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Liquid chromatographic determination of toxigenic secondary metabolites produced by *Fusarium* strains ☆

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Abstract

Various liquid chromatographic methods used in the analysis of mycotoxins (zearalenone, trichothecenes and fumonisins) produced by *Fusarium* species were compared in this work. The results demonstrate the suitability of modern clean-up procedures employing multifunctional MycoSep and immunoaffinity columns although these methods are more expensive than conventional methodologies for clean-up. HPLC with both fluorescence and photodiode array detection is a suitable technique for the analysis of toxic secondary metabolites produced by *Fusarium* species; different derivatisation strategies have been studied to improve the sensitivity of the technique because of the low concentration of these metabolites in contaminated food. The utility of the proposed methodology was assessed in cereal cultures of various *Fusarium* strains.

Keywords: *Fusarium* spp.; Derivatisation, LC; Mycotoxins; Zearalenone; Trichothecenes; Fumonisins

1. Introduction

Species of the genus *Fusarium* occur widely in nature as saprophytes and plant parasites; they are found in a great variety of plants and agricultural products [1]. In addition to the losses caused by infection of plants by these fungi before or during harvest, some species are capable of producing mycotoxins in affected products. The wide range and frequent presence of naturally occurring *Fusarium* toxins in cereals reveal an increasing need for research on the toxigenic potential of *Fusarium* spp. grown on plants [2–4] to assess the extent of mycotoxin hazard to man and animals.

Zearalenone {6-[(10*S*)-10-hydroxy-6-oxo-*trans*-1-undecyl]-β-resorcylic acid lactone} (ZEA) is a widely distributed estrogenic secondary metabolite that is produced by various members of the genus *Fusarium* [5], colonising maize, sorghum, wheat, barley oats and other cereal grains [6]. This mycotoxin induces hyperestrogenism in farm animals, especially in female swine [7] and cows [8]. Several toxic effects have been also reported in humans following consumption of damaged cereals such as esophagitis and oesophageal cancer in China and in South Korea [9]. A ZEA derivative, *trans*-α-zearalenol (α-ZOL) has

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been observed to occur in naturally contaminated cereal grains. Its diastereomer, *trans*-β-zearalenol (β-ZOL) is also known as a metabolite related to ZEA.

Trichothecenes are a family of closely related sesquiterpenoids. Most of them have a double bond at position C9–10, a 12,13 epoxide ring and a variable number of hydroxyl and acetoxy groups. Although the number of characterised trichothecenes is large, only a few of these have been detected so far in naturally contaminated cereals and commodities, mainly belonging to both type A and type B trichothecenes. Type A trichothecenes belong to the group that does not contain a carbonyl function at C8 conjugated with a double bond in C9–10, which prevents these compounds from being detected by UV absorption and include T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS). Type B trichothecenes are characterised by a carbonyl group at C8 and include, among others, deoxynivalenol (DON), nivalenol (NIV), and their derivatives [10].

The fumonisin mycotoxins consist of a group of seven structurally related analogues [11], but only the fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) have been reported to occur naturally at significant concentrations in corn and corn-based products [12] and other substrates [13]. The amount of mycotoxins produced can vary over a considerable range, from less than 1 μg/kg to more than 1 mg/kg, and is dependent on factors such as temperature, duration of grown period, substrate and strain of fungal species [12].

Analysis of contaminants in grains and plant products is not simple. Many variables must be carefully controlled, such as suitable sampling from large quantities of starting material, and handling, storage, preparation and subsampling of initial samples to ensure that analysis is carried out on a truly representative sample of the test material [14]. Analysis is further complicated by possible heterogeneity of the starting material and the presence of contaminants in the storage or delivery systems. On the other hand, during the analysis, variables such as precision, accuracy and detection limit need to be assessed to assure that quantities measured are representative of the starting material and those analytical errors are minimised [15,16].

High-performance liquid chromatography (HPLC) has become an almost universal method for determination of mycotoxins. Nevertheless, some of them lack a significant UV chromophore and are not inherently fluorescent, so that sensitive detection at the low levels necessary for the analysis of naturally contaminated food samples requires derivatisation of the sample extracts. Alternative principles such as refractometry, photoreduction–fluorescence [17] or displacement chromatography with UV absorbers [18], impose severe limitations together with constraints on mobile phase selection. Both ZEA and type B trichothecenes can be detected by reversed-phase HPLC–UV or photodiode array detection but type A trichothecenes and fumonisins do not absorb. A derivatisation reagent was previously developed to obtain fluorescent derivatives of type A trichothecenes [19] and this procedure could be also applied to type B trichothecenes. On the other hand, several procedures have been developed to detect fumonisins by HPLC–fluorescence detection [20–22].

The aim of this work was to develop a sensitive, reproducible, reliable analytical method to separate and quantify mycotoxins in cereal samples by HPLC with fluorescence detection.

2. Experimental

2.1. Chemicals and reagents

Trichothecene mycotoxins, including T-2 toxin, HT-2 toxin, NEO, DAS, DON, NIV, 15-acetyldeoxynivalenol (15-AcDON), and 3-acetyldeoxynivalenol (3-AcDON) and also ZEA, α-ZOL, β-ZOL and FB_2 were supplied by Sigma (Steinheim, Germany) while the FB_1 standard was supplied by CSIR (Pretoria, South Africa). They were individually dissolved in acetonitrile at a concentration of 1 mg/ml and stored at −20 °C and brought to room temperature before use.

