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Food Chemistry 92 (2005) 559–568
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Determination of type B trichothecenes and macrocyclic lactone mycotoxins in field contaminated maize

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Received 1 June 2004; received in revised form 11 October 2004; accepted 18 October 2004

Abstract

A sensitive, reliable liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for determining some commonly found mycotoxins produced by *Fusarium* strains in maize was evaluated and applied to field samples. The selected substances were: trichothecenes B (nivalenol, deoxynivalenol, fusarenon X, 3- and 15-acetyldeoxynivalenol) and some macrocyclic lactones (zearalenone, α- and β-zearalenol, zearalanone, α- and β-zearalanol). Analytes were extracted from a 1 g sample by homogenization with acetonitrile/water (75:25, v/v, 25 mL final volume). 5 mL of crude extracts was cleaned-up on Carbograph-4 cartridges. Two fractions were obtained and were analyzed by HPLC-electrospray ionization (ESI) in negative mode. Recoveries for spiked maize samples were in the range 79–106% and method detection limits (MDLs) were $\leqslant$6 ng/g for all compounds, except fusarenon X (12 ng/g). 25 random maize samples were analyzed both by the ELISA-based methods specific for deoxynivalenol and zearalenone and by this method for trichothecenes B and macrocyclic lactones. Results were comparable for zearalenone ($R^2 = 0.982$), but disagreed for deoxynivalenol. Finally, a total of 78 freshly harvested maize samples, collected from central and northern Italy during 2002, and divided in two different experiments, were analyzed by the developed method. Data show that there exists a phenomenon of random contamination from the target fusariotoxins just before harvest and an increase of trichothecene B and zearalenone abundance on field crop possibly related to damp climate, temperature range and delayed harvest period. Deoxynivalenol was the most abundant (up to 3430 ng/g) and frequent mycotoxin (40%) detected, followed by acetyldeoxynivalenol. Derivatives of zearalenone were present in traces and β-zearalanol was never found.

Keywords: *Fusarium*; Mycotoxins; Trichothecenes B; Zearalenone; Electrospray; HPLC; Maize; Food analysis

1. Introduction

Agricultural products, mostly grains, can be infested by filamentous and microscopic fungi. The *Fusarium* genus is the most frequent fungal contaminant of field corn (*Zea mays L.*), especially in temperate regions (Bottalico, 1998; Creppy, 2002). This genus is able to produce toxic secondary metabolites, namely mycotoxins, that affect food safety (Kuiper-Goodman, 1995). *Fusarium graminearum*, *F. culmorum*, *F. crookwellense* and *F. poe* (Bottalico, 1998) are the most important, and produce trichothecenes B, nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUS X), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). These are tricyclic sesquiterpenes having a carbonylic group at position C-8. *Fusarium* spp., mainly *F. graminearum*, *F. culmorum*, *F. crookwellense* and *F. equiseti* (Bottalico, 1998),

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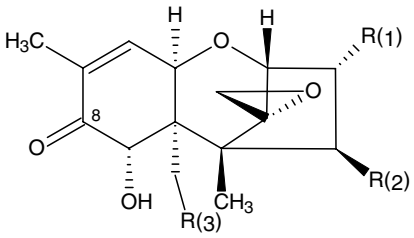
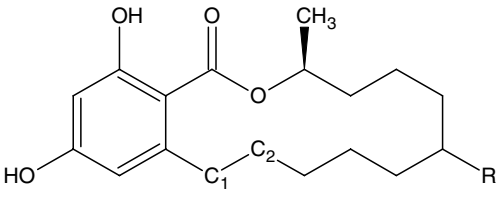
doi:10.1016/j.foodchem.2004.10.008



	MW	R(1)	R(2)	R(3)
NIV	312	OH	OH	OH
DON	296	OH	H	OH
FUS X	354	OH	OAc	OH
3-ADON	338	OAc	H	OH
15-ADON	338	OH	H	OAc



	MW	R	C_1—C_2
ZON	318	=O	C=C
α–**ZOL**	320	····OH	C=C
β–**ZOL**	320	◄OH	C=C
ZAN	320	=O	C—C
α–**ZAL**	322	····OH	C—C
β–**ZAL**	322	◄OH	C—C

Fig. 1. Structures and molecular weights (MW) of investigated type B trichothecenes and zeranols.

can also produce zearalenone (ZON), a macrocyclic lactone derivative of resorcilic acid. Indeed, *Fusarium* spp. have been found to generate at least five metabolites: α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL) and zearalanone (ZAN) (Erasmuson, Scahill, & West, 1994; Richardson, Hagler, & Mirocha, 1985). In this paper, we call ZON and its derivatives "zeranols", as van Bennekom, Brouwer, Laurant, Hooijerink, and Nielen (2002) have proposed. Structures of both trichothecenes B and zeranols (fusariotoxins) are given in Fig. 1. Dietary exposure to fusariotoxins can cause a variety of adverse health effects in farm animals and humans (Creppy, 2002). Trichothecenes B lead to feed refusal, vomiting, anemia, hemorrhage, and immunosuppression (D'Mello, Placinta, & Macdonald, 1999). Zeranols have shown estrogenic and carcinogenic properties (Minervini, Dell'Aquila, Maritato, Minoia, & Visconti, 2001). Infestation of corn by *Fusaria* starts in the field giving rise to biosynthesis and accumulation of fusariotoxins. Field contamination depends strongly on several factors including climatic conditions (rainfall, temperature, humidity), genetic susceptibility of maize cultivars to fungal infection, soil type, maize kernel damage by birds and, lastly, nutritional factors (Bakan, Melcion, Richard-Molard, & Cahagnier, 2002; Mubatanhema, Moss, Frank, & Wilson, 1999). Much effort has been focused on the development of quick and reliable methods for the quantitation of fusariotoxins at trace levels in cereals and cereal-based food and feedstuffs.

