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ScienceDirect
ELSEVIER
Food Chemistry 105 (2007) 700–710
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Mycotoxins produced by *Fusarium* genus in maize: determination by screening and confirmatory methods based on liquid chromatography tandem mass spectrometry

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Received 20 September 2006; received in revised form 27 November 2006; accepted 27 December 2006

Abstract

A confirmatory method for fusariotoxin analysis in maize meal, based on liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS), was developed, and compared with a previously published screening method, based on the same technique. By eluting selectively from a Carbograph-4 clean-up cartridge trichothecenes, fumonisins and macrocyclic lactones, and optimizing LC–MS/MS conditions for every chemical class, a sensitive and reliable determination was performed. Method quantification limits for confirmatory and screening methods were in the range 0.001–0.019 mg/kg and 0.003–0.125 mg/kg, respectively.

Maize samples collected from four different hybrids grown in five experimental field trials were analyzed with both screening and confirmatory procedures. In most of the samples, fumonisin B_{1-3} were revealed with a concentration above 2 mg/kg. Zearalenone was found at a higher level than 0.5 mg/kg in three samples, and nine samples were found positive for this toxin only with the confirmation method, being contaminated at levels below 0.008 mg/kg. Among trichothecenes B only deoxynivalenol was found twice at a concentration over 1 mg/kg, whereas fusarenon X was never revealed. Trichothecenes A were present at a concentration lower than 0.015 mg/kg.
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Keywords: Fumonisin components; FB_{1-3}; Macrocyclic lactones; Trichothecenes; Maize; LC/ESI-MS/MS

1. Introduction

Fusarium subspecies are common contaminants of maize (*Zea mays* L.) causing root, stalk, and ear rot (Leslie, 1996). Infection may be associated with yield reduction, but *Fusarium* spp. may also infect the plant without ear-rot symptoms (Munkvold & Desjardins, 1997). In addition, the *Fusarium* spp. can produce a wide array of structurally different mycotoxins including thricothecenes, fumonisins, and macrocyclic lactones (Placinta, D' Mello, & Macdonald, 1999; Munkvold, 2003).

These toxins are worthy of interest because of increasing evidence of their involvement in human and animal diseases (CAST, 2003). For example, thricothecenes lead to food refusal, emesis, anaemia, haemorrhage, immunosuppression, neurotoxic effects, and for T-2 toxin, a possible carcinogenic effect. Macrocyclic lactones have shown estrogenic and carcinogenic properties. Among the 15 fumonisin analogues isolated and characterized, fumonisin B1 (FB_1) is typically found at the highest levels and is toxicologically the most abundant (Musser & Plattner, 1997). When consumed by animals, FB_1 causes diseases such as leukoencephalomlacia in horses (Kellerman et al., 1990) and pulmonary edema in pigs (Harrison, Colvin, Greene, Newman, & Cole, 1990). These mycotoxins have been classified as cancer promoters (Gelderblom, Kriek, Marasas, & Thiel, 1991), and they are

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doi:10.1016/j.foodchem.2006.12.057

linked to neural tube defects in rat and human embryos (Sadler et al., 2002, & references therein). Moreover, in the case of contemporaneous exposure to more than one toxin, even if at levels generally considered ineffective, there could be unexpected adverse effects, caused by an additive or synergic effect (Speijers & Speijers, 2004, & references therein).

The contamination of maize by these fungi and their related mycotoxins is a worldwide problem (Shephard, Thiel, Stockenstrom, & Sydenham, 1996), so the demand for maize form cultivars carrying resistance to *Fusarium* spp. as well as resistance to mycotoxin production is high. Strategies to reduce mycotoxins include cultivar practices and crop management, host plant resistance through breeding and/or genetic engineering and biocontrol, e.g. cytotoxigenic strains (Munkvold, 2003). The most effective and economical way to keep mycotoxin contamination under control is the development and use of resistant hybrids. Rapid and reliable screening methods are also required for their identification and quantitation in grain cereals to ensure safety and compliance with the current (Food and Agricultural Organization) and forthcoming legislation. Among screening tests for fusariotoxins, ELISA (enzyme-linked immunosorbent assay) methods are widely utilized (Krska & Josephs, 2001; Krska, Baumagartner, & Josephs, 2001). ELISA-based kits are simple to use and allow analysis of many samples per day (Gilbert, 1999). However, they are available only for some fusariotoxins, are generally expensive, and may suffer from cross-reactivity phenomena giving rise to false positive results that must then be confirmed by gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS).

Recently, we have developed a multiresidue method based on liquid chromatography/electrospray tandem mass spectrometry (LC/ESI-MS/MS) for the simultaneous identification and quantification of the major mycotoxins that can arise from the *Fusarium* species in maize grain samples, even if not in the optimum LC/MS conditions (Cavaliere, Foglia, Pastorini, Samperi, & Laganà, 2005). We also reported that an important advantage of this screening method was the possibility of performing more sensitive and accurate confirmatory analysis on the same extract, by optimizing the clean-up and LC/MS conditions for each mycotoxin chemical type.

Accordingly, in the present work the confirmatory method for fusariotoxin analysis was developed by suitably modifying two our previously published procedures (Cavaliere, D'Ascenzo, et al., 2005; Faberi, Foglia, Pastorini, Samperi, & Laganà, 2005). Moreover, this method was compared with the screening method, analyzing with both procedures a series of maize samples collected from five Italian experimental field trials where different cultivars were grown.