For HPLC–fluorescence detection derivatisation of trichothecenes, 4-dimethylaminopyridine (DMAP) was supplied by Sigma and coumarin-3-carboxylic acid and thionyl chloride were purchased from Aldrich (Gillingham, UK). The *o*-phthaldialdehyde (OPA) derivatizing reagent was prepared by adding 5

ml of an 0.1 *M* sodium tetraborate aqueous solution and 50 μl of 2-mercaptoethanol (Sigma) to 1 ml of methanol containing 40 mg of OPA (Fluka, Buchs, Switzerland). The 4-fluoro-7-nitrobenzofurazan (NBD-F) derivatizing reagent was 50 m*M* NBD-F (Sigma) in ethanol.

Organic solvents were HPLC grade from Merck (Darmstadt, Germany). Water was prepared with a Waters Milli-Q system (Waters, Milford, MA, USA).

2.2. Fungi and samples

Five *Gibberella fujikuroi* strains (Gf2, Gf6, Gf7, Gf20 and Gf47), three strains of *Fusarium sporotrichioides* (FSp1, FSp3 and FSp6), two strains of *F. graminearum* (FG1 and FG6) and two strains of *F. culmorum* (FCu1 and FCu4) were used to inoculate cereal samples. These strains are held in the collection of the Department of Microbiology, University of Valencia and maintained in potato-dextrose agar.

Cereal (corn, rice or wheat) samples were purchased commercially and used without grinding. They were previously tested for absence of mycotoxins. A 50 g amount of sample was placed in a 250-ml Erlenmeyer flask with 22.5 ml of deionised water. The flasks were plugged with cotton, covered with aluminium foil and autoclaved for 20 min at 120 °C. The substrate was inoculated with pieces of potato-dextrose agar single-spore cultures of each strain and maintained at 20 °C for 3 weeks. The cultures were dried at 45 °C for 48 h and then finally ground to powder with a laboratory mill.

2.3. Extraction

Ground cultures (5 g) were blended for 5 min in a high-speed blender (Ultraturrax T25, IKA, Stauffen, Germany) using a PTFE flask with methanol–1% aqueous NaCl (80:20, v/v) (2×25 ml) for ZEA or acetonitrile–water (84:16, v/v) for trichothecenes. After filtering through Whatman No. 4 filter paper, the filtrate was defatted with hexane (2×10 ml) and extracted with dichloromethane (3×15 ml). The combined dichloromethane extracts were dried over anhydrous sodium sulfate and evaporated to dryness in rotary evaporator prior to clean-up procedure.

For fumonisin determination, culture samples were finely ground to powder in a laboratory mill and thoroughly mixed. A 5 g amount of the mixed samples was blended in a high-speed blender with 25 ml of acetonitrile–water (50:50, v/v) for 5 min and filtered through Whatman No. 4 filter paper.

2.4. Sample clean-up

2.4.1. Silica and Florisil solid-phase extraction (SPE) cartridges

Using a gas-tight syringe, a Sep-Pak Florisil or silica cartridge (1 g) (both from Waters) was activated with 5 ml of chloroform followed by 5 ml of chloroform–methanol (90:10, v/v). The sample residue was dissolved in 2×1.5 ml of chloroform–methanol (90:10, v/v), loaded onto the SPE column and forced slowly through the cartridge. The column was washed with 20 ml of chloroform–methanol (90:10, v/v). The 23 ml of eluate was collected into a 100-ml round-bottom flask and evaporated to dryness in a vacuum evaporator at 45 °C. The evaporated sample was dissolved twice in 0.5 ml of methanol and transferred to a 4-ml reaction vial. The vial was placed into a water bath kept at 45 °C. The solvent was removed under a gentle stream of nitrogen.

2.4.2. C_{18} cartridge

A 2-ml volume of the sample filtrate for fumonisin analysis was added to 5 ml of 1% aqueous KCl and passed through the pre-conditioned cartridge. The column was washed with 3 ml of 1% aqueous KCl, followed by 2 ml of acetonitrile–1% aqueous KCl (1:4, v/v). Mycotoxins were eluted from the column with 2 ml of methanol–water (7:3, v/v). The solvent was evaporated to dryness under a gentle stream of nitrogen at 45 °C.

2.4.3. Strong anion-exchange (SAX) cartridge

A 2-ml volume of the sample filtrate for fumonisin analysis was diluted to 4 ml with methanol–water (7:3, v/v) and passed through a pre-conditioned cartridge. The column was washed with 8 ml of methanol–water (7:3, v/v) followed by 3 ml of methanol. Mycotoxins were eluted from the SAX cartridge with 14 ml of 1% acetic acid in methanol. The solvent was evaporated to dryness under a gentle stream of nitrogen at 45 °C.

2.4.4. MycoSep 225 column

A 5-ml volume of the filtrate sample extract in acetonitrile–water (84:16, v/v) was placed into the culture tube of a Mycosep 225 column (Romer Labs., Union, MO, USA). The rubber flange end of the clean-up column was pushed slowly into the culture tube, creating a tight seal between the rubber flange and the glass wall of the culture tube. As the column was pushed farther into the tube, the extract was carefully forced through the frit, one-way valve and packing material (it should not take <25 s). A 2-ml volume of the purified extract was transferred using a micropipette to a vial. The solvent was evaporated to dryness under a gentle stream of nitrogen at 45 °C.

