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Simultaneous determination of *Fusarium* mycotoxins in wheat grain from Morocco by liquid chromatography coupled to triple quadrupole mass spectrometry

Jesus Blesa [a], Juan-Carlos Moltó [a], Samira El Akhdari [b], Jordi Mañes [a], Abdellah Zinedine [c,*]

[a] Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain
[b] Etablissement Ibn Al-Khatib, Salé Tabriquet, Académie Régionale d'Education et de Formation, Rabat-Salé-Zemour-Zaer, Morocco
[c] Department of Biology, Faculty of Sciences, Chouaib Doukkali University, BP 20, El Jadida 24000, Morocco

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ABSTRACT

In the present study, eighteen (18) mycotoxin produced by the genus *Fusarium* (fumonisins (FB1, FB2 and FB3), type-B trichothecenes (NIV, DON, FUS-X, 3Ac-DON and 15Ac-DON), type-A trichothecenes (NEO, DAS, T2 and HT2), zearalenone (ZEA), beauvericin (BEA), and enniatins (ENA, ENA_1, ENB and ENB_1)) were monitored in different samples of wheat grain commercialized in Morocco. A liquid chromatography coupled to triple quadrupole mass spectrometry method previous matrix solid phase dispersion extraction was used for sample analysis. A total of eighty (80) samples of durum wheat were collected in different local markets from several areas in Morocco. Analytical results showed that 54 out of 80 total wheat samples (68%) were contaminated. The mycotoxins found were ENA, ENA_1, ENB and ENB_1, DON and BEA. The rest of investigated mycotoxins were below the limits of quantification. In positive samples, enniatins levels ranged between 2.5 and 2570 μg/kg. DON levels ranged between 121 and 1480 μg/kg and BEA levels were between 5.4 and 16 μg/kg. Among enniatins, ENB was predominant with a frequency of 61%. Co-presence of DON, enniatins and BEA from durum wheat from Morocco was found for the first time.

1. Introduction

In Morocco, agriculture plays an important role in the national economy. Its contribution to Gross Domestic Product (GDP) ranges between 15% and 17%, and it employs just over 30% of the working population. Cereals represent one of the most important dietary items in the country. Wheat contributes almost 74% on average of the gross value of cereals, followed by barley (23%), maize (2%) and other cereals (1%) (Ministry of Agriculture, 2000). Cereals are grown in the various agro-climatic zones of the country in rotation with other annual crops (legumes, industrial crops and fodder crops). The principal cereal-growing areas are in the rain-fed plains and plateaux of "Chaouia", "Abda", "El-Haouz", "Tadla", "El-Gharb" and "Sais", where the vast majority of farms grow cereals (Aït El Mekki, 2006, pp. 1–21). The production of cereals in Morocco fluctuates annually due to rainfall conditions. Cereals imports develop according to the volume of national production. Currently imports are made from France, USA, Ukraine, Russia, Canada, Argentina and Brazil. Total cereals demand of Morocco could amount to 138 million quintals by 2020 on a nutritional basis for a population of 40 million inhabitants.

Mycotoxins are abiotic hazards produced by certain fungi that can grow on a variety of crops including cereals (Marin, Ramos, Cano-Sancho, & Sanchis, 2013), the production of these toxic substances is greatly influenced by environmental factors, being the most important temperature, relative humidity, insect damage, drought and inadequate storage conditions leading nutritional losses and representing a significant hazard to the food chain (Rodríguez-Carrasco, Ruiz, Font, & Berrada, 2013).

Among fungi, around 100 identified and characterized species are known to produce mycotoxins. Species of the genus *Fusarium* are able to produce several mycotoxins in cereal grains and animal feed. Theses toxins include fumonisins (FB1, FB2 and FB3), zearalenone (ZEA) and its metabolites, deoxynivalenol (DON) and its derivatives the 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON), HT-2 and T-2 toxins, diacetoxyscirpenol (DAS), neosolaniol (NEO), fusarenon-X (FUS-X) and nivalenol (NIV), enniatins (A, A1, B, B1), fusaproliferin (FUS) and

* Corresponding author. Tel.: +212 6 61 66 65 56.
E-mail address: zinedineab@yahoo.fr (A. Zinedine).

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beauvericin (BEA), etc. *Fusarium* mycotoxins have shown both acute and chronic toxicity in animals. Spontaneous outbreaks of *Fusarium* mycotoxicoses have been reported in Europe, Asia, Africa, New Zealand, and South America. Moreover, chronic intake of these mycotoxins is reported on a regular and more widespread basis due to their global occurrence. Recently, Cortinovis, Pizzo, Spicer, and Caloni (2013) reviewed the effect of *Fusarium* toxins on reproductive function in domestic animals. Indeed, authors reported that several studies *in vitro* revealed the direct effect of these mycotoxins on ovarian cells, altering oocyte maturation, inhibiting granulosa cell proliferation, and differentiated functions such as gonadotropin-supported steroid production. While *in vivo*, these toxins can alter follicular growth and atresia, ovulation, and puberty onset. Due to their toxicity, the presence of many *Fusarium* toxins was regulated in foods. Nowadays, maximum levels for DON and ZEA ranging from 200 to 1750 μg/kg and from 20 to 400 μg/kg are set, respectively, in unprocessed (or processed) cereals and/or derivatives (European Commission, 2007). More recently, the European authorities have recommended the presence of the sum of the toxins T-2 and HT-2 in cereals and cereal products at values that varied between 15 and 2000 μg/kg (European Commission, 2013).