2. Materials and methods

2.1. Reagents and chemicals

Standards of trichothecenes A: diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), monoacetoxyscirpenol (MAS), neosolaniol (NEO), T-2 toxin (T-2); of trichothecenes B: nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUS X), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON); of two fumonisins (FBs): fumonisin B_1 (FB_1), fumonisin B_2 (FB_2); and of macrocyclic lactones: zearalenone (ZON), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL); and of the internal standards (ISs): zearalanone (ZAN), verrucarol (VER), and diclofenac were purchased from Sigma–Aldrich (Milan, Italy). Pure crystalline fusariotoxins were individually dissolved in acetonitrile at concentrations of 1 mg/mL, stored at −20 °C in amber glass vials, and brought to room temperature before use. Standard solutions are more stable in acetonitrile than in methanol for long-term storage (Josephs, Krska, MacDonald, Wilson, & Pettersson, 2003). However, NIV was prepared in acetonitrile at 0.020 mg/mL and FB_1 in acetonitrile/water (50:50, v/v) at 1 mg/mL because of their low solubility. Composite working standard solutions were prepared by combining suitable aliquots of each individual standard stock solution and diluting them with a suitable amount of solvent. These solutions were kept at 4 °C and renewed weekly.

All organic solvents were HPLC grade from Carlo Erba (Milan, Italy) and used as received. Ultra-pure water was produced by a Milli-Q system (Millipore Corporation, Billerica, MA, USA). Hydrochloric acid, formic acid and ammonia were purchased from Merck (Darmstadt, Germany).

2.2. Equipment

Bakerbond Octadecyl (40 μm) was supplied by J.T. Baker (Deventer, The Netherlands) and Carbograph-4 by LARA (Rome, Italy). Carbograph-4 is a graphitized carbon black (GCB) with a surface area of 210 m^2/g and particle size range of 120–400 mesh, similar to Carboprep 200 (Restek) and EnvicarbX (Supelco). C_{18} cartridges were prepared by filling 6 mL polypropylene tubes with 100 mg of the adsorbent placed between two polyethylene frits. Immediately prior to use these cartridges were activated with 5 mL of acetonitrile/water (75:25, v/v). Carbograph-4 cartridges were prepared by placing 500 mg of the adsorbent inside 6 mL polypropylene tubes between two polyethylene frits. Before processing samples, Carbograph-4 cartridges were attached to a vacuum manifold apparatus and washed sequentially with 10 mL of dichloromethane/methanol (80:20, v/v) containing 50 mmol/L formic acid, 5 mL of methanol, 20 mL of acidified water (10 mmol/L HCl) and 10 mL of water. Tubes, frits and the vacuum manifold were from Supelco (Bellefonte, PA, USA). PTFE syringe filters (0.45 μm; 15 mm diameter) were purchased from Chemtek Analytica (Bologna, Italy).

2.3. Fusariotoxin analysis

2.3.1. Screening method

A previously reported procedure was employed (Cavaliere, Foglia, et al., 2005), with the difference that a second

internal standard was added. Briefly, 1 g of corn meal was homogenized for 15 s with 10 mL of acetonitrile/water (75:25, v/v). The homogenized sample was transferred on the top of a 6 mL cartridge containing 100 mg of C_{18} adsorbent and the extract was collected into a 25 mL volumetric flask placed in a vacuum manifold. The extraction vessel was washed twice with 7 mL of the extracting mixture and these washings were also passed through the cartridge and collected. The volume was then adjusted to 25 mL and a 5 mL aliquot, diluted to 500 mL with water, was used for clean-up on a Carbograph-4 (500 mg) 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. Fusariotoxins were eluted with 1 mL of methanol followed by 8 mL of dichloromethane/methanol (80:20, v/v) acidified with 50 mmol/L formic acid. Both ISs were added and the extract was evaporated to about 100 μL, diluted with 100 μL of the starting LC mobile phase, filtered, and 20 μL were analyzed by LC–MS/MS.

2.3.2. Confirmatory method

The confirmatory method was devised by suitable modifications of two of our published methods for determining trichothecens B, macrocyclic lactones (zeranols) and fumonisins B (Cavaliere, D'Ascenzo, et al., 2005; Faberi et al., 2005). The confirmatory method differs from the screening one starting from the analyte clean-up cartridge elution step. Fusariotoxins were fractionated sequentially and collected in three 1.4 cm i.d. round-bottom glass vials. Trichothecens A and B were eluted with 8 mL of methanol (fraction A). Then, macrocyclic lactones were recovered with 12 mL of dichloromethane/methanol (80:20, v/v) (fraction B) and finally fumonisins were recovered with 8 mL of the same dichloromethane/methanol mixture acidified with formic acid, 50 mmol/L (fraction C). The three fractions were spiked with the proper IS solution (VER for fraction A, ZAN for fraction B and diclofenac for fraction C) and evaporated at 40 °C under a gentle flow of nitrogen. Fraction A, evaporated to dryness, was reconstituted with 250 μL of water/acetonitrile/methanol (90:7:3, v/v), fraction B and C, both evaporated to about 50 μL, were reconstituted respectively with 250 μL of water/acetonitrile/methanol (50:35:15, v/v) and 350 μL of water/methanol (50:50, v/v) containing 25 mmol/L formate buffer (pH = 3.8). All solutions were forced trough PTFE syringe filters. Twenty milliliters of each of the three final solutions were injected into the HPLC column. In particular, fraction A was analyzed two times in different chromatographic conditions for trichothecenes A and B, respectively.