2.4.5. ZearalaTest column

A 10-ml volume of the filtrate was collected and mixed with 90 ml distilled water. A 10-ml volume of diluted extract was passed through the ZearalaTest immunoaffinity column (Vicam, Watertown, MA, USA) at a flow-rate of about 1 drop/s, followed by 2×5 ml distilled water at 1–2 drops/s flow-rate. ZEA was then eluted with 1.5 ml methanol and collected in a clean vial. The eluted extract was then evaporated under a gentle stream of nitrogen at 50 °C.

2.5. Derivatisation

2.5.1. Coumarin-3-carbonyl chloride

The procedure has been previously described [19]. A 10-μl volume of DMAP solution in toluene (6.5 mg/ml) was added to a derivatisation vial containing evaporated samples or standards. A 10-μl volume of the coumarin reagent was added. The mixture was heated at 80 °C for 20 min in a heater block. Then it was cooled in ice water and cleaned up by liquid–liquid extraction between toluene and 0.05 *M* dihydrogenphosphate buffer, pH 5.5. The cooled reaction mixture was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 75 μl of acetonitrile–water (65:35, v/v), acidified with acetic acid to reach a final concentration of 0.75% (v/v) (HPLC mobile phase). The solution was filtered through a 0.20-μm filter and 20 μl was injected into the liquid chromatograph.

2.5.2. OPA reagent

The residue was dissolved with 200 μl methanol. An aliquot of the sample (100 μl) was derivatised with 200 μl of OPA reagent, mixed and injected into the liquid chromatograph [20].

2.5.3. NBD-F reagent

A 100-μl volume of the sample was evaporated to dryness under a moderate stream of N_2 and redissolved in 100 μl of 0.04 *M* sodium tetraborate buffer, pH 8.3 (adjusted with 1 *M* HCl) in a 2-ml vial. A 50-μl volume of NBD-F reagent was added, the reaction mixture was heated for 1 min in an aluminium block at 60 °C, then cooled with ice and added to 100 μl of mobile phase (A) [21].

2.6. HPLC analysis

The HPLC system consisted of a Waters 600 quaternary pump, a Waters 474 scanning fluorescence detector and a photodiode array detector Waters 996. Signals were processed with Millennium software. Chromatographic separations were performed on a stainless steel LiChrospher 100 C_{18} reversed-phase column (250×4 mm, 5 μm particle size) connected to a guard column (4×4 mm, 5 μm particle size) filled with the same stationary phase. The column was kept at room temperature. The mobile phase was degassed by passing through a vacuum-degassing device (Waters) at a flow-rate of 1 ml/min.

For ZEA determination methanol–water (65:35, v/v) was used as mobile phase. The compounds were detected by a fluorescence (excitation wavelength, λ_{ex} 274 nm/emission wavelength, λ_{em} 440 nm) and a photodiode array detector, recording the chromatograms at 236 nm.

For fluorescence detection of trichothecenes, the mobile phase was acetonitrile–water (65:35, v/v), acidified with acetic acid to reach a final concentration of 0.75% (v/v). The excitation and emission wavelengths of the fluorometer were set at 292 and 425 nm, respectively. For UV detection of type B trichothecenes, the mobile phase was water–acetonitrile (90:10, v/v) and chromatograms were recorded at 221 nm.

The mobile phases for determination of fumonisins were (A) methanol–0.05 *M* sodium di-

Table 1
Influence of the clean-up procedure on the level of zearalenone found in contaminated ground corn

Clean-up procedure	Level found (μg/g)	Recovery (%)
C_{18}	36.6	91.5
Florisil	38.1	95.3
Silica	6.8	16.9
ZearalaTest	36.6	91.8

hydrogenphosphate adjusted to pH 5.0 with 2 *M* NaOH (50:50, v/v) and (B) acetonitrile–water (80:20, v/v). The gradient program was 100% A for 5 min and then changed to 50% A+50% B with a final hold of 15 min.

3. Results and discussion

3.1. Clean-up procedure

The most frequently used method for the clean-up of samples is SPE. More recently, analyte selective columns have become popular in mycotoxin analysis as a very selective and time-saving one-step sample clean-up tool offering high extraction capacity and an almost complete removal of matrix compounds [22–24].

3.1.1. Zearalenone

Ground cultures were extracted with methanol–1% aqueous NaCl (80:20, v/v). The extracts were cleaned up by the SPE procedures indicated in the Experimental section. Results are shown in Table 1. The chromatograms obtained after clean-up with Florisil were quite free from extraneous peaks. The response obtained for the sample purified by this procedure was also quite high, especially with fluorescence detection. The C_{18} cartridges afforded responses comparable to those obtained with Florisil, but the chromatograms showed more peaks from co-extracted materials. Silica cartridges were inappropriate, because they adsorbed a great proportion of ZEA. Immunoaffinity clean-up provided cleaner extracts, which could be analysed with less interference than did the other cleaning columns, although recovery data are similar to those obtained with Florisil or C_{18} cartridges, in agreement with Zöllner et al. [25]. Thus, among the clean-up procedures assayed, the ZearalaTest immunoaffinity column seems to give the best results in agreement with Rosenberg et al. [26]. However, it showed lower sample capacity than the SPE cartridges. Successive clean-up steps done by passing the eluate from one cartridge through another one filled with a different sorbent material resulted in very low recovery of the toxin and proved to be unsuitable (data not shown).

