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Further data on the presence of *Fusarium* emerging mycotoxins enniatins, fusaproliferin and beauvericin in cereals available on the Spanish markets

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ABSTRACT

In this work, 64 samples of cereals purchased from local markets in the Valencian community (Spain) were investigated for the presence of six emerging mycotoxins: enniatins ENs (ENA, ENA1, ENB and ENB1), beauvericin (BEA) and fusaproliferin (FUS). Samples were extracted with a mixture of water/acetonitrile (85/15, v/v) by using an Ultra-turrax homogenizer. Mycotoxins were then identified and quantified with a liquid chromatography (LC) with diode array detector (DAD). Positive samples were confirmed with an LC–MS/MS. Analytical Results showed that the frequencies of contamination of samples with ENs, BEA and FUS were 73.4%, 32.8% and 7.8%, respectively. ENA1 was the most mycotoxin found and levels ranged from 33.38 to 814.42 mg/kg. ENB levels ranged between 2.23 and 21.37 mg/kg. ENB1 levels varied from 4.34 to 45.94 mg/kg. All samples were free of ENA. BEA levels ranged from 0.51 to 11.78 mg/kg and FUS levels varied between 1.01 and 6.63 mg/kg. It could be concluded from this study that the high contamination levels found especially for ENs could be of a negative impact on the population. This is the first paper on the presence of emerging mycotoxins in cereals available in Spain.

1. Introduction

Mycotoxins are toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. Mycotoxins are common contaminants of many grains like wheat, barley, maize and rice, and they can evoke a broad range of toxic properties including carcinogenicity, neurotoxicity, as well as reproductive and developmental toxicity (Jestoi, 2008). *Fusarium* species produce many mycotoxins that are correlated with hepatotoxicity, teratogenicity and carcinogenicity in humans and animals. Among these substances, fumonisins, zearalenone, trichothecens, T-2, HT-2 toxins, etc. are of a great concern because of their negative impact on human and animal health and the economic losses engendered. The estimated losses in wheat and barley attributable to the *Fusarium* mycotoxins in USA alone are about 2900 million US dollars a year (Windels, 2000). The presence of these compounds in foods and feeds was well worldwide documented in the literature (D'Mello et al., 1999; Soriano and Dragacci, 2004; Zinedine et al., 2007).

Fusarium species are also responsible for the production of another group of bioactive compounds called emerging or "minor" mycotoxins. This group including enniatins (ENA, ENA1, ENB and ENB1), fusaproliferin and beauvericin is not well investigated by scientists. Indeed, few worldwide data are published about the occurrence of theses toxins in commodities from the Nordic countries (Norway, Finland) and Italy (Jestoi, 2008), however little information is available in the literature about their their presence in foods especially in the South of Europe and the rest of the world.

Enniatins (ENs) are produced by strains of some species of *Fusarium*, other fungal genera including *Alternaria, Halosarpheia* and *Verticillium* were also described to produce ENs (Supothina et al., 2004). ENs were discovered from cultures of *F. orthoceras Appl. and Wr. Var. Enniatum* later renamed *F. oxysporum*. A list over the at least 23 different compounds, which have been described as naturally occurring enniatin analogues, may be found in the literature (Feifel et al., 2007). ENs represents six-membered cyclic depsipeptides, which are commonly composed of three D-a-hydroxyisovaleric acid (Hiv) residues linked alternatively to three L-configured N-methyl amino acid residues to give an 18 membered cyclic skeleton (Zhukhlistova et al., 1999).

ENs are of high interest because of their wide range of biological activity. This bioactivity has long been assumed to be associated with their ionophoric properties (Uhlig et al., 2009). ENs inhibit the enzyme acyl-CoA:cholesterol acyl transferase (ACAT) (Tomoda

Abbreviations: BEA, beauvericin; ENA, enniatin A; ENA1, enniatin A1; ENB, enniatin B; ENB1, enniatins; ENs, enniatins; FUS, fusaproliferin.