2.3.3. LC–MS/MS analysis

Liquid chromatography was performed using Perkin–Elmer series 200 micropumps (Norwalk, CT, USA) coupled with a Perkin–Elmer autosampler. The analytes were chromatographed on an Alltima C_{18} column (250 × 2.1 mm i.d., 5 μm particle size) from Alltech (Deerfield, IL, USA) with a SecurityGuard ODS, 4 × 2 mm i.d. precolumn supplied by Phenomenex (Torrance, CA, USA), both thermostated by an oven (Timberline Instruments, Inc., Boulder, CO, USA). An API 2000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Concord, Ontario, Canada), coupled with a TurboIonSpray source, was used. Biosystems/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing.

2.3.4. LC/ESI-MS/MS screening method

Fusariotoxins were analyzed as reported in our previous work (Cavaliere, Foglia, et al., 2005). Briefly, analytes were separated using gradient elution with water/methanol, both containing 10 mmol/L formic acid and adjusted to pH 3.8 with ammonia, at a flow rate of 200 μL/min, and at 45 °C. Methanol was linearly increased from 20% to 50% in 10 min, then to 80% in 15 min, and finally brought to 100% and held constant for 10 min.

2.3.5. LC/ESI-MS/MS confirmatory method

Trichothecenes A were separated and detected in the same LC/ESI-MS/MS conditions described above for the screening analysis. Trichothecenes B and macrocyclic lactones were analyzed under the optimized conditions already published (Cavaliere, D'Ascenzo, et al., 2005). Briefly, trichothecenes B analysis was performed using a gradient separation. 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. Macrocyclic lactones were separated in isocratic conditions. The mobile phase composition was set at 53% of acetonitrile/methanol (70:30, v/v) in water. Fumonisins were separated, as previously reported (Faberi et al., 2005), using gradient elution with water as mobile phase A and methanol as mobile phase B, both containing 25 mmol/L formic acid. After an isocratic step at 60% B for 3 min, B was linearly increased to 90% in 5 min, then brought to 100% and held constant for 10 min. The flow rate was 200 μL/min in every case.

2.3.6. MS/MS analysis

The mass spectrometer was operated in both positive an negative ESI ionization mode. Depending on the behaviour of the analytes, the protonated or deprotonated molecules or adducts were selected by the first quadrupole, fragmented, and two suitable transition pairs, when available, were selected for acquisition in multi reaction monitoring (MRM) mode.

Ionization and mass spectrometric conditions were optimized by infusing, at a flow rate of 10 μL/min, fusariotoxin 5 ng/μL standard solutions.

Gas and temperature tuning parameters used for analysis, mass calibrations and resolution adjustments on the

Table 1

LC–MS/MS conditions and precursor/product ion pairs for studied compound acquisition in ESI-MRM mode

Analytes	Confirmatory method[a]							Screening method						
	Period	Ionization polarity	Retention time (min)	Declustering potential (V)	Relative collision energy (%)[b]	Precursor ion (*m/z*)	Product ions (*m/z*)	Period	Ionization polarity	Retention time (min)	Declustering potential (V)	Relative collision energy (%)[a]	Precursor ion (*m/z*)	Product ions (*m/z*)
NIV	1[c]	−	6.1	−92	11	311	281	1	−	5.54	−80	13	357	311, 281
DON	2	−	7.9	−70	15	295	265, 138	2	−	7.85	−54	13	341	295, 265
FUS X	3	−	9.7	−90	27	353	187, 59	3	+	9.94	48	10	372	355, 247
3-ADON	4	−	11.9	−62	12	337	307, 173	5	+	12.98	100	13	339	231, 203
15-ADON	4	−	12.1	−75	11	337	219, 150	5	+	12.98	100	11	339	297
NEO	1[d]	+	10.50	52	14	400	305, 215	3	+	10.50	52	14	400	305, 215
VER (IS)	2	+	11.85	45	8	284	249, 231	4	+	11.85	45	8	284	249, 231
MAS	3	+	14.92	56	11	342	265, 107	6	+	14.92	56	11	342	265, 107
DAS	4	+	17.67	60	13	384	307, 247	7	+	17.67	60	13	384	307, 247
HT-2	5	+	21.31	80	14	442	263, 215	8	+	21.31	80	14	442	263, 215
T-2	6	+	23.82	60	14	484	245, 215	9	+	23.82	60	14	484	245, 215
β-ZAL	1[e]	−	10.3	−110	29	321	277, 161							
β-ZOL	1	−	11.0	−115	29	319	174, 160							
α-ZAL	1	−	14.8	−110	29	321	277, 161							
α-ZOL	1	−	16.1	−115	29	319	174, 160	10	−	25.06	−115	29	319	174, 160
ZAN (IS)	2	−	24.1	−110	29	319	205, 161	10	−	25.40	−110	29	319	205, 161
ZON	2	−	25.5	−110	29	317	175, 131	10	−	25.92	−110	29	317	175, 131
FB_1	1[f]	+	8.2	98	38	722	352, 334	8	+	21.71	98	38	722	352, 334
FB_3	1	+	8.9	130	36	706	354, 336	9	+	24.23	130	36	706	354, 336
FB_2	1	+	9.6	130	36	706	354, 336	11	+	26.58	130	36	706	354, 336
FB_4	1	+	10.2	130	36	690	338, 320							
Diclofenac (IS)	1	+	12.0	25	25	296	215							

[a] Confirmatory method was devised by selective elution from Carbograph-4 clean-up cartridge (three fractions) and optimized LC–MS/MS conditions (four injections); see text for details.

[b] Expressed as % with respect to the maximum voltage difference value between the high-pressure entrance quadrupole (Q0) and collision cell quadrupole (RO2) (+ or −130 V) permitted by the instrument.

[c] First fraction, first injection.

[d] First fraction, second injection.