Various techniques have been used for the extraction of mycotoxins from maize matrices and for the subsequent purification of the extracts (Krska, 1998; Mateo, Mateo, Hinojo, Llorens, & Jiménez, 2002; Pallaroni & von Holst, 2003). Among the clean-up procedures applicable for trichothecene or zeranol analysis, the most frequently used method is solid-phase extraction (SPE), employing several adsorbent materials, such as charcoal-alumina (Romer, 1986), ion-exchange resin (Mateo et al., 2002), silica (Mateo et al., 2002), Florisil (Mateo et al., 2002; Tanaka, Yoneda, Inoue, Sugiura, & Ueno, 2000), graphitized carbon black (GCB) (Laganà et al., 2003), MycoSep columns (Mateo et al., 2002; Schothorst & Jekel, 2001) and immunoaffinity columns (Zöllner, Jodlbauer, & Lindner, 1999). So far, thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC) and enzyme-linked immunosorbent assay (ELISA) have been applied for determination of fusariotoxins (Krska & Josephs, 2001; Krska, Baumagartner, & Josephs, 2001). ELISA kits for DON and ZON are now commercially available and they are commonly used by Italian public institutions as rapid screening tests to check DON and ZON contents in grains (Haouet & Altissimi, 2003). A drawback is the event of both false positive and false negative results, therefore, confirmation by HPLC-based procedures of doubtful and/or positive ELISA results is required (Anklam, Stroka, & Boenke, 2002). Coupling of HPLC and mass spectrometry (MS) provides a powerful tool in mycotoxin analysis. Multiple reaction monitoring (MRM) with tandem mass spectrometry (MS/MS) enables selective and accurate analyses over a wide linear range (Zöllner et al., 1999). In recent years, the initial, enthusiastic idea that atmospheric pressure ionization followed by tandem MS is a panacea for complex analytical problems has been revised. More and more experimental evidence (Bogialli, Curini, Di Corcia, Nazzari, & Samperi, 2003a; Kebarle & Tang, 1993; Zöllner, Berner, Jodlbauer, & Lindner, 2000) proves that, especially for multicomponent analysis in complex samples, the matrix effect can weaken the

ionic signal to a large, unforeseeable extent. The yield of protonation (or deprotonation) of the analytes during electrospray ionization can be decreased by competition effects due to the co-presence of matrix components. As a consequence, sensitivity decreases and, unless ideal internal standards are available, time-consuming internal calibrations are required to achieve accuracy (Bogialli, Curini, Di Corcia, Nazzari, & Sergi, 2003b). Selective extraction methods (Matuszewski, Constanzer, & Chavez-Eng, 1998), suitable chromatography (Bogialli et al., 2003a, 2003b), or both of them (Matuszewski et al., 1998) could minimize analyte ion suppression.

We now report the modification of a previously published HPLC-MS/MS method for determining trichothecenes B in maize (Laganà et al., 2003) in order to include zeranols. A simultaneous extraction of the target fusariotoxins was followed by a specially designed fractionation in the clean-up step resulting in two fractions that were then analyzed separately. The multi-analyte method was evaluated in terms of accuracy, precision and method detection limits (MDLs). Furthermore, we applied this method to evaluate performances of two ELISA-based methods, respectively, for analysis of DON and ZON. Finally, this study also includes application of the HPLC-MS/MS method developed to freshly harvested maize samples collected from representative Italian fields and from two experimental fields.