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et al., 1992). They are also known as phytotoxins and are associated with plant diseases characterized by wilt and necrosis (Burmeister and Plattner, 1987). Other studies reported the bioactivity of ENs against *Mycobacterium* sp. and *Plasmodium falciparum* (Nilanonta et al., 2000; Supothina et al., 2004). Hiraga et al. (2005) showed that ENs have been identified as inhibitors of major drug efflux pumps in *Saccharomyces cerevisiae*.

Beauvericin (BEA) is a cyclic lactone trimer containing an alternating sequence of three N-methyl L-phenylalanyl and three D-a-hydroxyisovaleryl residues. BEA was first isolated from the culture of the insect-pathogenic fungus *Beauverina bassiana* (Hamill et al., 1969). Recently, Moretti et al. (2007) reported the production of BEA by *F. bulbicola*, *F. denticulatum*, *F. lactis*, *F. phyllophilum*, *F. pseudocircinatum* and *F. succisae*. BEA affected the electromechanical and physiological properties of isolated smooth and heart muscle preparations (Lemmens-Gruber et al., 2000). BEA is a specific cholesterol acyltransferase inhibitor (Tomoda et al., 1992). BEA is toxic to several human cell lines (Logrieco et al., 2002) and can induce apoptosis and DNA fragmentation (Ojcius et al., 1991). This mycotoxin inhibited the L-type Ca^{2+} current in the NG108-15 neuronal cell line and increased the intracellular calcium by increasing the formation of cation selective channels in lipid membrane (Wu et al., 2002; Kouri et al., 2003). Previous studies have shown that BEA induced cell death can be prevented by administration of intracellular calcium chelator-BAPTA/AM (Jow et al., 2004) in human lymphoblastic leukemia CCRF-CEM cells, indicating that the intracellular Ca^{2+} plays an important role in cell death signaling.

Fusaproliferin (FUS) is a bicyclic sesterterpene consisting of five isoprenic units, which was originally isolated from a pure culture of *Fusarium proliferatum* (Randazzo et al., 1993). Moretti et al. (2007) reported that FUS could be produced by *F. antophilum*, *F. begoniae*, *F. bulbicola*, *F. circinatum*, *F. concentricum*, *F. succisae* and *F. udum*. FUS is also produced by *Fusarium subglutinans* (Ritieni et al., 1995; Meca et al., 2009). FUS is produced through the isoprenoid pathway via common terpene intermediates originating from acetyl-CoA subunits. Preliminary studies indicated that FUS has been found to be toxic in the brine shrimp (*Artemia salina*) larve bioassay (Ritieni et al., 1995) and mammalian cells (Logrieco et al., 1996) and causes teratogenic effects on chicken embryos (Ritieni et al., 1997a).

Even if recent published papers have focused on the toxicity of emerging mycotoxins, so far there is limited data worldwide on the co-occurrence and contamination levels of these metabolites of *Fusarium* spp. in foods. The jomain purpose of this work was to investigate for the first time the presence of FUS, BEA and the four ENs (ENA, ENA1, ENB and ENB1) in cereals available in supermarkets of the Valencian community in Spain.

2. Material and methods

2.1. Chemical and reagents

Acetonitrile was purchased from Fisher Scientific (Madrid, Spain). Deionized water (<8 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

The stock standard solution of BEA was purchased from Sigma–Aldrich (St. Louis, USA). Standard solutions of FUS and ENs (ENA, ENA1, ENB and ENB1) were gently given by Professor Alberto Ritieni, Department of Food Science, University of Naples "Federico II", Italy. All stock solutions were prepared by dissolving 1 mg of the mycotoxin (BEA, FUS or ENs) in 1 ml of pure methanol, obtaining a 1 mg/ml (1000 μg/ml) solution. These stocks solutions were then diluted with pure methanol in order to obtain the appropriated work solutions. All solutions were stored in darkness at 4 °C until the HPLC analysis. Acetonitrile, methanol, water, all of HPLC grade, were purchased from Merck (Whitehouse Station, NJ, USA).