[e] Second fraction.

[f] Third fraction.

resolving quadrupoles were the same reported in our previous paper (Cavaliere, Foglia, et al., 2005).

LC/ESI-MS/MS parameters for mycotoxin detection for both screening and confirmatory methods are summarized in Table 1.

2.4. Quantitation

Fusariotoxins were quantified using external calibration for the confirmatory method and a matrix matched procedure for the screening method, since the latter, as reported in Table 2, showed some matrix effect. For the external calibration, standard solutions were prepared at seven concentration levels by diluting suitable volumes of the working standard solution. Matrix matched calibration was performed by spiking analyte-free samples after clean-up with known and appropriate volumes of the working standard and internal standard solutions, as previously reported (Cavaliere, Foglia, et al., 2005). For each analyte the summed ion currents profile of both fragment ions was extracted from the LC-MRM dataset, and the plot of peak area versus injected amount was obtained by measuring the resulting peak areas. All samples were run in duplicate and the results averaged.

Total recovery was assessed by spiking analyte-free maize meal samples, applying the extraction and clean-up procedures, measuring the peak areas, calculating the peak area ratios relative to the IS added after clean-up, and comparing these data with those obtained by spiking the extracts of the same corn meal sample after clean-up. The concentration of FB_3, for which a standard was not available on the market, was evaluated as recently reported (Faberi et al., 2005).

2.5. Plant materials

Four hybrids (Matea, Tevere, Cotos, and Eleonora), widely used for cultivation in Northern Italy and representing four maturity groups (from short-season to full season), were used in this study. The four hybrids were evaluated at four plant densities in field trials grown in 2004, near Caleppio (LO), Luignano (CR), Vigone (TO), Rottaia (PI), and Palazzolo (UD). The experimental design was a randomized complete block with treatments in a split plot arrangement. Plant densities were assigned to main plots and cultivars to each subplot. Each experiment had four replications. Plot size at all locations was six rows 6 m long and spaced 0.75 m. All subplots were overplanted and plants thinned to give densities equivalent to 6.5, 6.8, 7.0 and 7.5 plants/m^2, respectively, for earlier to late hybrids. At each location, the level of fertilization and weed control practices were those currently used to grow maize hybrids.

Table 2
Matrix effect, recovery, precision, and method quantification limit (MQL) obtained with the confirmation method and with the screening method ($n = 6$)

Compound	Confirmatory method			Screening method[a]		
	Relative peak area[b] (RSD %)	Recovery[c] ± (RSD %)	MQL[d] (mg/kg)	Relative peak area[b] (RSD %)	Recovery[e] ± (RSD %)	MQL[f] (mg/kg)
NIV	0.78 (4)	80 ± 6	0.010	0.53 (8)	83 ± 10	0.070[g]
DON	0.84 (7)	91 ± 5	0.004	0.80 (6)	85 ± 9	0.125[g]
FUS X	0.94 (3)	95 ± 3	0.019	0.77 (12)	88 ± 6	0.025
3-ADON	0.91 (5)	98 ± 6	0.007	0.86 (9)	99 ± 8	0.021
15-ADON	1.21 (9)	111 ± 8	0.008	0.82 (9)	95 ± 11	0.082[g]
NEO	0.89 (4)	93 ± 8	0.004	0.83 (5)	90 ± 8	0.016
VER (IS)	0.90 (5)			nd		
MAS	0.98 (7)	89 ± 7	0.001	0.86 (6)	89 ± 5	0.003
DAS	1.06 (6)	81 ±10	0.002	0.93 (7)	97 ± 7	0.005
HT-2	0.90 (11)	86 ± 2	0.002	0.84 (10)	91 ± 7	0.012
T-2	0.98 (3)	85 ± 3	0.002	0.86 (7)	92 ± 8	0.015
β-ZAL	0.92 (6)	95 ± 6	0.003	nd	nd	nd
β-ZOL	0.85 (9)	92 ± 5	0.004	nd	nd	nd
α-ZAL	0.88 (10)	102 ± 7	0.004	nd	nd	nd
α-ZOL	0.81 (8)	100 ± 5	0.004	0.64 (10)	93 ± 9	0.005
ZAN (IS)	1.09 (7)			nd		
ZON	0.90 (4)	96 ± 4	0.003	0.68 (8)	91 ± 10	0.010
FB1	0.98 (9)	90 ± 10[h]	0.004	0.92 (5)	85 ± 5[i]	0.009
FB2	0.96 (5)	93 ± 7	0.002	0.86 (6)	83 ± 5[i]	0.007
Diclofenac (IS)	0.95 (8)			nd		

[a] From reference Kellerman et al., 1990.
[b] Peak area of the analytes injected from a maize extract relative to that of the analyte injected from a standard solution. Spiking level was 0.050 mg/kg.
[c] Spiking level was 0.020 mg/kg.
[d] Spiking level was 0.100 mg/kg.
[e] Spiking level was 0.025 mg/kg.
[f] Spiking level was 0.100 mg/kg.
[g] Spiking level was 10 mg/kg.
[h] Spiking level was 0.5 mg/kg.
[i] Spiking level was 5 mg/kg.

Irrigation was applied throughout the summer to avoid water stress.

Random ears (10), for mycotoxin analysis, were hand-harvested from the central rows from each subplot beginning about 28 days after black layer maturity (Ritchie & Haway, 1982) and after 15 days. The harvested ears were immediately dried at 40 °C in a forced air dryer for 24 h. The ears were machine-shelled and grain samples of at least 1 kg from each experimental unit were mixed twice in a sample splitter. Replicate samples (4 × 500 g) were mixed and ground using a Cyclotec mill and one third selected for extraction as described below. Samples were conserved in seeded plastic bags at 0 °C until chemical analysis.