3.1.2. Trichothecenes

Ground cultures were extracted with acetonitrile–water (84:16, v/v). The extracts were purified as indicated in the Experimental section. Results are shown in Table 2. Several problems were found when silica or Florisil cartridges were used. Low effectiveness was observed, especially for NIV, due to the low solubility of this polar trichothecene in the less polar solvents used with these cartridges. The high polarity of NIV (due to its four hydroxyl groups) also favours interactions with the active groups of silica. Moreover, different evaporation and dissolution steps are needed when using these pro-

Table 2
Influence of the clean-up procedure on the recovery of trichothecenes in contaminated ground corn

Clean-up procedure	Type A trichothecenes								Type B trichothecenes							
	T-2 toxin		HT-2 toxin		NEO		DAS		DON		NIV		3-AcDON		15-AcDON	
	Level found (μg/g)	Recovery (%)	Level found (μg/g)	Recovery (%)	Level found (μg/g)	Recovery (%)	Level found (μg/g)	Recovery (%)	Level found (μg/g)	Recovery (%)	Level found (μg/g)	Recovery (%)	Level found (μg/g)	Recovery (%)	Level found (μg/g)	Recovery (%)
Silica	17.5	87.6	16.9	84.3	17.5	87.5	17.9	89.3	13.3	66.5	6.2	31.2	11.8	59.2	12.3	61.5
Florisil	18.3	91.5	17.8	88.9	18.1	90.6	17.7	88.4	15.9	79.7	8.2	41.1	14.4	71.8	14.9	74.8
MycoSep 225	16.9	84.3	15.9	79.6	17.1	85.4	16.6	82.9	17.9	89.6	15.1	75.6	14.5	72.6	15.2	76.2

cedures, critical steps that are time-consuming and may cause significant losses of toxins.

Although efficient removal of impurities was obtained with both silica and Florisil cartridges (colourless solutions, clean chromatograms), lower recoveries were obtained with the first one. Similar problems were found by Weingaertner et al. [27].

The MycoSep 225 column is simpler to use and provides a faster clean-up method, giving chromatograms comparable to methods consisting of several purification steps. Only one evaporation step is required. Weingaertner et al. [27] and Romer [28] pointed to potential binding of analytes to active sites of the sorbent. Good recoveries were obtained for type B trichothecenes. Recoveries were somewhat lower for type A trichothecenes compared with the results provided by silica or Florisil. Recovery was lower for the more polar NIV.

3.1.3. Fumonisins

Ground corn cultures were extracted with acetonitrile–water (50:50, v/v). Prior to chromatography, the fumonisin extracts must be purified to remove matrix impurities and to concentrate mycotoxins. For fumonisins, this could be achieved either by SPE on reversed-phase (C_{18}) or SAX cartridges or by immunoaffinity columns. Both C_{18} and SAX cartridges provide similar purification results (data not shown), but the use of SAX cartridges requires monitoring of the pH of the sample extract (pH must be above 5.8 for adequate retention) and careful control of elution flow-rates at not more than 1 ml/min for reproducible recoveries [29].

On the other hand, large variations in recovery from C_{18} cartridges have been noted which were surmised to be due to interaction of the fumonisins with active sites of sorbent [30]. The sequential use of first a C_{18} and then a SAX cartridge has been applied to the clean-up of the extracts but better purification and recovery results were not obtained. As this last technique is more time consuming and an additional concentration step is necessary, passage through one cartridge is more suitable for fumonisin determination.

As an alternative to SPE, some authors have proposed the use of immunoaffinity columns containing antibodies reactive with fumonisins; they provide a more selective purification of sample extracts [31]. Care must be exercised in the use of these columns because they have a limited capacity which should not be exceeded. Compared with SPE columns, cleaner chromatograms with similar recovery data were reported [32–35].

3.2. Evaluation of calibration lines and limits of detection

The limits of detection for ZEA were obtained by spiking ground cereal samples, previously found to contain undetectable levels of the toxin, with low levels (0.25–0.005 μg/g) of ZEA. Then, the sample was extracted, purified through ZearalaTest column and injected into the liquid chromatograph. The λ_{ex} 236/λ_{em} 420 nm combination was not the best choice for fluorescence detection and signals were higher with the λ_{ex} 274 nm/λ_{em} 440 nm combination, in agreement with spectral data [23,36]. Calibration lines were linear in the range 2–2000 ng (injected amounts) of ZEA using both fluorescence and photodiode array detectors, with correlation coefficients of 0.993 and 0.9983, respectively. The first detector was more sensitive, but the second permits the display of the whole UV spectrum, which provides additional information to confirm the identity of the mycotoxin. As the UV spectra of ZOLs and ZEA are quite similar [36], their separation is necessary. The limits of detection (signal to noise = 3) using fluorescence (λ_{ex} 236 nm/λ_{em} 420 nm) and photodiode array detectors (236 nm) were 10 ng/g corn and 30 ng/g corn, respectively. Similar results were obtained with the other substrates assayed. The limit of detection by fluorescence detection decreased to 4 ng/g sample by setting λ_{ex} and λ_{em} at 274 nm and 440 nm, respectively. This last detection limit agrees with the data reported by Schumacher et al. [37] using HPLC–fluorescence detection and is below the value given by Ware et al. [38].