2. Materials and methods

2.1. Reagents and chemicals

Standards of mycotoxins, 3α,4β,7α,15-tetrahydroxy-12,13-epoxytrichothec-9-en-8-one (NIV), 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one (DON), 3α,7α,15-trihydroxy-4β-acetoxy-12,13-epoxytrichothec-9-en-8-one (FUS X), 3α-acetoxy-7α,15-dihydroxy-12,13-epoxytrichothec-9-en-8-one (3-ADON), 15α-acetoxy-3α,7α-dihydroxy-12,13-epoxytrichothec-9-en-8-one (15-ADON), 2,4-dihydroxy-6-[10-hydroxy-6-oxo-trans-1-undecyl]benzoic acid μ-lactone (ZON) 2,4-dihydroxy-6-[6α,10-dihydroxy-trans-1-undecyl]benzoic acid μ-lactone] (α-ZOL), 2,4-dihydroxy-6-[6β,10-dihydroxy-trans-1-undecyl]benzoic acid μ-lactone (β-ZOL), 2,4-dihydroxy-6-[10-hydroxy-6-oxo-undecyl]benzoic acid μ-lactone (ZAN), 2,4-dihydroxy-6-[6α-10-dihydroxyundecyl]benzoic acid μ-lactone (α-ZAL), 2,4-dihydroxy-6-[6β-10-dihydroxyundecyl]benzoic acid μ-lactone (β-ZAL) and the internal standards, 3,17α-dihydroxy-1,3,5(10)-estratriene (17 α-estradiol) and 6-[2-ethoxy-1-naphatamido] penicillin (nafcillin sodium salt monohydrate) were purchased from Sigma–Aldrich (Milan, Italy). They were individually dissolved in acetonitrile at concentrations of 1 mg/mL, stored at −20 °C, and brought to room temperature before use. Standard solutions are more stable in acetonitrile than in methanol for long time storage (Pettersson & Åberg, 2003; Shepherd & Gilbert, 1988). Only NIV was prepared at 0.020 mg/mL because of its low solubility. Composite working standard solutions were prepared by combining suitable aliquots of each individual standard stock solution and diluting with a suitable solvent. These solutions were kept at 4 °C and renewed weekly. All organic solvents were HPLC grade from Carlo Erba (Milan, Italy) and were used as received. Water was treated with a Waters Milli-Q system. Formic acid was purchased from Merck (Darmstadt, Germany). Carbograph-4 was supplied by L.A.R.A. S.r.l. (Rome, Italy) and no particular precautions were taken in its storing. This graphitized carbon black is similar to Carboprep 200 (Restek), and Envicarb X (Supelco). Its surface area is 210 m^2/g and the particle size range is 120–400 μm. Carbograph-4 cartridges were prepared by filling 6×1.3 cm i.d. polypropylene tubes (Supelco, Milan, Italy) with 500 mg of adsorbent material, placed between two polyethylene frits (Supelco). Before processing sample, Carbograph-4 cartridges were attached to a vacuum manifold apparatus (Supelco) and washed with 10 mL of dichloromethane/methanol (80:20, v/v), 5 mL of methanol, 20 mL of water 10 mmol/L HCl (Carlo Erba) and 10 mL of water. Oasis HLB™ cartridges (200 mg) were purchased from Waters (Milford, USA) and were utilized according to the instructions given by the manufacturer. Pure cellulose filter papers (Whatman no. 1) used in the pre-ELISA treatment sample were purchased from Carlo Erba.

2.2. Sample preparation

A previously reported procedure (Laganà et al., 2003) was slightly modified. Briefly, 1 g of finely ground maize (80–170 mesh) was homogenized with 10 mL of acetonitrile/water (75:25, v/v) for 15 s, using an Ultra Turrax apparatus, and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a volumetric flask (25 mL). The maize residue was washed twice with 7 mL of acetonitrile/water (75:25, v/v) and the centrifugation was repeated every time. The supernatants were collected into the flask and the volume adjusted to 25 mL. Afterwards a 5-mL aliquot of the extract was diluted to 1 L with water and passed through the pre-conditioned Carbograph-4 cartridge. The cartridge was washed with 10 mL of water and the residual water content was further decreased by slowly passing 0.3 mL of methanol through the cartridge. Trichothecenes B were eluted with 5 mL of methanol (fraction A). Then the cartridge was washed with 10 ml of methanol containing 50 mmol/L formic acid and 5 mL of neutral methanol and washings were discarded. Macrocyclic lactones were eluted with 15 mL of dichloromethane/methanol (80:20,

v/v) (fraction B). Each fraction was evaporated to dryness at 40 °C under a gentle flow of nitrogen. Fraction A residue was redissolved in 250 μL of methanol/water (10:90, v/v) at 0.1 ng/μL of nafcillin, as internal standard, while residue of fraction B was redissolved in 250 μL of acetonitrile/methanol/water (35:15:50, v/v/v) at 0.2 ng/μl of α-estradiol, as internal standard. 50 μL of the two final solutions was injected into the HPLC column.

For recovery studies, mycotoxin-free samples were artificially fortified as follows. 1 g of finely ground maize was soaked in 1 mL of acetone solution containing a suitable amount of each analyte, then the sample was placed for 2 h in a ventilated oven at 40 °C. Finally, the spiked sample was extracted and analyzed.