2.6. Statistical analysis

Standard analyses of variance and mean procedures were used to analyze data for all traits. Analyses on the basis of subplots as the experimental units were done assuming the five locations as five random environment and treatment effects (that is effects due to genetic strains and harvest dates) considered as fixed factors. *F*-Values were calculated for the main effects and interactions in accordance with the expected mean squares. Simple correlation coefficients between fumonisin variables were calculated using hybrid mean values in environments.

3. Results and discussion

3.1. Analytical methodologies

The LC–MS/MS multiresidue method evaluation has been reported elsewhere (Cavaliere, Foglia, et al., 2005); it was used as described with the only difference consisting in the introduction of the second IS verrucarol for trichothecens quantification. Confirmatory methods were, in part, already published methods for analysis of specific fusariotoxin classes (Cavaliere, D'Ascenzo, et al., 2005; Faberi et al., 2005), and only the variations from these procedures will be discussed.

3.2. Clean-up

The confirmatory method was devised starting from the ability of some graphitized carbon blacks, like Carbograph, to establish a particular kind of interaction. As formerly reported, Carbograph can behave both as reversed-phase and as anion-exchanger sorbent and, besides, have a particular affinity for aromatic compounds with respect to aliphatic ones (Andreolini, Borra, Caccamo, Di Corcia, & Samperi, 1987); actually, it was possible to separate trichothecenes in the methanol fraction from macrocyclic lactones in the dichloromethane/methanol (80:20, v/v) fraction (Cavaliere, D'Ascenzo, et al., 2005), whereas the fumonisins, being acidic compounds, were eluted later with an acidified dichloromethane/methanol mixture (Faberi et al., 2005). To avoid the effect of overlapping among fractions, only 5 mL of the entire extract were submitted to clean-up. As regards the fraction containing trichothecenes dissimilarly from our original work in which only type B were analyzed, the methanol volume was increased from 5 to 8 mL to improve the recovery of the less polar compounds such as MAS, HT2 and T2 toxins. In addition, a washing step with acidified methanol before zeranols recovery was omitted to avoid a partial loss of fumonisins. In any case, this fractionation procedure gave cleaner extracts than collecting a single fraction and drastically reduced ion suppression during ESI ionization process. In Table 2 the signal suppression effect expressed, for each compound, as ratio between analyte peak areas obtained injecting a fortified maize extract and those obtained from standard solutions having the same concentration is reported.

3.3. Analysis by LC–MS/MS

Tricothecenes A and B were collected in the same fraction, but they behave very differently in the ESI ionization source. Type B give the most intense response in negative mode, using a water/methanol or water/acetonitrile solvent systems whereas type A are ionized better in a water/methanol system containing ammonium ion. For this reason, they were analyzed in different chromatographic runs using different solvent systems. NEO, although classified as type B, behaves as type A trichothecene from a mass-spectrometric point of view.

3.4. Accuracy, precision, linear dynamic range and method quantification limits (MQLs)

Some modifications regarding instrumentation, extraction and clean-up procedure have been made to the procedures already reported for selectively determining trichothecenes B, zeranols and fumonisins; in addition, type A trichothecenes were not included, then recoveries, precisions and MQLs were determined for the confirmatory method, and results are summarized in Table 2. Values previously reported were substantially confirmed. As far as fumonisins are concerned, lower recovery rates were expected, the extraction step being performed with a not acidified solvent mixture (Cavaliere, D'Ascenzo, et al., 2005). On the other hand, this experiment was conducted at higher spiking level than the previous one (10 mg/kg for FB_1 and 5 mg/kg for FB_2), as suggested by the high contamination levels found; this fact might explain the discrepancy. Recoveries, precision and MQLs of the screening method (Cavaliere, Foglia, et al., 2005) were also reported in Table 2 for rapid comparison. Note that, as the quantitation with the screening method was carried out by matrix matched calibration, matrix suppression effects were not included in the recoveries.

The ESI-MS/MS responses were linearly related to injected amounts from MQLs to 100 ng ($R^2 > 0.985$).

Maize samples contaminated at concentration level over linearity range were appropriately diluted and reanalyzed.

3.5. Effect of sampling

The sampling strategy described in the experimental section provides four replicate samples for each experimental sample. The sampling strategy validation being outside the purposes of this study, we combined the replicates to have two composite samples for each location.

In analyzing cereal samples, usually ten or more g per sample, are extracted to ensure representativity, and part of the extract is submitted to the subsequent analytical steps. That amount is hardly compatible with our extraction method, which uses a high solvent/sample ratio and a statical–dynamical extraction mode. Our reasoning was that if a sample is homogeneous enough, a sub-sample as small as 1 g may be representative. In fact, analyzing six times 1 g sub-samples of a sample naturally contaminated, FB_1, FB_2, and FB_3 mean values and RSD obtained were respectively $14.40 \pm 8\%$, $5.80 \pm 11\%$ and $2.60 \pm 12\%$, and in the same sample mean values of 0.043 and 0.127 were found for DAS and ZON, both with a RSD of 6%. This means that the between sub-sample precision is not statistically different from the between day precision ($p < 0.05$).

3.6. Comparison of screening and confirmatory methods for determination of contamination levels

A total of 40 samples were analyzed in duplicate by both the screening and the confirmation methods. Replication includes the extraction step whereas two aliquots of the same 25 mL extract were used for a set of screening and confirmatory analysis. Results are summarized in Table 3. Because the screening method has a higher MQL than the confirmatory one, two columns of positive sample number were reported. For example, ZON was confirmed six times at level >0.010 mg/kg and found positive 10 times for concentration level from 0.003 to 0.010 mg/kg.