2.2. Sampling

Samples of cereals ($n = 64$) including wheat, barley, corn, bread mill, rice and mixture of cereals were collected from supermarkets in the Valencian community (Spain). All samples were milled and then divided with a subsample divider. A 200 g subsample was collected in a plastic bag and kept at −20 °C until analysis.

2.3. Mycotoxin extraction procedure

The method used for mycotoxins analysis (BEA, ENs and FUS) was described by Jestoi (2008). Briefly, 3 g of cereal samples were extracted with 20 ml of a mixture of water/acetonitrile (85/15, v/v) using an Ultra Ika T18 basic Ultraturrax (Staufen, Germany) for 3 min. The extract was centrifuged at 4500*g* for 5 min and then the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland) and then re-dissolved in 2 ml of extraction solvent. This final solution was filtered through a 25 mm/0.45 μm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before the injection into the LC-DAD system for analysis.

2.4. LC-DAD analysis

LC analyses of BEA, ENs and FUS were performed using a Shimadzu LC system equipped with LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Gemini (150 × 4.6 mm, 5 μm) Phenomenex column was used. HPLC conditions were set up using a constant flow at 1.0 ml/min and acetonitrile/water (70:30 v/v) as starting eluent system. The starting ratio was kept constant for 5 min and then linearly modified to 90% acetonitrile in 10 min. After 1 min at 90% acetonitrile, the mobile phase was taken back to the starting conditions in four minutes. BEA and ENs were detected at 205 nm, while FUS was detected at 261 nm. All samples were filtered through a 0.22 μm syringe filter Phenomenex prior to injection (20 μl) onto the column. Mycotoxin identification was performed by comparing retention times and UV spectra of purified extracted samples to pure standards. Quantification of mycotoxins was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

2.5. LC–MS/MS confirmation

LC analysis of BEA, FP and ENs was carried out with a TQ mass spectrometer Quattro LC from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters, Milford, MA) consisted of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and a Mass Lynx NT software 4.1 was used for data acquisition and processing. The autoinjector was programmed to inject 20 μl into the Luna C18 column (150 × 4.6 mm, 5 μm) Phenomenex maintained at 30 °C. The analytical separation for LC–MS/MS was performed using gradient elution with water as mobile phase A, and acetonitrile as mobile phase B, both containing 0.5% formic acid. After an isocratic step of 65% B for 3 min, it was linearly increased to 75% B in 4 min and held constantly for 3 min. Flow rate was maintained at 0.3 ml min. Analysis was performed in positive ion modes. The ESI source values were as follows: capillary voltage, 3.20 kV; source temperature, 125 °C; desolvation temperature, 350 °C; desolvation gas (nitrogen, 99.95% purity) flow, 700 l/h. Ideal fragmentation conditions were accomplished varying the cone voltage and collision energies for each compound.

For the detection of FP and BEA the precursor ion were m/z 445 and 801 being the product ions selected were m/z 427–409 and 784–756. For EN B the precursor ion was m/z 640, and the product ions m/z 196 and 527. For EN B1 the precursor ion was m/z 654, and the product ions m/z 196 and 228, for EN A the precursor ions was m/z 682, and the product ions m/z 210 and 555, for EN A1 the precursor ion was m/z 668, and the product ions m/z 210 and 541 (Sorensen et al., 2009).

3. Results and discussion

3.1. Method performance

Mean recoveries of fortified cereal samples ($n = 3$) at levels of ENS (0.3–50 μg/g), FUS (0.3–50 μg/g) and BEA (0.3–50 μg/g) were respectively 84.6%, 70.5% and 88.6% with a relative standard deviations of 3.5%, 4.6% and 3.2% (Table 1). The values obtained for recoveries and relative standard deviations of the method used are in agreement with the EU Commission Directive 2002/26/EC for methods of analysis of mycotoxins in foodstuffs (European Commission, 2002). Intra-day ($n = 5$) and inter-day (5 different days) variation values at were 2.5–8.6%, 2.0–10.4% and 2.5–8.3%, respectively for ENs, FUS and BEA. These values are below 15% which is the maximum variation for certification exercises for several mycotoxins. The detection limit (LOD) and the limit of quantification (LOQ) values were calculated according to $s/n = 3$ and

s/n = 10, respectively. The LOD and the LOQ for ENs, FUS and BEA are represented in Table 1.

