products, and pasta (Chandrika & Shahidi, 2006).

Wheat can be easily grown in different climatic regions, and then it is exposed to many pathogenic fungi, of which some are toxigenic fungi, such as *Fusarium, Aspergillus* and *Penicillium*. These fungal genera are producers of, mycotoxins, which are a hazard for both human and animal health (Mankeviciene, Butkutė, Dabkevičius, & Suproniene, 2007). Wheat kernels can be contaminated during pre or/and post-harvest with mycotoxins. The knowledge of the occurrence of specific fungal genera in different growing areas will help to predict mycotoxin content in harvested grains.

The most well-known mycotoxins, aflatoxins (AFs), ochratoxin A and some *Fusarium* toxins (deoxynivalenol, zearalenone, *fumonisins),* have been classified by IARC (1993) and regulated by European Union (EU, 2006a, 2010). On the other hand, there are other mycotoxins, such as enniatins and beauvericin, which have not been classified nor legislated yet.

Mycotoxin existence depends on several factors, such as fungal strains presence, climatic and geographical conditions, cultivation technique and susceptibility level of host plants and crop management practices (Logrieco, Bottalico, Mulé, Moretti, & Perrone, 2003; Pancaldi et al., 2010; Rubert, Soriano, Mañes, & Soler, 2013). Several surveys have been conducted on the levels of mycotoxins in wheat all over the world such as USA, Canada, Serbia, Italy or Jordan (Gallo, Lo Bianco, & Bognanni, 2008; Jelinek, Pohland, & Wood, 1989; Roscoe et al., 2008; Salem & Ahmad, 2010; Skrbic, Zivancev, Mladenovic, & Godula, 2012), while only one survey on aflatoxins (AFs) presence has been carried out on different Syrian's food commodities, in Lattakia (Syria) (Haydar et al., 1990).

Since toxicity has not been evaluated for all the mycotoxins found in food, the total effect of these naturally occurring

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contaminants on human health cannot be estimated. In fact, for the most well-documented toxins, the tolerable daily intakes (TDI), established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA), remain temporary (t-TDI), provisional (PTWI) or provisional maximum levels (PMTDI), due to the lack of information on toxicology and exposure (Leblanc, Tard, Volatier, & Verger, 2005).

The analysis of mycotoxins is challenging as they are usually present in low concentrations in complex matrices, and they may occur in various combinations produced by a single or by several fungal species. During the last decade, liquid chromatography–mass spectrometry (HPLC–MS/MS) has been established as a powerful tool for unambiguous identification of mycotoxins in food (Turner, Subrahmanyam, & Piletsky, 2009). In addition, alternative extraction methods have recently emerged, such as QuEChERS and MSPD (Rubert, James, Mañes, & Soler, 2012a; Rubert, Soler, & Mañes, 2011; Rubert et al., 2012b; Shephard et al., 2012). A recent review, focused on recent trends in matrix solid phase dispersion (MSPD), has described the key factors for the success of MSPD and its application in food and animal tissues, highlighting its feasibility, flexibility, versatility, low costs and rapidity (Capriotti, Cavaliere, Laganà, Piovesana, & Samperi, 2013). To our knowledge, MSPD–HPLC–QTRAP® is still scarcely used as a routine analytical technique for mycotoxin analysis.

The aims of this work were to obtain the first report on the incidence and levels of mycotoxins in Syrian and Italian wheat grains for human and animal consumption by the application of a mycotoxin analytical method based on MSPD extraction method followed by HPLC–MS/MS using a 3200 QTRAP® instrument and to validate the method to analyze mycotoxins in wheat grain.