As far as thricotecenes B are concerned, only DON was found twice at a >1 mg/kg concentration, both in Cotos hybrid (Caleppio and Palazzolo locations), whereas FUS X was never revealed. 3-ADON and 15-ADON determination with the screening method was affected by a very high inaccuracy. This drawback was to some extent to be expected considering that the two isomers co-elute in the chromatographic conditions devised for the screening analysis and, although the transitions selected (only one for 15-ADON) were selective for the two compounds, they were not so specific as to avoid any interference. Finally, we found that the co-presence of the two isomers, contrarily to what is reported in literature (Berger, Oehme, & Kuhn, 1999), was the rule and not the exception.

Tricothecens A were detected in some samples in trace amount and the inaccuracy of the screening method appears to be very high. Both findings were not surprising considering that (i) in general, trichothecens A did not represent a contamination problem for maize grown in temperate zone (Visconti, Lattanzio, Pascale, & Haidukowski, 2005); (ii) concentration levels were very close to the MQL of the screening method.

Table 3
Method comparison for contamination levels

Compound	Positive[a]			Range (mg/kg)	
	Screening	Confirmation		Screening	Confirmation
NIV	0	2	0[b]	–	0.010–0.017
DON	3	7	3	0.208–2.63	0.005–2.09
FUS X	0	0	0	–	–
3-ADON	3	4	3	0.050–0.310	0.020–0.540
15-ADON	3	3	3	0.77–1.95	0.210–1.50
NEO	1	2	0	0.018	0.004–0.015
MAS	9	13	8	0.003–0.023	0.001–0.015
DAS	18	24	16	0.005–0.009	0.002–0.008
HT-2	1	8	0	0.012	0.002–0.008
T-2	3	8	0	0.015–0.019	0.002–0.013
β-ZAL	nd	0	–	–	–
β-ZOL	nd	3	–	–	0.003–2.34
α-ZAL	nd	0	–	–	–
α-ZOL	3[c]	4	4	0.006–0.040	0.005–0.020
ZON	7	16	6	0.010–1.68	0.003–1.51
FB1	40	40	40	0.270–69.40	0.368–64.15
FB2	40	40	40	0.250–42.20	0.193–37.09
FB3	40	40	40	0.030–13.30	0.040–16.69

[a] 40 samples analyzed.
[b] Positive for concentration ⩾ screening method MQL.
[c] One false negative in screening method revealed by confirmation method.

Among macrocyclic lactones, ZON was confirmed at a higher level than 0.5 mg/kg in three samples (all of them was Cotos hybrid and also DON contamination was found) and nine samples were found positive only with the confirmation method, being contaminated at levels of less than 0.008 mg/kg. α-ZOL presents the only case of false negative of the screening method evidenced with confirmation method (0.09 mg/kg). β-ZOL, β-ZAL and α-ZAL were not included in our screening method since coeluted with fumonisins and, moreover, were occasionally found at levels of few μg/kg (Cavaliere, D'Ascenzo, et al., 2005). By the specific fraction devised for analyzing macrocyclic lactones, we found in three samples only levels > MDL < MQL for α- and β-ZAL; on the contrary, in one case (hybrid Matea, location Palazzolo) β-ZOL was detected at concentration of about 2.3 mg/kg, whereas in that sample the concentration of ZON was only 0.44 mg/kg, the relative chromatograms are reported in Fig. 1. Because casual contamination or wrong identification could be reasonably excluded, this result, although isolated, may indicate that some strains of *Fusarium* spp., probably *Fusarium greminearum* (Richardson, Hagler, & Mirocha, 1985) can produce large amounts of this metabolite.

Finally, FB_{1-3} contaminated, at a relatively high concentration, all 40 samples, so we could make a comparison by regression lines. Indicating on the x axis the results coming from the confirmatory method and on the y axis those