3.2. Occurrence of ENs, BEA and FUS in analyzed samples

Results from the occurrence of ENs in analyzed samples are represented in Table 2. As shown, the frequencies of contamination of analyzed samples with ENs were 89.2%, 61.9% and 50% for corn, wheat and barley, respectively. The mean values of total ENs (ENA, ENA1, ENB and ENB1) found were 167.70, 225.37 and 148.16 mg/kg in samples of corn, wheat and barley, respectively. Other samples found contaminated in this survey were one (1) sample of rice (814.42 mg total ENs/kg), one (1) sample of oat flour (388.38 mg total ENs/kg), one (1) sample of bread mill (37.47 mg total ENs/kg) and four (4) samples out of five mixture of cereals (mean total ENs value at 191.03 mg/kg). The maximum levels of total ENs found in this study were 822.37 and 823.66 mg/kg, respectively, in rice and corn samples.

Concerning the distribution of ENs in positive cereals samples, ENA1 was present in 47 out of 64 total samples (73.4%) with maximum values of 813.01 and 814.42 mg/kg, respectively, in rice and corn. ENA1 levels ranged from 33.38 to 814.42 mg/kg. ENB was present in five samples (7.8%) with a maximum value of 21.37 mg/kg found in a sample of barley. ENB levels ranged from 2.23 to 21.37 mg/kg, ENB1 was present in three samples (4.6%) with a maximum level found in a sample of barley. ENB1 levels varied from 4.34 to 45.94 mg/kg. ENA was not detected in any analyzed sample.

The presence of BEA in analyzed samples is represented in Table 3. BEA was present in 21 out 64 total samples (32.8%) while the frequencies of contamination of wheat, corn and barley were 42.8%, 21.4% and 50%, respectively. The maximum contamination levels of BEA were found in a sample of corn (9.31 mg/kg) and a sample of rice (11.78 mg/kg). The mean BEA levels in samples of wheat, corn and barley are respectively 2.3, 5.72 and 4.87 mg/kg.

Data about FUS levels in analyzed samples is presented in Table 3. As shown, 5 out of 64 total samples (7.8%) were positive for FUS. This mycotoxin contaminates only three samples of wheat, one sample of corn and one sample of rice. The maximum FUS level was found in a sample of wheat with 6.63 mg/kg. A LC-DAD chromatogram of a positive sample of corn contaminated with ENs, FUS and BEA is represented in Fig. 1.

Up until now, only a limited number of studies have been published which have determined the emerging mycotoxins in commodities especially from the Nordic countries. In the literature, there is more information about the traditional mycotoxins. This is partly due to the fact that the role of BEA, ENs and FUS as mycotoxins has been appreciated only during the last few decades (Jestoi, 2008).

Our findings showed high incidence of ENs especially ENA1 in corn, wheat and barley. Previous works have reported high contamination levels of ENs in cereals. The presence of ENs has been reported in grains from Norway, Finland and Italy (Jestoi, 2008). Indeed, in Norway, ENs were found to contaminate barley, wheat and oats with maximum value of 5.8 mg ENB/kg in wheat (Uhlig et al.,

Table 1
Mean recoveries, inter-day and intra-day variations, LOD and LOQ of the analytical method.

Mycotoxin	Mean recovery ± RSD (%)	Inter-day variation (%)	Intra-day variation (%)	LOD (mg/kg)	LOQ (mg/kg)
FUS	70.5 ± 4.6	2.0	10.4	0.210	0.600
BEA	88.6 ± 3.2	2.5	8.3	0.170	0.500
ENA	85.9 ± 4.4	3.3	9.2	0.215	0.600
ENA1	82.3 ± 3.3	2.1	7.4	0.140	0.400
ENB	84.1 ± 3.7	2.0	8.7	0.145	0.400
ENB1	86.4 ± 2.6	2.7	9.1	0.165	0.500

Table 2
Presence of enniatins (ENA, ENA1, ENB, ENB1) in analyzed samples.