The limits of detection of trichothecenes were obtained by injections of blank and spiked cereal samples. The calibration curves were linear in an interval from 0.01 to 1000 ng, with correlation coefficients higher than 0.999. For type A trichothecenes (derivatised with coumarin-3-carbonyl chloride) the detection limits (at signal-to-noise ratio = 3) were 10 ng/g sample for T-2 toxin and about 15 ng/g for the remaining toxins. For all type B

trichothecenes, the limits of detection were about 10 ng/g when photodiode array detection was used, and 20 ng/g for 3-AcDON, 40 ng/g for NIV and 15-AcDON, and 50 ng/g for DON when fluorescence detection was used. Photodiode array detection provided greater responses for underivatised type B trichothecenes than fluorescence detection for the respective coumarin-3-carbonyl derivatives. These data could be explained by incomplete derivatisation of type B trichothecenes when coumarin-3-carbonyl chloride was used to obtain fluorescent derivatives. Type A trichothecenes could be derivatised with coumarin-3-carbonyl chloride more completely, which may be explained by the different polarities of the two types of trichothecenes. This method has the advantage to provide a good chromatographic separation of the peaks corresponding to the four type B trichothecenes but it is necessary to lower the limits of detection to the level obtained for type A trichothecenes [19].

The analytical method proved to be linear for fumonisin in the range from 10 to 4000 ng. The detection limits in corn cultures (at a signal-to-noise ratio=3) were 5 ng/g sample for FB_1 and FB_2 when OPA derivatives were analysed, and 40 ng/g sample when NBD-F was used for derivatisation. Taking into account that OPA derivatives are more stable during the first minutes after the addition of the fluorescent reagent and that cleaner chromatograms were obtained with OPA, this method is more suitable for fumonisin determination.

3.3. Recovery

After the comparative study of extraction solvents and SPE clean-up procedures, the combination that produced the best results was chosen for recovery studies on spiked cereals.

Samples of ground cereals were spiked with standard solutions of ZEA to provide fortification levels ranging from 0.005 to 25 μg/g and the samples were analysed as described in the Experimental section. The results of these assays appear in Table 3. The recovery ranged from 91.5 to 116% (mean=102.4%; RSD=8%) for spiking levels in the range 0.025 to 25 μg/g. The 0.005 μg/g spiking level was still detected (with fluorescence detection) but quantification was not reliable. Photodiode array detection was less sensitive; it also responded to coeluting UV absorbers from the matrix and it was not able to detect ZEA at or below 0.025 μg/g level.

Table 3
Recovery of zearalenone from spiked cereals

Spiking level (μg/g)	HPLC–fluorescence		HPLC–UV	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
25	91.5	2.2	82.3	3.0
12.5	94.4	3.4	88.4	3.6
6.25	100.5	1.3	93.6	2.4
2.5	101.8	4.4	95.3	4.3
0.25	103.6	7.7	117.6	6.3
0.025	106.2	10.6	–	
0.005	109.5	15.4	–	

Number of replicates at each spiking level=3.

Ground cereals were spiked with standard solutions of type A and type B trichothecenes. The contamination levels ranged from 0.025 to 12.5 μg/g. After solvent evaporation and thorough mixing, the samples were extracted with acetonitrile–water (84:16, v/v) as described in the Experimental section. The results of these assays are shown in Table 4. Among type A trichothecenes, recoveries were 54.6–69.5% for T-2 toxin, 51.6–64.2% for HT-2 toxin, 53.1–67.6% for NEO and 52.9–67.0% for DAS. Among type B trichothecenes, recoveries were 52.5–67.1% for DON, 40.1–50.6% for NIV, 41.9–53.2% for 3-AcDON and 41.7–54.6% for 15-AcDON. These low recoveries were due to the low efficiency of the liquid–liquid partition step necessary to eliminate the excess of the derivatisation reagent (about 70%). Higher levels of trichothecene recovery can be reached by repeating this purification step at the cost of more time and sample handling.

Recovery values from blended cereals spiked with fumonisin standards by using C_{18} clean-up and OPA derivatisation vary from 97 to 105% over a range from 10 to 500 μg/g of FB_1 and FB_2 (Table 5).

3.4. Analysis of mycotoxins in cereal cultures of Fusarium

Fig. 1 displays a HPLC–fluorescence chromatogram obtained from the analysis of a corn sample inoculated with strain FCu4. The optimised method

Table 4
Recovery of trichothecenes from spiked cereals

Spiking level (μg/g)	Type A trichothecenes								Type B trichothecenes							
	T-2 toxin		HT-2 toxin		NEO		DAS		DON		NIV		3-AcDON		15-AcDON	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
12.5	54.6	2.5	51.6	3.1	53.1	3.6	52.9	2.4	52.5	3.3	39.7	4.6	41.9	3.7	41.7	4.1
5.0	56.2	4.1	52.9	5.0	55.9	5.2	54.8	2.9	54.9	4.6	41.4	6.9	44.9	5.6	45.2	6.6
2.5	60.5	4.8	54.6	6.3	57.4	7.0	57.0	5.6	57.9	5.1	43.6	8.1	47.8	6.8	47.2	8.0
1.3	62.9	6.4	57.9	7.9	60.1	8.1	60.5	8.0	60.7	6.0	46.1	9.9	50.3	8.4	51.6	9.5
0.5	66.2	7.2	61.2	9.1	63.8	8.6	64.2	8.2	63.0	6.9	48.3	10.5	51.5	9.2	51.4	10.6
0.3	68.4	7.9	62.9	9.9	66.1	9.2	65.8	8.9	64.4	8.6	49.4	12.6	52.6	10.5	52.9	11.7
0.0	69.5	9.2	64.2	11.3	67.6	10.3	67.0	9.2	67.1	9.5	49.6	14.3	53.2	11.3	54.6	12.8

Number of replicates at each spiking level=3.

