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Simultaneous analysis of twenty-six mycotoxins in durum wheat grain from Italy

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ABSTRACT

A multi-mycotoxin analysis method based on liquid chromatography coupled to triple quadrupole mass spectrometry was validated and applied for the determination of twenty-six mycotoxins, including eight trichothecenes, three fumonisins, sterigmatocystin, ochratoxin A, four aflatoxins, zearalenone, five "emerging" mycotoxins and three Alternaria mycotoxins in 74 durum wheat samples from central Italy. Eighty-eight percent of the analysed samples contained one or more mycotoxins, with enniatin B showing the highest contamination levels (78% of samples with levels ranging from 23 to 1826 ng/g), followed by enniatin B1 (10–1384 ng/g) and deoxynivalenol (48–2267 ng/g). This work gives an important contribution in terms of mycotoxin analysis and occurrence in durum wheat and in assessing the mycotoxicological risk posed by both traditional and emerging mycotoxins for the final consumers of durum wheat derived products.

1. Introduction

Mycotoxins are toxic fungal metabolites, which can be frequently found in a wide range of food commodities in the world. Many analytical methods used for mycotoxin detection are specific for one or a closely related group of them. The validation and application of a multi-mycotoxin analytical method for the simultaneous detection of a large number of mycotoxins is an important step to perform a wide monitoring of unprocessed and processed products because mycotoxins do not usually occur singularly but as a mix, determining synergistic negative effects on food safety and final consumer's health (Streit et al., 2013).

Agriculture plays a very important role in the Italian national economy and, especially because of the importance of the pasta industry, durum wheat (*Triticum durum* Desf.) represents one of the most relevant agricultural commodities in Italy. With a production of 3,710,634 tons and a cultivated surface of 1,080,837 ha in 2013, durum wheat was the most cultivated small grain cereal in Italy (AGRIT Bulletin, 2013). In Europe, in 2011, Italy ranked at the fourth place for durum wheat production, after France, Germany and Spain while it ranked at the 20th position in the world (EUROSTAT, 2012).

Half of the Italian durum wheat production is obtained in the southern regions such as Apulia, Sicily, Basilicata and Sardinia. The remaining part is obtained in the central area of the country, in particular in The Marche, Emilia-Romagna, Tuscany, Latium, Abruzzo and Umbria regions. Durum wheat cultivation in the Northern area is very limited and restricted to the northern part of the Emilia-Romagna region (AGRIT Bulletin, 2013). However, in the last decade, durum wheat cultivation gradually expanded to the Northern area of the Italian peninsula.

The global quality of durum wheat depends on the complex interaction among different factors such as: agronomic quality (grain yield), milling quality (semolina yield, ash content, humidity, impurity of grains), technological quality (protein content, gluten quantity and quality) as well as hygienic and sanitary quality (related to fungal microorganisms that infect/contaminate durum wheat in the field and during storage). Grain safety is considered one of the most important aspects to obtain high quality durum wheat, and consequently pasta, also in relation to the ability of a wide range of fungal microorganisms to biosynthesize mycotoxins (Fagnano et al., 2012).

However, the susceptibility of this crop to fungal infections, exacerbated by the relatively recent introduction of durum wheat cultivation from southern Italy into new disease favourable environments such as those of central and northern Italian regions, has determined higher contamination risks by mycotoxigenic fungal species such as *Fusarium* spp. *Alternaria* spp., *Aspergillus* spp. and

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Penicillium spp. (Covarelli et al., 2014, 2015; Pestka, 2010). The most important mycotoxins mainly found to contaminate this crop are *Fusarium* toxins such as deoxynivalenol (DON), T-2 and HT-2 toxins, zearalenone (ZEA), aflatoxins (AFs) and ochratoxin A (OTA) which have been classified by IARC (1993) and legally regulated by the European Union during the past 10 years. However, other "emerging" mycotoxins, such as enniatins (ENs) and beauvericin (BEA), have not been legislated yet and are currently under risk evaluation including "in vivo" and "in vitro" studies of toxicity (Juan, Manyes, Font, & Juan-García, 2014; Juan-García, Manyes, Ruiz, & Font, 2013). On the other hand, *Alternaria* mycotoxins have frequently been detected in vegetables (tomatoes), legumes (lentils) and fruits such as grapes and strawberries (Ostry, 2008; Scott, 2001). There is a remarkable lack of information about the natural contamination of cereals by these mycotoxins; but small-spored *Alternaria* species such as: *Alternaria alternata* (Fr.) Keissler, *Alternaria tenuissima* (Kunze) Wiltshire, *Alternaria triticina* and *Alternaria infectoria* E.G. Simmons represent the most predominant spp. found on wheat, oats and barley (Mercado Vergnes, Renard, Duveiller, & Maraite, 2006). These *Alternaria* species have frequently shown to produce alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), tenuazonic acid (TeA) and tentoxin (TEN) in laboratory cultures (Li, Toyazaki, & Yoshizawa, 2001; Patriarca, Azcarate, Terminiello, & Fernández Pinto, 2007). So far, surveys on naturally contaminated grains have rarely been reported for example, in Argentina (Azcarate, Patriarca, Terminiello, & Fernández Pinto, 2008) and in China (Li & Yoshizawa, 2000), AOH, AME and TeA were detected in damaged wheat. Quantitative data on the AOH, AME and TeA content in unprocessed cereals in Europe are still limited. Low levels of AOH, AME and ALT and slightly higher concentrations of TeA have been detected in some small grain cereal samples in Germany (Müller, Waydbrink, Peters, Umann, & Seyfarth, 2002), and in Czech Republic (Ostry, Skarkova, Nedelnik, Ruprich, & Moravcova, 2005).