2.3. Mycotoxin analysis by HPLC-MS/MS and ELISA

Liquid chromatography was performed using a Perkin–Elmer binary LC pump 250 (Perkin–Elmer, Norwalk, CT, USA) provided with a Rheodyne 7125 injector and a 50-μL loop. The analytes were chromatographed on an Alltima C_{18} column (250 × 4.6 mm i.d., 5 μm particle size) from Alltech (Deerfield, IL, USA) with a Supelguard 20 × 4.6 mm i.d. precolumn supplied by Supelco (Milan, Italy). A PE-Sciex (Concord, Ont., Canada) API 365 bench-top triple-quadruple mass spectrometer, coupled with ESI source, was used. Analyses were performed using two different mobile phase settings for the separation of trichothecenes B and macrocyclic lactones. A gradient separation was used for trichothecenes B. The initial composition of the mobile phase was 10% of acetonitrile/methanol (70:30, v/v) and the remaining 90% of water. The gradient was programmed to linearly increase the amount of organic phase up to 45% in 10 min, then immediately to 80% and held constant for 7 min. 3-ADON and 15-ADON, beside having the same MS/MS spectrum, cannot be separated in these conditions and then we determined them as total ADON. Therefore, the acronym ADON will denote the sum of both isomers. Recently, Razzazi-Fazeli, Böhm, Jarukamjorn, and Zentek (2003) obtained an adequate separation of the above-mentioned isomers by employing a Polar-RP column. Macrocyclic lactones were separated in isocratic conditions. The mobile phase composition was set at 50% of acetonitrile/methanol (70:30, v/v) in water. In both cases, to purge the column after each chromatographic run, the amount of acetonitrile/methanol was increased to 95% and held for 5 min. The flow rate was 1 mL/min, and the column effluent was split (using a PEEK tee) to allow only a flow of 0.2 mL/min into the ESI interface.

The interface was operated in negative (NI) mode. A turbo-gas of nitrogen heated at 350 °C was used for ESI working. MS and MS/MS spectra were preliminarily recorded by connecting the Harvard infusion pump directly to the source. In order to optimize the tuning parameters (ion optic potential, energy and pressure values) standard solutions of about 10 ng/μL for each compound, in acetonitrile/methanol (70:30, v/v) 50% in water, were infused at 10 μL/min. From MS/MS full scan spectra, suitable transition pairs were selected for the acquisition in multiple reaction monitoring (MRM). HPLC-(ESI)MS/MS parameters for detection of each mycotoxin are summarized in Table 1.

Two competitive enzyme immunoassays, RIDASCREEN® DON and RIDASCREEN®FAST Zearalenon, both provided by R-Biopharm AG (Darmstadt, Germany), were utilized for the analysis, respectively, of DON and ZON. The instructions given by the manufacturer were strictly followed for the correct use of the kits. The results of the analyses were obtained photometrically at $\lambda = 450$ nm using a Microplate Reader 550 (BIO-RAD) spectrophotometer. Recovery rate in cereal is between 85–110% for DON and 64–97% for ZON. Detection limits of the methods are, respectively, 17 and 18.5 ng/g. Upper limit of linearity is 500 ng/g for both kits.

Table 1
HPLC-MS/MS conditions and precursor ion/product ion pairs for the acquisition in negative ESI(MRM) mode of the target compounds

Analytes[a]	Period	Retention time (min)	Orifice potential (V)	Collision energy (eV)	Precursor ion (*m/z*)	Product ions (*m/z*)
NIV	1	7.9	−13	10	311	281
DON	2	10.2	−26	17	295	265, 247, 138
FUS X	3	11.9	−31	37	353	187, 59
ADON	4	14.5	−26	14	337	307, 173, 59
β-ZAL	1	10.3	−46	43	321	277, 161, 91
β-ZOL	1	11.0	−29	45	319	160, 144, 130
α-ZAL	2	14.8	−46	43	321	277, 161, 91
α-ZOL	2	16.1	−28	46	319	174, 160, 130
ZAN	3	23.3	−51	38	319	205, 161, 107
ZON	3	24.7	−30	40	317	175, 160, 135

[a] NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; ADON, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol; β-ZAL, β-zearalanol; β-ZOL, β-zearalenol; α-ZAL, α-zearalanol; α-ZOL, α-zearalenol; ZAN, zearalanonel ZON, zearalenone.

2.4. Sample collection and field experiments

Researchers from *Istituto Sperimentale per la Cerealicoltura* (ISC – Rome, Italy) conducted sampling during the 2002 harvest season and kindly provided us with 78 maize samples. 46 maize samples (from three hybrid types belonging to FAO 400, 500 and 600 classes) were from 12 villages scattered in the countryside across central and northern Italy. They were harvested from private farms "in open field". In this case management practices were determined by the farmer co-operator according to local practice and needs. We divided the samples into three groups depending on their geographical origin which is linked to area climatic conditions: 1 – central Italy, 2 – Po Valley, and 3 – north-western Italy (Fig. 2). Area 1 is hilly with little rain, but muggy, area 2 is a wide valley between Apennines and Alps and very humid, area 3, placed in the North of Po Valley, is drier than area 2, but very rainy. Meteorological data relating to the months between April and October 2002 were acquired from weather reports edited by UCEA (Ufficio Centrale di Ecologia Agraria) for the Department of Agriculture. These data came from 10 stations located through the whole area studied. In the April–October period mean rainfall index in area 1 was around 77 mm/month with mean humidity 53–83%, in area 2 around 103 mm/month with humidity 75–81% and in area 3 around 120 mm/month with humidity in the range 52–75%. Besides this, the most important data are the abundant and unusual rainfalls occurred in Italy during the July–August period.