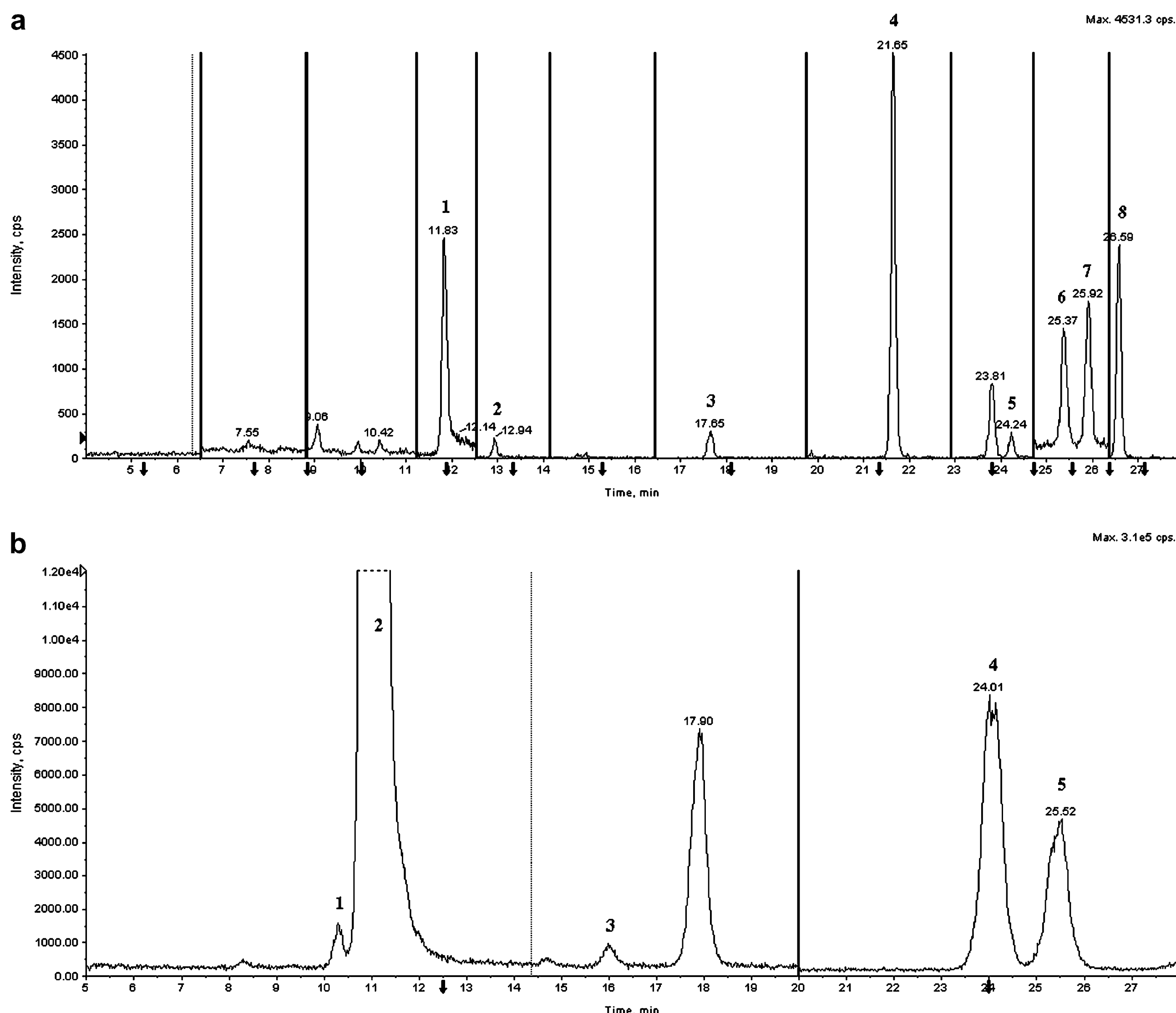

Fig. 1. LC/ESI-MS/MS chromatograms obtained on analyzing a maize sample (hybrid Matea, location Palazzolo): (a) by screening method. 1, verrucarol (IS); 2, 3-acetyldeoxynivalenol (0.050 mg/kg); 3, diacetoxyscirpenol (0.009 mg/kg); 4, fumonisin B_1 (0.270 mg/kg); 5, fumonisin B_3 (0.030 mg/kg); 6, zearalanone (IS); 7, zearalenone (0.050 mg/kg); 8, fumonisin B_2 (0.250 mg/kg); (b) by confirmatory method, for determining macrocyclic lactones. 1, β-zearalanol (below MQL); 2, β-zearalenol (2.34 mg/kg); 3, α-zearalenol (0.005 mg/kg); 4, zearalanone (IS); 5, zearalenone (0.044 mg/kg).

coming from the screening method, the lines of regression of y on x were: $y = 1.0067x + 171$ ($R^2 = 0.9899$), $y = 1.0104x + 53$ ($R^2 = 0.9755$), and $y = 1.1376x + 105$ ($R^2 = 0.8531$) for FB_1, FB_2, and FB3, respectively. The reported data show a good agreement between methods with a slight tendency to overestimate contamination in the screening method respect to the confirmatory one.

3.7. Fumonisin contamination of field samples

In maize, significant and consistent differences among inbred lines and hybrids that differ with respect to fumonisin accumulation have been identified, some of which contained only a small amount of fumonisins (Clements, Maragos, Pataky, & White, 2004, & references therein). Moreover, it has been shown that weather conditions influence fumonisin contaminations of maize (Payne, 1992), although *Fusarium* spp. are found in a wide range of climate conditions. Therefore, because fumonisins were the only toxins present in most of the samples at high concentration, we performed a statistical analysis of the contamination level in the maize samples collected from the experimental trials.

The analysis of variance (not shown) indicated that variation in ear harvest time had no significant effect upon the fumonisins measured and their components and were thus considered as additional replications. Significant differences were observed among hybrids and locations for all variables. In contrast, hybrid x location interactions for all the variables examined, with the exception of FB_3, were not significant. However, for FB_3 this interaction was not large relatively to mean squares for hybrids and locations; therefore, means presented in Tables 4 and 5 were combined over locations and hybrids, respectively.

Table 4
Mean values and ranges (subplot means) of fumonisin contamination (mg/kg based on dry weight) in maize kernels from different hybrids grown in Italy, combined across locations, during summer 2004

Hybrids	Fumonisin level (mg/kg)				
	FB_1	FB_2	FB_3	Total fumonisin	
				Means	Range
Matea	12.12	6.01	2.34	20.47	0.60–73.53
Tevere	20.74	10.86	4.61	36.21	1.40–122.93
Cotos	12.09	3.63	1.68	17.40	2.62–35.06
Eleonora	15.26	6.45	2.43	24.14	3.42–35.22
Overall mean	*15.05*	*6.74*	*2.76*	*24.55*	
Range	*0.37–64.15*	*0.19–37.09*	*0.04–16.69*	*0.60–122.93*	
LSD (0.05)	*2.08*	*1.02*	*0.36*	*3.07*	