Samples	Number	Enniatins ENs (mg/kg)								
		Positive samples	ENA		ENA1		ENB		ENB1	
			Mean	Maximum level	Mean	Maximum level	Mean	Maximum level	Mean	Maximum level
Wheat	21	13	ND	ND	225.37	634.85	ND	ND	ND	ND
Corn	28	25	ND	ND	167.70	813.01	4.47	6.31	4.34	4.34
Barley	4	2	ND	ND	148.16	361.57	21.37	21.37	45.94	45.94
Other										
Mixture of cereals	5	4	ND	ND	191.03	268.54	ND	ND	ND	ND
Bread mill	4	1	ND	ND	37.47	149.90	ND	ND	23.94	23.94
Oat flour	1	1	ND	ND	388.38	388.38	ND	ND	ND	ND
Rice	1	1	ND	ND	814.42	814.42	7.95	7.95	ND	ND

ND: not detected.

Table 3
Presence of *Beauvericin and Fusaproliferin* in analyzed samples.

Samples	Number	Beauvericin (mg/kg)			Fusaproliferin (mg/kg)		
		Positive samples	Mean	Maximum value	Positive samples	Mean	Maximum value
Wheat	21	9	2.3	3.50	3	3.12	6.63
Corn	28	6	5.72	9.31	1	2.47	2.47
Barley	4	2	4.87	6.94	–	ND	ND
Other							
Mixture of cereals	5	1	3.12	3.12	–	ND	ND
Bread mill	4	1	0.51	0.51	–	ND	ND
Oat flour	1	1	4.18	4.18	–	ND	ND
Rice	1	1	11.78	11.78	1	3.17	3.17

ND: not detected.