In the same period, the ISC institute provided us with a further 32 samples coming from corn cultivated in two experimental fields in Lazio, one in Northern Rome, and the other in Torreinpietra (a village to the West of Rome). The former is located in a valley in which humidity stagnation can occur, while the Torreinpietra one is near the sea. Field experimental design was carried out employing controlled farming conditions (same irrigation, fertilizing, etc.). In both sites the area selected for sowing was divided into equal parcels each of 9.6 m^2; three replicate plots were planted for each maize hybrid. 5 random ears of corn were sampled from each parcel and then three sub-samples were put together in a single sample for analysis. 8 different maize hybrids belonging to three different FAO precocious flowering groups (FAO 300, 400 and 500) were sowed. In particular, the 32 maize samples were divided into two batches (16×2), belonging to the different sampling sites: Northern Rome and Torreinpietra. Each batch was, in its turn, divided into two sets (8×2), depending on different sowing and harvest days: the first sowed on 8 April and harvested on 9 September 2002 ("normal harvest"), the second sowed on 30 April, and harvested on 1st October 2002 ("delayed harvest"). Samples were sent to our laboratory as soon as collected and were ground, sieved and extracted on arrival and analyzed in order to avoid external factors that could favor mold growth and mycotoxin development.

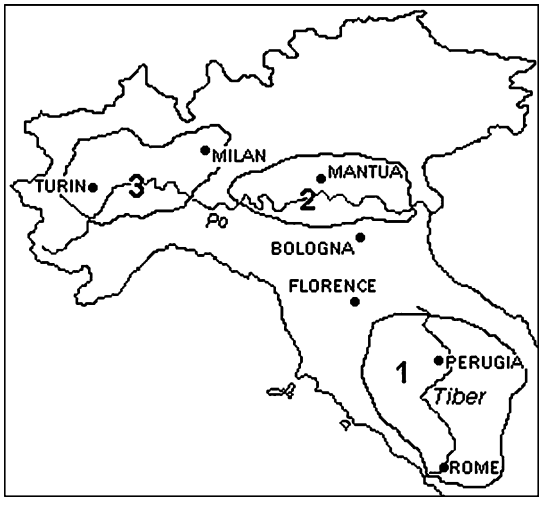


Fig. 2. 1, 2 and 3 corn sown sampling areas across central and northern part of Italy.

2.5. Quantitation and statistical evaluation

Analytes were quantified by external calibration procedure. Standard solutions were prepared at 8 levels by using appropriate volumes of the working standard solution. For each analyte, the peak area versus injected amount chart was obtained by measuring the resulting peak area and relating this area to that of the internal standard. The response of ESI-MS/MS was linearly related to injected amounts up to 200 ng ($R^2 > 0.985$). Correction for recovery was not done. Statistical evaluations were performed by ANOVA ($p = 0.05$).

3. Results and discussion

3.1. Extraction and clean-up procedure

The extraction procedure, as described in Section 2.2, was optimized after evaluating the performance of different techniques, such as pressurized liquid extraction (PLE), matrix solid phase dispersion-extraction (MSPDE), ultrasonication and homogenization-extraction, with different mixtures of solvents. Recoveries were obtained by spiking, at a level of 100 ng/g, analyte-free samples, before and after the extraction step, and following the rest of the procedure. In this way, the effect of extraction on total recovery can be isolated and evaluated by comparing the absolute peak areas for the same compound in samples spiked *ante* and *post* extraction. The best compromise for simultaneous quantitative extraction in a short time was reached by homogenizing in acetonitrile/water (75:25, v/v) a

previously ground and sieved maize sample (Laganà et al., 2003). All analytes were extracted from maize with recoveries above 92%.

For the extract clean-up, two adsorbents suitable for extraction of both polar and non-polar compounds, such as Oasis HLB™ and Graphitized Carbon Black (Carbograph-4), were tested. Apparently low recoveries, especially for NIV, DON and ZON, were obtained with both materials. By comparing samples spiked before and after clean-up, we found that a strong matrix effect was responsible for this behavior. As formerly reported, GCBs can behave as both reversed-phase and ion-exchanger sorbents and, besides, have a particular affinity for aromatic compounds with respect to aliphatic ones (Andreolini, Borra, Caccamo, Di Corcia, & Samperi, 1987; Crescenzi, Di Corcia, Passariello, Samperi, & Turnes Carou, 1996). Indeed, it was possible to separate, according to the procedure reported in Section 2, trichothecenes B in the methanol fraction from zeranols in the dichloromethane/methanol (80:20, v/v) fraction, whereas acidic compounds were retained. This clean-up procedure gave cleaner extracts than collecting a single fraction and drastically reduced ion suppression during ESI ionization process. When the intermediate acidic washing step was omitted, a decrease in ZON response was still found. To avoid the effect of breakthrough on the SPE cartridge, only 5 mL of the entire extract, added to 1 L of water, was submitted to clean-up. Analytes did not show degradation or insolubility phenomena during the concentration step and the reconstitution of dried extract before HPLC-MS/MS analysis. Recoveries, obtained by comparing analyte-free samples spiked at level of 100 ng/g before and after clean up, were better than 85%.