2. Materials and methods

2.1. Chemical and reagents

Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Solid-phase used for matrix solid-phase dispersion (MSPD) extraction was Sepra C18-E (50 μm, 65 Å) endcapped silica-based C_{18} from Phenomenex (Torrance, USA). Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade reagent formic acid (purity > 98%), and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

The standards of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), sterigmatocystin (STER), α-zearalenol (αZOL), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenon X (FUSX), neosolaniol (NEO), diacetoxyscirpenol (DAS), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), beauvericin (BEA) were purchased from Sigma Aldrich (Madrid, Spain). T-2 toxin (T-2) and HT-2 toxin (HT-2) stock solutions (in acetonitrile) were purchased from Biopure referenzsubstanzen GmBH (Tulln, Austria). Fumonisin B_3 (FB_3) was supplied by the PROMEC unit (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa). Enniatins (ENs): ENA_1, ENA, ENB and ENB_1 were purchased by Enzo Life Science (Lausen, Switzerland).

The standard solutions were prepared and kept at −20 °C (Rubert et al., 2012a,b). All working standard solutions were prepared before the use by diluting the stock solution with acetonitrile.

2.2. Sampling

A total of 86 wheat grain samples collected during 2009 and 2010 seasons were analysed for the presence of mycotoxins. Forty samples (40) of durum and soft wheat were collected from different areas of Syria including Deir Ezzor (11), Damascus rural (19), Daraa (3) and Al Hassakeh (7). Forty-six samples (46) of durum wheat were collected from different Italian areas, Emilia-Romagna (12), Toscana (5), Marche (12), Umbria (7), Lazio (3), Basilicata (3) and Sicilia (4). Fig. 1 shows the regions in Italy and Syria where the wheat samples were collected.

According to EU guidelines (EU, 2006b), three incremental samples of at least 1 kg were collected to obtain an aggregate sample of 3 kg total weight. After homogenization, samples were packed in a plastic bag and kept at −20 °C in a dark and dry place until analysis. Just before analysis, a subsample of 200 g was mixed thoroughly using an Oster® food processor (Professional Series Blender model BPST02-B00) to obtain wheat flour.

2.3. Extraction procedure

Sample preparation was optimized in a previous study (Rubert, Soler, & Mañes, 2012c). A MSPD extraction method was applied to wheat grain. Samples (200 g) were prepared using an Oster® food processor (Professional Series Blender model BPST02-B00), mixing the sample thoroughly. Portions of 1 g were weighed and placed into a glass mortar (50 mL) and were gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 15 mL of elution solvent which was a mixture of acetonitrile/methanol (50/50, v/v) with 1 mM ammonium formate by applying a slight vacuum. Then, the extract was transferred to a 25 mL conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 mL with methanol/water (50/50, v/v) and filtered through a 13 mm/0.22 μm nylon filter purchased from Membrane Solutions (Texas, USA) before the injection into the liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) system.

For spiked samples (EU., 2002), 1 g of "blank" sample (sample in which it was corroborated that none of the analytes were present) was spiked with 0.1 mL of a working mixture of the mycotoxins at the appropriate concentration. Spiked samples were then left to stand for 3 h at room temperature before the extraction to allow the solvent to evaporate and to establish equilibration between the spiked mycotoxins and wheat flour samples. Ten replicates were prepared at each spiking level.

2.4. HPLC–MS/MS method

The 3200 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) was coupled to Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA). Separation of analytes was performed with a reversed-phase analytical column (Gemini C_{18}, 150 mm, 2 mm i.d, 5 μm; Phenomenex) maintained at 35 °C. As mobile phase, 5 mM ammonium formate and 0.1% formic acid in water (A) and 5 mM ammonium formate in methanol (B) were used. The gradient was as follows: at the start 5% of solvent B and after the percentage of solvent B was linearly increased to 95% in 10 min. The percentage of solvent B was kept for 5 min. Finally, the column was equilibrated to initial conditions for 10 min. The flow rate was 250 μl min^{-1} and the injection volume was 10 μl. The 3200 QTRAP® mass spectrometer was equipped with a Turbo V™ Ion Spray (ESI) interface. The QTRAP® analyzer combines a fully functional triple-quadrupole and ion trap mass spectrometer within the same instrument. The analyses were performed using Turbo V™ Ion Spray in positive ionization mode (ESI+). The operation conditions were previously optimised (Rubert et al., 2012a,c). The operation conditions for ESI+ were as follow: ion spray voltage