Table 5
Recovery of fumonisins from spiked cereals

Mycotoxin added (μg/g)	FB1		FB2	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
10	105.0	5.9	104.0	6.3
20	101.5	5.0	102.0	4.5
50	99.4	2.6	101.2	3.6
100	99.3	2.3	98.8	4.1
200	99.0	2.7	99.1	3.1
500	97.3	3.4	97.0	2.5

Number of replicates at each spiking level=3.

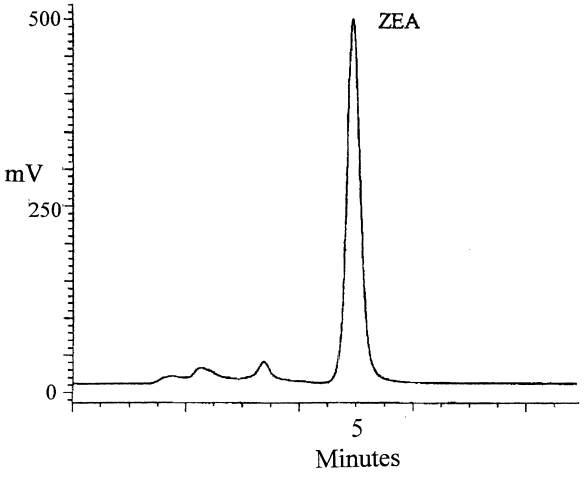

Fig. 1. HPLC–fluorescence chromatogram of a corn sample inoculated with FCu4 strain. Concentration of zearalenone is shown in Table 4.

was applied to the analysis of ZEA in cereal cultures inoculated with four strains of *Fusarium* species reported to be producers of this mycotoxin. *F. graminearum* (FG1 and FG6) provided high values whereas *F. culmorum* (FCu4) gave the highest yield (Table 6; Fig. 1); suitable dilution rates were made for the accurate analysis of high ZEA concentrations in order to maintain its level within the calibration range.

The proposed analytical method was tested for its suitability for determining type A trichothecenes in different cereal samples previously inoculated with several strains of *Fusarium sporotrichioides*. Fig. 2a displays a chromatogram obtained from the analysis of a corn sample inoculated with strain FSp3, derivatised with coumarin-3-carbonyl chloride. There is no peak interfering with any of the four trichothecenes studied. This was found for other samples of contaminated rice and corn as well. Table 6 lists the levels of the four trichothecenes found in the samples of inoculated cereals. The method proposed is useful for the determination of T-2 toxin, HT-2 toxin, NEO and DAS in corn and rice contaminated with *F. sporotrichioides* and, probably, with other type A trichothecene-producing fungal species. This method has a higher sensitivity and is more linear than alternative, published methods for type A trichothecenes, such as GC–electron-capture detection (ECD) [39], and needs shorter analysis time (DAS, NEO, T-2 and HT-2 toxin can be eluted, detected and quantified in only 12 min, other related trichothecenes may require somewhat longer times [40]).

Table 6
Mycotoxin levels (mg/kg) found in cereal cultures of the stains of *Fusarium* studied in triplicate

Fusarium strain	Substrate	Type A trichothecenes										Type B trichothecenes											
		Zearalenone		T-2 toxin		HT-2 toxin		DAS		NEO		DON		NIV		3-AcDON		15-AcDON		FB1		FB2	
		mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)	mg/kg	RSD (%)
FCu1	Rice											ND		ND		ND		ND					
	Wheat											ND		ND		ND		ND					
FCu4	Corn	1430	1.7																				
	Rice	404	2.1																				
	Wheat	4870	1.2																				
FG1	Corn	245	4.1									0.359	7.9	1.893	5.2	1.2	3.9	0.36	6.2				
	Rice	113	3.5									1.101	4.3	0.074	8.9	2.76	4.1	1.35	5.1				
FG6	Rice	58	3.4									2.685	3.8	8.267	1.6	0.124	6.4	tr					
	Wheat	46	4.3									tr.		2.859	3.1	tr.		tr					
FSp1	Corn			0.221	4.6	0.12	6.2	0.039	8.4	0.04	7.8												
	Rice			2.44	5.7	0.065	5.4	tr		tr													
FSp3	Corn			1.31	3.6	0.047	8.7	0.286	6.3	0.073	6.5												
	Wheat			1.85	4.7	0.045	7.6	0.254	4.3	0.321	4.1												
FSp6	Rice			0.03	8.9	0.12	10.3	0.055	8.7	0.031	9.9												
	Wheat			0.039	9.6	0.058	9.3	0.03	9.6	tr													
Gf2	Corn																			2680	2.6	185.2	4.3
	Rice																			3006	2.1	254	6.1
Gf6	Corn																			11.1	8.7	1.6	12.5
	Rice																			ND		ND	
	Wheat																			ND		ND	
Gf7	Corn																			1340	3.6	91.9	5.4
	Wheat																			671	6.2	45.9	5.1
Gf20	Corn																			1515	4.6	569.2	4.2
	Rice																			675	5.1	116	6.2
Gf47	Corn																			1264	5.8	99.5	5.4
	Rice																			1348	3.6	105.1	4.3

tr=Traces (<2×limit of detection), ND=not detected.