3.2. Analysis of mycotoxins by HPLC-MS/MS

Fractions A and B (250 μL each) containing, respectively, trichothecenes B and zeranols, were analyzed separately by HPLC-MS/MS. For trichothecenes B we adopted the gradient conditions discussed in a previous work (Laganà et al., 2003). Berger and Plattner have used verrucarol, a trichothecene obtained by hydrolysis of macrocyclic precursors, as internal standard (Berger, Oehme, & Kuhn, 1999; Plattner & Maragos, 2003). Until now, it is not clear if it could be found naturally. We used, as internal standard for type B trichothecene analysis, nafcillin, a compound not structurally related with trichothecenes. Nafcillin was added to fraction A before injection into the HPLC, to control and compensate for variation of the instrument response in multiple runs during the day. Then, quantitation was performed using external calibration, as done by other authors (Huopalahti, Ebel, & Henion, 1997; Langseth & Rundberget, 1998; Mateo et al., 2002). ZON and the other macrocyclic lactones were eluted along an isocratic run, with the aim of separating the pairs of isomers α-ZOL from β-ZOL and α-ZAL from β-ZAL.

Trichothecenes B showed the best sensitivity if analyzed in negative ESI with neutral phase (Laganà et al., 2003). Whereas ESI gave similar or slightly less sensitivity for zeranols than APCI, ESI interface was selected for both analyses (Huopalahti et al., 1997; Laganà, Fago, Marino, & Santarelli, 2001; Plattner & Maragos, 2003).

3.3. Accuracy, precision and method detection limits

Calibration curves were obtained, as reported in Section 2, for a set of six analyte-free samples spiked after clean-up. Samples were fortified to have a concentration range of 0.004–4 ng/μL in 250 μL of final extract. The procedure was repeated three times. All calibration curves show good linearity with correlation coefficients R^2 not lower than 0.923 for trichothecenes B and not lower than 0.967 for ZON and its metabolites. Furthermore, slope and intercept do not differ significantly ($p = 0.05$) from calibration in standard solution (external calibration procedure) for all the compounds except NIV (−11%). Therefore, the method appears to suffer slightly from ion suppression phenomena by virtue of sample clean-up efficiency and good chromatography.

Accuracy and precision were evaluated by recovery experiments. Standards were added to analyte-free maize samples at three levels (25, 100 and 3000 ng/g) and the spiked samples were worked up and analyzed by HPLC-MS/MS as described in Section 2. Recoveries obtained for the three spiking levels do not differ significantly and were above 88% (RSD% ⩽ 12%) for all target compounds, except for NIV (recovery = 79%). MDLs ($S/N = 3$) were estimated with a sample spiked

Table 2
Recoveries, relative standard deviations (RDSs) and method detection limits (MDLs) obtained from artificially contaminated maize samples

Analytes[a]	Recovery[b]±RSD[c] (%)	MDL[d](ng/g)
NIV	79 ± 8	6
DON	89 ± 10	2
FUS-X	95 ± 6	12
ADON	97 ± 3	3
β-ZAL	103 ± 10	3
β-ZOL	89 ± 5	3
α-ZAL	106 ± 12	4
α-ZOL	100 ± 10	5
ZAN	97 ± 10	6
ZON	95 ± 7	3

[a] NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; ADON, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol; β-ZAL, β-zearalanol; β-ZOL, β-zearalenol; α-ZAL, α-zearalanol; α-ZOL, α-zearalenol; ZAN, zearalanone; ZON, zearalenone.
[b] Spiking level was 100 ng/g.
[c] RSD was calculated for six samples.
[d] Spiking level was 25 ng/g.

at level of 25 ng/g. They were in the range 2–6 ng/g, except FUS X value (12 ng/g). Results are reported in Table 2. When necessary, both accuracy and precision may be improved by internal calibration (Bogialli et al., 2003a, 2003b).

3.4. Comparison between ELISA and HPLC-MS/MS methods

Table 3 shows data obtained from 25 randomly selected samples of freshly harvested maize and analyzed by both techniques, ELISA for DON and ZON, and HPLC-MS/MS for trichothecenes B and zeranols. Concerning ZON, only 7 samples were found contaminated at level above MDL value of the ELISA-based method (18.5 ng/g). Regression analysis (x = HPLC-MS/MS) gave $y = 0.93x + 17$ and $R^2 = 0.982$. Cross-reaction event with α-ZOL, β-ZOL and ZAN has been reported for this commercial ELISA test kit (Krska & Josephs, 2001). We did not find any overestimation that could be due to cross-reactivity of ELISA antibody with metabolites of ZON, probably because they were present at very low concentration in all samples (no more than 15 ng/g as total). Conversely, in the case of DON, the HPLC-MS/MS method evidenced eight cases of false positives and one case of overestimation (Table 3). This fact can be ascribed partly to cross-reaction phenomena of the antibody towards other trichothecenes B. Cross-reactivity rates reported in the ELISA kit instructions are: NIV 4%, 3-ADON > 100%, 15-ADON 19% and FUS X < 1%. Indeed, the same maize samples, analyzed by HPLC-MS/MS, were proved to be randomly contaminated by NIV and ADON. An other drawback of ELISA method is the restricted linear dynamic range that is 17–500 ng/g. DON concentration values in the samples 9–10 exceeded the upper limit of these range, but it has not been possible to reanalyze both samples. By HPLC-MS/MS the linear dynamic ranges are from MDLs until at least 5.000 ng/g, keeping in mind that 1/5 of the 1 g extract is cleaned-up and then 1/5 of the purified extract is analyzed.