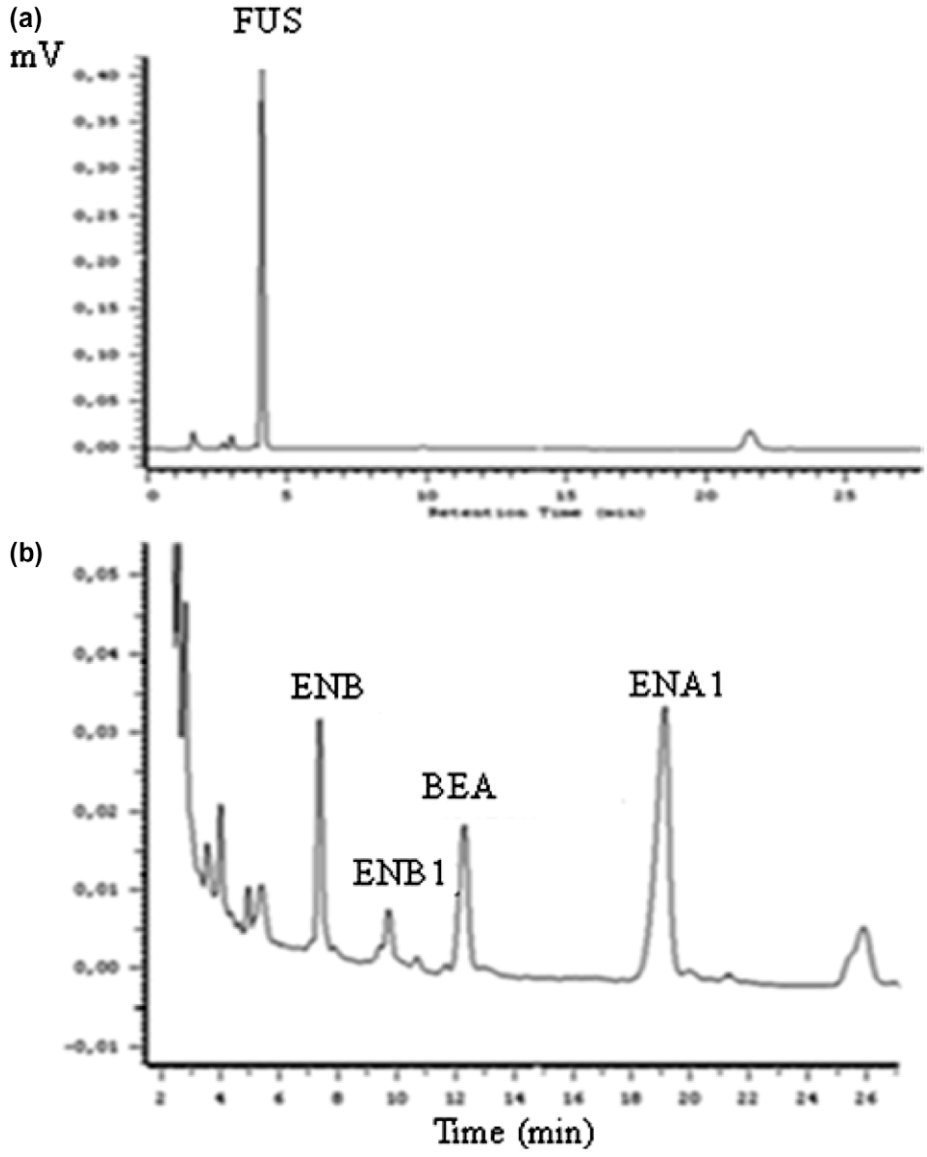

Fig. 1. LC-DAD chromatograms of a positive sample of corn naturally contaminated with: (a) FUS and (b) ENs, and BEA.

2006). The contamination of wheat, barley oats and rye with ENs was also reported in Finland with a maximum value of 18.3 mg ENA1/kg in wheat (Jestoi et al., 2004). In Italy, grain based products were found contaminated with ENs with a maximum value of (Jestoi et al., 2004).

Concerning the presence of BEA and FUS in cereals and grains from Spain, data obtained showed a weak contamination levels found in our study comparing to previous studies from other countries. Data available from Italy showed that FUS was found to contaminate maize samples up to 500 mg/kg (Ritieni et al., 1997b). In another study from USA, Munkvold et al. (1998) reported high contamination levels of animal feeds with FUS with levels up to 30 mg/kg. Logrieco et al. (1993) reported high levels of BEA up to 60 mg/kg in maize from Poland, while Ritieni et al. (1997b) reported high levels of BEA up to 520 mg/kg in maize from Italy.

The cereal samples, which were analyzed in this survey, were from most of the grain producing areas of Spain. As far as we know, this is the first report about the presence of emerging mycotoxins in cereals produced and commercialized in Spain. Levels of contamination of analyzed samples with ENA1 seem very high comparing to the other ENs levels (ENA, ENB and ENB1), BEA and FUS levels. The question is why ENs levels are quite higher than FUS and BEA levels in Spanish grains comparing to the Nordic countries (Finland and Norway) and to Italy. These results suggest that Spanish climatic conditions could be favorable to high ENs production in grains by *Fusarium* species and that ENA1 could be produced with a major ratio. This hypothesis, which is totally in disagreement with results obtained from Norway, needs to be cheeked. Indeed, it was suggested by Uhlig et al. (2006) that under Norwegian growing conditions, *Fusarium* species produced ENs in a distinct concentration ratio (ENB > ENB1 > ENA1 > ENA).

4. Conclusion

The presence of emerging mycotoxins (ENs, BEA and FUS) was determined in samples of cereals commercialized in Spain. The percentage of contamination of total samples with ENs, BEA and FUS were 73.4%, 32.8% and 7.8%, respectively. The high levels found in analyzed samples were 814.42 mg/kg for ENA1, 11.78 mg/kg for BEA and 6.63 mg/kg for FUS. Corn and wheat seems the commod-

ities the most affected than barley. However, we have observed that rice could be a best substrate for emerging mycotoxins production since higher contamination levels were found in the limited analyzed samples. A large number of samples from different areas in the country need to be investigated for the presence of emerging mycotoxins. It could be concluded that because of the absence of international legislation limits for this group of toxins, and due to the toxicological effects recently described, more research are needed to completely assess the situation especially on the moulds species and factors responsible for their production.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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