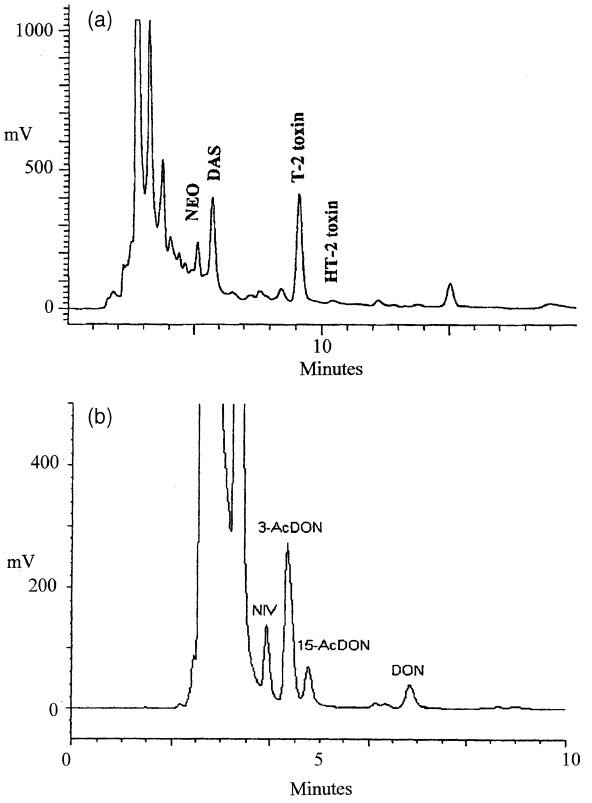

Fig. 2. HPLC–fluorescence chromatogram of (a) a corn sample inoculated with FSp3 strain and (b) a corn sample inoculated with FG1 strain. Concentrations of trichothecenes are shown in Table 4.

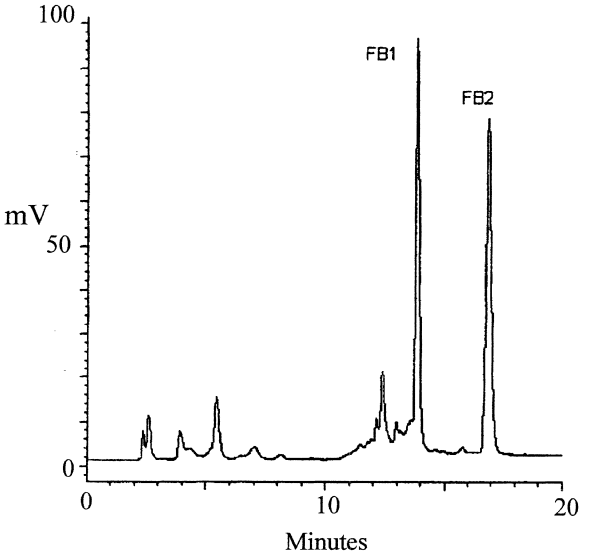

Fig. 3. HPLC–fluorescence chromatogram of a corn sample inoculated with Gf20 strain. Concentration of each fumonisin is shown in Table 4.

The optimised method was applied to the analysis of type B trichothecenes in corn, wheat and rice cultures inoculated with three strains of *Fusarium* (see Experimental section). The results are listed in Table 6. Fig. 2b shows the chromatogram of a corn sample inoculated with FG1 strain after derivatisation with coumarin 3-carbonyl chloride. In agreement with previous reports [9,42], our data show that *F. culmorum* is unable to produce type B trichothecenes, although more experiments must be made to confirm this conclusion. Both strains of *F. graminearum* can produce these mycotoxins. Analysis of naturally contaminated cereals was simulated by adding trichothecene containing cultures to uncontaminated samples at low ratios (usually, about 10%). The entire procedure worked satisfactorily, because the levels found for the different type B trichothecenes agreed within 10% with the predicted concentrations in the spiked samples.

This method was also used to analyse cereal samples inoculated with strains of *Gibberella fujikuroi* and cultured as described in the Experimental section. Results are shown in Table 6. Fig. 3 shows the HPLC–fluorescence chromatogram of a corn sample inoculated with Gf20 strain, derivatised with OPA. All strains produced variable levels of both fumonisins. The range for FB_1 and FB_2 was 0 to 3006 mg/kg and 0 to 569 mg/kg. The Gf2 strain was the highest fumonisin producer (about 3000 mg FB_1/kg). Rice cultures of *G. fujikuroi* gave cleaner extracts with less small fine particles than cultures grown in corn. Their extracts filtered also faster and appeared to contain lower amounts of pigments than extracts obtained from corn. In general, yields of fumonisins from rice cultures were similar to yields obtained from corn cultures.