Table 3
Comparison of data obtained analyzing maize samples with both ELISA and HPLC-MS/MS methods

Sample (#)	ELISA	HPLC-MS/MS	
	DON	DON	$\Sigma_i X_i$[a]
Trichothecenes B (ng/g)			
1	191	–	433
2	27	–	–
3	–[c]	–	29
4	61	–	660
5	–	–	23
6	29		–
7	–	–	7
8	154	20	48
9	>500	2060	5560
10	>500	1050	2020
11	42	–	2620
12	20	–	487
13	74	–	427
14	44	–	–
15–25	–	–	–
	ZON	ZON	$\Sigma_i Y_i$[b]
Zeranols (ng/g)			
1	–	8	8
2	54	46	46
3	97	86	101
4	385	384	395
5	–	10	18
6	251	223	238
7	379	414	429
8	59	68	68
9	184	161	174
10	–	6	6
11–25	–	–	–

[a] $\Sigma_i X_i$: sum of deoxynivalenol (DON), nivalenol, fusarenon X, 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol.

[b] $\Sigma_i Y_i$: sum of zearalenone (ZON), α- and β-zearalenol, zearalanone, α- and β-zearalanol.

[c] < MDL.

Therefore, results obtained from ELISA and HPLC-MS/MS for ZON were in good agreement. Moreover, simultaneous presence of ZON and its metabolites in significant amounts was never found even by other authors (Müller, Reimann, Schumacher, & Schwadorf, 1997; Schollenberger, Jara, Suchy, Drochner, & Müller, 2002), so that it is unlikely that cross-reaction may occur. On the contrary, for DON the HPLC-MS/MS should be used to confirm positive samples detected by the ELISA-based method. In addition, by HPLC-MS/MS NIV, DON, ADON and FUS X can be unequivocally identified from the mass spectrum and quantified simultaneously. This is important if the different toxicity of trichothecenes is taken into account.

3.5. Maize samples collected from Italian fields

The second part of this study was achieved by collecting 46 maize samples "in open field" directly from some Italian private farms across the midlands and northern part of Italy. The authors' intent was to sample corn grown in a large variety of geographical locations to investigate the occurrence extent of some fusariotoxins in the field. These data are still partial because, as explained in Section 2, the exceptionally high rainfall index leveled differences among the selected sampling areas and probably amplified the occurrence levels.

The peculiarity of this work lies in evaluating the occurrence of some fusariotoxins produced by fungi on corn grown just before harvest. For this reason these data cannot be compared with other analogues in the literature as well explained by Müller and co-workers (2002). Some authors conducted specific studies on mycotoxin contamination of freshly harvested cereals: on fumonisins in Brazilian corn (Almeida et al., 2002),

Table 4
Incidences, mean of positives and concentration ranges (ng/g) of target fusariotoxins in maize samples collected from different farms placed in the north-centre of Italy

Analytes[a]	Central Italy			North-western Italy			Po valley		
	Incidence (pos/tot)	Mean (ng/g)	Range (ng/g)	Incidence (pos/tot)	Mean (ng/g)	Range (ng/g)	Incidence (pos/tot)	Mean (ng/g)	Range (ng/g)
NIV	6/31	607	12–2440	1/11		200	0/4	–	–
DON	6/31	712	5–2060	11/11	1330	45–3430	3/4	387	68–967
FUS X	5/31	137	26–420	1/11	–	34	0/4	–	–
ADON	5/31	711	6–3500	7/11	31	7–95	0/4	–	–
ZON	6/31	93	8–384	7/11	228	41–969	0/4	–	–
α-ZOL	1/31	–[b]	9	4/11	14	7–33	0/4	–	–
β-ZOL	3/31	8	6–11	5/11	20	8–33	0/4	–	–
ZAN	1/31	–	13	1/11	–	12	0/4	–	–
α-ZAL	0/31	–	n.d.[c]	0/11	–	n.d.	0/4	–	–
β-ZAL	2/31	7	6–8	0/11	–	n.d.	0/4	–	–

[a] NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; ADON, sum of 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol; ZON, zearalenone; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol; ZAN, zearalanone; α-ZAL, α-zearalanol; β-ZAL, β-zearalanol.
[b] Not calculated.
[c] n.d. < MDL.

fumonisin B1, moniliformin and ZON in a Zimbabwean one (Mubatanhema et al., 1999), trichothecenes B and moniliformin in Polish wheat (Grabarkiewicz-Szczęsna, Kostecki, Goliński, & Kiecana, 2001), and *Fusarium* toxins in German barley (Müller et al., 1997). Other authors analyzed fusariotoxin levels in genetically modified maize grown in France, Spain (Bakan et al., 2002) and the United States (Hammond et al., 2004). Data obtained in the present study are summarized in Table 4. As far as trichothecenes B is concerned, over 40% of the samples were found contaminated by DON, which was the most abundantly detected mycotoxin (up to 3430 ng/g), followed by ADON (26%, maximum value 3500 ng/g). The frequencies of NIV (15%) and FUS X (13%) contamination were less than DON's and, in particular when FUS X was found, the levels were no more than 420 ng/g. In addition, co-occurrence of DON and ADON was observed in 23%, NIV and FUS X in 10%, while NIV and DON, FUS-X and DON co-occurred only in 2%. The prevalence of DON resulting from our gross screening across Italian fields is not surprising and may be attributed, partially, to the large diffusion of *Fusarium* strains in temperate regions producing, as described by some authors (Bottalico, 1998; Mubatanhema et al., 1999; Müller et al., 1997), high contents of DON. This fact is further amplified by a wet 2002 summer. It has to be noted that the highest mean value of DON (1330 ng/g) and the highest incidence (100%) were found in the samples coming from the northern-western Italy (area 3), where, as a general rule, fluctuations over the day–night cycle (up to 15°C) are higher than in areas 1 and 2.