Since the total effect of all these naturally occurring contaminants on human health has not yet been established, due to the lack of information on their toxicology and exposure, the tolerable daily intakes (TDI) established by the FAO/WHO Joint Expert Committee on Food Additives (JECFA) are represented by the temporary TDI (t-TDI), provisional tolerable weekly intakes (PTWI) or provisional maximum TDI (PMTDI) and they all depend on the type of mycotoxin (Leblanc, Tard, Volatier, & Verger, 2005; JECFA, 2013). Recently, the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2001a, b, 2010 and 2011; EC, 2010) and the Scientific Committee on Food (SCF, 2002) have proposed a TDI for DON, and a PMTDI for single and combined mycotoxins, so that there is a PMTDI for NIV, one for the sum of DON, 3AcDON and/or 15AcDON and another one for the sum of T-2 and HT-2 toxins, while EFSA proposed a PTWI for OTA (EFSA, 2006).

In this work, an optimized method developed in our laboratory by Juan, Ritieni, and Mañes. (2012) was validated and applied for the simultaneous determination of 26 mycotoxins in 74 durum wheat samples harvested in central Italy, including DON, 3-acetyl-deoxynivalenol (3AcDON), 15-acetyl-deoxynivalenol (15AcDON), nivalenol (NIV), neosolaniol (NEO), diacetoxyscirpenol (DAS), T-2 and HT-2 toxins, fumonisins (F) B1, B2 and B3, sterigmatocystin (STG), OTA, aflatoxins (AF) B1, B2, G1 and G2, zearalenon (ZEA), beauvericin (BEA), enniatins (EN) A, A1, B and B1, alternariol (AOH), alternariol methyl ether (AME) and tentoxin (TEN).

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were supplied by PanReac AppliChem (Castellar del Vallés, Spain).

Anhydrous magnesium sulphate was obtained from Alfa Aesar GmbH & Co (Karlsruhe, Germany), sodium chloride was purchased from Merck (Darmstadt, Germany), formiate ammonium and formic acid were obtained from Sigma Aldrich (St. Louis, USA).

The certified standards of AFB1, AFB2, AFG1, AFG2, OTA, STG, ZEN, NIV, DON, 3-AcDON, 15-AcDON, DAS, NEO, T-2 and HT-2 toxin, FB1, FB2, FB3, BEA, ENNs (A, A1, B, B1), AOH, AME, and TEN were purchased from Sigma Aldrich (Madrid, Spain).

The individual stock solutions of AFB1, AFB2, AFG1, AFG2, OTA, STG, ENs (A, A1, B, B1), BEA, AOH, AME, and TEN at 500 μg/mL were prepared in acetonitrile, whereas ZEN, FB1, FB2, FB3, NIV, DAS, NEO, DON, 3-AcDON, 15-AcDON, T-2 and HT-2 toxin were prepared at 1000 μg/mL in methanol. A working mixed standard solution at 5 and 10 μg/mL were prepared immediately before use by diluting the individual stock solution in methanol. This solution was used to prepare the calibration curves, matrix matched calibration curves and for repeatability (intraday) and reproducibility (interday) studies.

2.2. Sampling

A total of 74 durum wheat samples, which included 28 varieties, were collected in 2013 immediately after their harvest from 39 different farms located mainly across the Umbria Region (central Italy). According to EU regulation (EC, 2006a), three incremental samples were collected immediately after wheat harvest of at least 1 kg 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 500 g was mixed thoroughly using a blender to obtain wheat flour and were divided into two bulks of 250 g each. For each sample, information about the location of origin and the variety was collected.

2.3. Extraction

Sample extraction was performed according to the method of Juan, Ritieni, and Mañes (2013) with slight modifications. Wheat samples (250 g) were previously finely milled with a blender. Representative sub-samples of 2 g each were weighed and placed into 50 mL polypropylene centrifuge tubes and 10 mL of an extraction solvent composed by a mixture of acetonitrile/water/formic acid (84:15:1 v/v/v) were added. The tubes were shaken using a horizontal shaking device (Edmund Buhler SM30 Control, Hechingen, Germany) (200 shakes min^{-1}) for 1 h and then centrifuged for 5 min at 3500 rpm, filtered with Whatman filter paper and 5 mL of the supernatant were 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 (70/30) (v/v) and filtered through a 13-mm/0.22 μm nylon filter purchased from Análisis Vínicos S.L. (Tomelloso, Spain) before LC–MS/MS analysis.

For the preparation of fortified samples, 2 g of durum wheat "blank" mixture sample were spiked with a working mixture of the mycotoxins at LQs, 2 LQs and 10 LQs concentration levels. Then, spiked samples were left to stand for 3 h at room temperature before the extraction to allow the evaporation of the solvent and to establish equilibration between mycotoxins and the sample. Three replicates were prepared for each spiking level.

2.4. LC-MS/MS

The analysis was performed using a LC-MS/MS system, consisting of a LC Agilent 1200 using a binary pump and an automatic injector, and coupled to a 3200 QTRAP® AB SCIEX (Applied Biosystems, Foster City, CA) equipped with a Turbo-V™ source (ESI)