Table 5
Mean values and ranges (subplot means) of fumonisin contamination (mg/kg based on dry weight) in maize kernels from different hybrids grown in Italy at several locations, during summer 2004

Locations	Fumonisin level (mg/kg)				
	FB_1	FB_2	FB_3	Total fumonisin	
				Means	Range
Caleppio	18.56	8.36	3.18	30.10	17.94–41.88
Luignano	22.27	9.19	4.05	35.51	14.05–41.88
Vigone	6.24	2.29	0.99	9.52	4.56–12.84
Rottaia	22.54	12.23	4.78	39.55	16.58–70.86
Palazzolo	5.65	1.63	0.81	8.09	1.95–17.11
Overall mean	*15.05*	*6.74*	*2.76*	*24.55*	
Range	*0.37–64.15*	*0.19–37.09*	*0.04–16.69*	*0.60–122.93*	
LSD (0.05)	*2.32*	*1.12*	*0.40*	*6.44*	

To give an overall view of the samples tested, the total fumonisin amount and the single components (FB_1, FB_2, and FB_3) means and their ranges of variation determined in the grain samples of the hybrids are shown in Table 4. The magnitude of the phenotypic variation was appreciably wide for each of the variables examined. The average level of the total fumonisin contamination in the samples from the four hybrids considered across locations was 24.55 mg/kg. The hybrid with the greatest amount of fumonisin contamination in grain had a concentration of 122.93 mg/kg, while grain from the hybrid with the lowest amount of fumonisin had a concentration of 0.60 mg/kg, with a more than 200-fold difference between the hybrid sample with the lowest and that with the highest concentration, indicating the occurrence of some samples at very high level of contamination (individual results are not shown).

It was reported that among several fumonisins identified, only FB_1, FB_2, and FB_3 appear to be produced in significant amount under both cultures and natural conditions (Cawood et al., 1991; Sydenham, Shephard, & Thiel, 1992); therefore, our investigation was restricted to those subtypes. In this study, fumonisin FB_1 was detected in all the samples at levels ranging from 0.37 to 64.15 mg/kg; this component was the predominant toxin subtype in all samples examined and had levels above 2 mg/kg for the majority of samples; this confirms that cultivation of maize in Italy has a high risk of FB_1 contamination (Ritieni et al., 1997). Note that FDA has published the "guidance for Industry" suggesting a limit of fumonisin concentrations between 2 and 4 mg/kg for maize flour and other milled maize products for human consumption (CFSAN, 2001). Our results also indicated that FB_1 represented, on the average, 60% of total fumonisins. FB_2 and FB_3 were also detected, with a less abundant trend of contamination, ranging from 0.19 to 37.09 and 0.04 to 16.69 mg/kg, respectively. These results are consistent with the ratios of FB_1, FB_2, and FB_3 observed in maize in previous reports (Ross et al., 1992, & references therein), with high contamination level and elevated positive sample incidence, typical parameters of country temperate climate (Soriano & Dragacci, 2004).

The mean of total fumonisin concentration and its components (FB_1, FB_2, FB_3) determined in the grain samples of the different locations are summarized in Table 5. At two locations, total fumonisin and single component levels were lower in comparison to the other locations. The highest total mycotoxin concentrations were found in Rottaia. At this location total fumonisin concentrations were more than 4-fold those detected in Vigone and Palazzolo. A similar trend was in general observed also for FB_1, FB_2, FB_3 components. Findings reported by various authors indicated that the levels of fumonisins in maize grain are influenced by environmental factors. Conditions such as high humidity, host water, and drought at or just before flowering are favorable for high levels of ear rot and fumonisin

contamination in maize (Abbas et al., 2002; Payne, 1992; Shelby, White, & Bauske, 1994). In the present study irrigation was used to minimize the effect of drought stress. Similarly, fertilization was used to minimize nutrition stress, and optimal planting and weed control methods were used to minimize population stress, leaving heat as a major uncontrolled source of stress, although additional unrecognized sources of stress may have been present. During the kernel development (i.e. between silking and black layer), that in Northern Italy occurs in June–August, heat stress, particularly nightime temperatures above 20 °C (Abbas et al., 2002; Payne, 1992), is a major factor in mycotoxin contamination. In 2004, Luignano, Caleppio, Rottaia experienced high temperatures, particularly during June and July, causing high amounts of fumonisins. In contrast, temperatures in Vigone and Palazzolo were only modestly above normal. This suggests that the environmental conditions found in the specific area of cultivation play an important role in the accumulation of fumonisins in maize.

On the other hand, even though Palazzolo was the least fumonisin contaminated location, there was three multiclass contamination cases, with contemporaneous presence of ZON, DON and their metabolites. The correlation coefficients between total fumonisin concentration and its components (significant at the 0.01 probability level) were higher than 0.851, indicating that the levels of total fumonisins were strictly correlated with their components (FB_1, FB_2, FB_3). Similar associations were found between fumonisin subtypes. Studies on the structure of fumonisins indicated that these components are metabolites of a polyketide pathway and that the structural genes responsible for biosynthesis are clustered, and specific regulatory genes have been found to reside in those gene clusters (Flaherty & Woloshuk, 2004).

4. Safety

Mycotoxins are dangerous compounds, consequently solutions and extracts should be handled with care. Gloves and protective clothing were worn as safety precautions during the handling of the compounds. Standards were handled in a glove box. Residues from analysis were disposed properly and glassware treated with 3% sodium hypochlorite before washing and reuse.

Acknowledgement

This work was supported by Ministero delle Politiche Agricole e Forestali, Rome, Italy, special grant "Riduzione della contaminazione da aflatossine; filiera mais – prodotti lattiero-caseari."

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