ZON was detected in 30% of the samples, usually at low levels, while its derivatives were rarely encountered, as reported in previous works (Bakan et al., 2002; Müller et al., 1997; Schollenberger et al., 2002). We found α- and β-ZOL in 28% of the samples with concentration values up to 33 ng/g. α-ZAL was never detected.

Although there was random contamination in the field samples collected, however the few samples did not allow us to find significant correlations between hybrid type and mycotoxin content.

3.6. Maize samples coming from experimental fields

Finally, we also tried to investigate the way in which field contamination, when it occurred, was dependent on weather and environmental factors. Although two different irrigations on maize crops in Lazio were scheduled, because of considerable summer rainfall, the second irrigation was eliminated. Thus, it was impossible to determine any difference in mycotoxin prevalence between well-watered and stressed maize. After a hot and dry June, in July and August, generally arid months in Mediterranean regions, abundant and unexpected rains had fallen, enhancing the harvests, but, probably encouraging mold growth.

Four sets (32 samples) of maize samples, kindly provided us by ISC of Rome, were collected from two experimental fields (Northern Rome and Torreinpietra) selected for their different microclimatic conditions (see Section 2). The results are summarized in Table 5. Quite similar low levels of mycotoxins were detected in both sets coming from Torreinpietra and in the first set from Northern Rome. Notably, in the second set of the Northern Rome samples, the level of mycotoxins, especially trichothecenes B, was seriously increased if compared with the first one. NIV varied from the mean value of 135 to 1920 ng/g, ADON from 14 to 2060 ng/g, DON and ZON from "not detected" to 2040 and 273 ng/g, respectively. Presumably this is due to the stagnant humidity

Table 5
Incidences, mean of positives and maximum levels (ng/g) of fusariotoxins found in maize samples coming from two experimental fields, located in Torreinpietra and in northern Rome, after the normal harvest and delayed harvest

Analytes[a]	Normal harvest			Delayed harvest		
	Incidence (pos/tot)	Mean (ng/g)	Max. (ng/g)	Incidence (pos/tot)	Mean (ng/g)	Max. (ng/g)
Torreinpietra						
NIV	1/8	–[b]	16	1/8	–	28
DON	1/8	–	19	1/8	–	17
FUS X	1/8	–	27	1/8	–	24
ADON	1/8	–	6	1/8	–	9
ZON	2/8	10	11	2/8	13	16
others	1/8	–	8	1/8	–	7
Northern Rome						
NIV	2/8	135	240	2/8	1920	3270
DON	0/8	–	n.d.[c]	2/8	2040	2140
FUS X	1/8	–	382	2/8	104	181
ADON	2/8	14	23	1/8	–	2860
ZON	0/8	–	n.d.	3/8	273	412
others	0/8	–	n.d.	6/8	9	13

[a] NIV, nivalenol; DON, deoxynivalenol; FUS X, fusarenon X; ADON, sum of 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol; ZON, zearalenone; others, sum of α- and β-zearalenol, zearalanone, α- and β-zearalanol.
[b] Not calculated.
[c] n.d. < MDL.

typical of the Northern Rome field, located in a valley, and to low minimum temperatures recorded in the 2nd and 3rd decade of September. The influence of temperature on simultaneous production of DON and ZON by *Fusarium* strains was investigated by other authors (Martins & Martins, 2002). In particular, ZON production is favored by cold weather in high moisture conditions (Hollinger & Ekperingin, 1999). Therefore, the maize harvested in October remained longer in the field between the end of summer and the beginning of autumn; the humidity and the rainfall contributed in mold development while thermic fluctuations favored fusariotoxin production. This trend was partially reflected by the data from the northern-western area (Table 4), where low minimum temperatures are usually reached before the normal harvest time. On the contrary, Torreinpietra, being near the sea, has a windier climate with higher minimum temperatures, therefore, has proved to be a site less susceptible to contamination when the corn harvest is delayed.

In conclusion, the simultaneous occurrence of mycotoxins assayed in a large part of just-harvested corn demonstrates the importance of improving farming and agronomic practice during cultivation. Indeed, the findings of the present study fortify the need for regular screening of cereals for *Fusarium* toxins, particularly in years with abundant precipitation late in the summer.

Acknowledgments

The authors are grateful to the "Istituto Sperimentale per la Cerealicoltura" (ISC – Rome) for providing maize samples and letting us use the results of mycotoxin analyses by ELISA-based method. A special acknowledgment is given to Maria Grazia D'Egidio and Fabrizio Quaranta, from ISC, for assistance during data processing.

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