The presence of *Penicillium* mycotoxins in cereals commercialized in Morocco was already documented (Zinedine & Mañes, 2009). However little information is available on the co-occurrence and on the multi-mycotoxin contamination of cereals by toxins of *Fusarium* species. Thus the principal objective of this work was to study the multi-mycotoxin presence [(FB1, FB2, FB3), type-B trichothecenes (NIV, DON, FUS-X, 3Ac-DON and 15Ac-DON), type-A trichothecenes (NEO, DAS, T2 and HT2), ZEA, BEA, ENA, ENA_1, ENB and ENB_1] in samples of wheat collected from different area in Morocco by using an in-house validated method that combines matrix solid phase dispersion extraction (MSPD), liquid chromatography (LC), electrospray ionization (ESI) and triple quadrupole tandem mass spectrometry (MS/MS).

2. Materials and method

2.1. Sampling

Eighty (80) samples of wheat were randomly collected from different local markets in different areas of Morocco. The samples were packaged in plastic bags, and then stored at 4 °C prior to analyses.

2.2. Chemicals

Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Deionized water (<18 mΩ cm^{-1} resistivity) was purified using the Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade formic acid (purity >98%) and ammonium formate (purity >97%) were obtained from Panreac (Barcelona, Spain). The solid phase used for MSPD was octadecylsilica (C18) (MFE-Pack 50 μm) from Analisis Vinicos (Tomelloso, Spain).

The standards of FB1, FB2, FB3, type-B trichothecenes (NIV, DON, FUS-X, 3Ac-DON and 15Ac-DON), type-A trichothecenes (NEO, DAS, T2 and HT2), ZEA, BEA, ENA, ENA_1, ENB and ENB_1 were purchased from Sigma Aldrich (Madrid, Spain). All stock solutions at a concentration of 100 μg ml^{-1} were prepared in acetonitrile. All solutions were kept in secure conditions at −20 °C. Mix working standard solution was prepared immediately before use by dilution and adequate mixing of stock solutions with methanol/water (80:20, v/v).

2.3. Apparatus and chromatographic conditions

A modification of a previously developed method of Rubert, Soler, and Mañes (2011) was used for the detection of 18 *Fusarium* mycotoxins. The triple quadrupole mass spectrometry detector (QqQ) was equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) that included an autosampler and a quaternary pump. Separation was attained on a Phenomenex (Madrid, Spain) Gemini-NX C18 (150 mm × 4.6 mm I.D., 3 μm particle size) analytical column, preceded by a security guard cartridge C18 (4 mm × 2 mm I.D.), using a gradient that started at 100% of A (5 mM ammonium formate and 0.1% of formic acid in water) and 0% of B (5 mM ammonium formate and 0.1% of formic acid in methanol), increased linearly to 100% B in 10 min. After, it was decreased linearly to 80% of B in 5 min and it was gradually decreased to 70% B in 10 min. Afterwards, the initial conditions were maintained for 5 min. Flow rate was maintained at 0.3 ml min^{-1}.

A QqQ mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Ver. 4.1 was used for MS/MS analyses. Parameters were optimized in positive (ESI+) ionization mode by continuous infusion of a standard solution (10 g ml^{-1}) via syringe pump at a flow rate of 20 l min^{-1}. The ESI source values were capillary voltage, 3.50 kV positive ionization mode; extractor, 1 V; RF lens 0.5 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 l h^{-1}; cone gas 50 l h^{-1} (nitrogen 99.99% purity). Cone voltages and collision energies were optimized for each analyte during infusion of the pure standard and the most abundant fragment ion chosen for the selected reaction monitoring. The analyzer setting were: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energies, 0.5; entrance and exit energies, 5 and 3; multiplier, 650; collision gas (argon, 99.99% purity) pressure 3.83 × 10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms.

At these conditions a good chromatographic separation was achieved in a 20 min run. The majority precursor ion was the protonated form except for DAS, T2 and HT2 where ammonium adducts were precursor ions selected. And ion ratio between qualify and quantify transitions was according to the EU guidelines (European Commission, 2002) (Table 1).

2.4. Sample extraction

Sample preparation was performed according to the method described previously by Rubert et al. (2011). Briefly, wheat subsamples (200 g) were mixed thoroughly using an Oster® food processor (model BPST02-B00). Portions of 1 g were placed into a glass mortar (50 ml) and gently blended with 1 g of C18 for 5 min using a pestle to obtain a homogeneous mixture. This homogeneous mixture was introduced into a 100 mm−9 mm I.D. glass column and eluted dropwise with 15 ml of a mixture of acetonitrile: methanol (50:50) (v/v) and 1 mM ammonium formate by applying a slight vacuum. The extract was then transferred to a 15 ml conical tube and evaporated to dryness at 45 °C with a gentle stream of nitrogen using a multi-sample TurboVap® LV (Zymark, Allschwil, Switzerland). The residue was reconstituted to a final volume of 1 ml with a mixture of methanol:water (80:20) (v/v). To fortify samples prior extraction, 1 g of "blank" sample was spiked with an appropriate volume 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 the wheat. To perform matrix-matched calibration, final reconstitution of the sample extract was doing