4. Conclusions

The results demonstrate the suitability of modern clean-up techniques for the analysis of the *Fusarium* mycotoxins. The clean-up procedures employing

multifunctional MycoSep and immunoaffinity columns are quick, reliable and rugged methods for the determination of these mycotoxins. Compared to the usual clean-up methods, they require less experience for the determination of these toxins. Furthermore, the use of immunoaffinity columns for clean-up provides a number of advantages which include: (a) provision of clean extracts due to the specificity of the antibodies for the single toxin or a group of related toxins that can be separated by HPLC; (b) optimal performances in terms of precision and accuracy within a wide range of concentrations which cover the field of practical interest; (c) speed, and (d) a considerable reduction in the use of dangerous solvents, highly desirable and necessary for environmental protection. Taking into account that similar recovery data were obtained, one of the major advantages of the conventional methods lies in the relative low material cost compared to the high cost for the multifunctional cartridges and immunoaffinity methods.

HPLC is well suited for the analysis of the toxic secondary metabolites produced by *Fusarium* species. For ZEA determination, fluorescence detection is more sensitive and thus preferred for the analysis of low levels of ZEA, while the use of photodiode array detection is appropriate for confirmation purposes. Trichothecenes could be detected by fluorescence after derivatisation with coumarin-3-carbonyl chloride, although photodiode array detection (at 221 nm) of type B trichothecenes seems preferable because lower limits of detection were reached and no derivatisation step was necessary. For type A trichothecenes, the method is more sensitive than other HPLC methods that use UV detection [41–43]. The OPA reagent proved useful for the determination of fumonisins in samples, providing better sensitivity than the NBD-F reagent.

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References

[1] Anonymous, Protection against trichothecenes, in: Reports of Committee on Protection against Mycotoxins, National Academic Press, Washington, DC, 1983, p. 227.
[2] V.R. Beasley, Trichothecene Mycotoxicosis: Pathophysiologic Effects, CRC Press, Boca Raton, FL, 1989.
[3] T.E. Möller, H.F. Gustavson, J. AOAC Int. 75 (1992) 1049.
[4] A. Visconti, A. Boenke, M.B. Doko, M. Solfrizzo, M. Pascale, Nat. Toxins 3 (1995) 269.
[5] C.J. Mirocha, S.V. Pathre, C.M. Christensen, in: J.V. Rodriccks, C.W. Hesseltine, M.A. Mehlman (Eds.), Mycotoxins in Human and Animal Health, Pathotox, Park Forest South, IL, 1977, p. 345.
[6] K.E. Richardson, W.M. Hagler, C.J. Mirocha, J. Agric. Food Chem. 33 (1985) 862.
[7] M.T. Liu, B.P. Ram, L.P. Hart, J.J. Pestka, Appl. Environ. Microbiol. 50 (1985) 332.
[8] M.A. Diekman, M.L. Green, J. Anim. Sci. 70 (1992) 1615.
[9] Y. Luo, T. Yoshizawa, T. Katayama, Appl. Environ. Microbiol. 56 (1990) 3723.
[10] R. Cole, R. Cox (Eds.), Handbook of Toxic Fungal Metabolites, Academic Press, New York, 1981, p. 152.
[11] B.E. Branham, R.D. Plattner, Mycopathologia 124 (1993) 99.
[12] M. Jimenez, T. Huerta, R. Mateo, Appl. Environ. Microbiol. 63 (1997) 364.
[13] J.J. Mateo, M. Jimenez, Food Microbiol. 17 (2000) 167.
[14] W. Langseth, T. Rundberget, J. Chromatogr. A 815 (1998) 103.
[15] M.J. Cardone, J. Assoc. Off. Anal. Chem. 660 (1983) 1257.
[16] M.J. Cardone, J. Assoc. Off. Anal. Chem. 660 (1983) 1283.
[17] M.S. Gandelman, J.W. Birks, Anal. Chem. 54 (1982) 2131.
[18] M.S. Gnanasambandan, H. Freiser, Anal. Chem. 54 (1982) 1282.
[19] M. Jimenez, J.J. Mateo, R. Mateo, J. Chromatogr. A 870 (2000) 473.
[20] G.S. Shephard, P.G. Thiel, S. Stockenström, E.W. Sydenham, J. AOAC Int. 79 (1996) 671.
[21] P.M. Scott, G.A. Lawrence, J. AOAC Int. 75 (1992) 826.
[22] M.W. Trucksess, J. AOAC Int. 80 (1997) 119.
[23] A. Visconti, M. Pascale, J. Chromatogr. A 815 (1998) 133.
[24] R. Krska, J. Chromatogr. A 815 (1998) 49.
[25] P. Zöllner, J. Jodlbauer, W. Linder, J. Chromatogr. A 858 (1999) 167.
[26] E. Rosenberg, R. Krska, R. Wissiack, V. Kmetov, R. Josephs, E. Razzazi, M. Grasserbauer, J. Chromatogr. A 819 (1998) 277.
[27] J. Weingaertner, R. Krska, W. Praznik, M. Grasserbauer, H. Lew, Fresenius J. Anal. Chem. 357 (1997) 1206.
[28] T. Romer, presented at the 5th International Seminar on *Fusarium* Mycotoxins, Taxonomy and Pathogenicity, Bari, 3–9 May 1995.
[29] E.W. Sydenham, G.S. Shephard, P.G. Thiel, J. AOAC Int. 75 (1992) 13.
[30] G.A. Bennet, J.L. Richard, J. AOAC Int. 77 (1994) 501.
[31] G.S. Shephard, J. Chromatogr. A 815 (1998) 31.

[32] G.W. Ware, P.P. Umrigar, A.S. Carman, S.S. Kuan, Anal. Lett. 27 (1994) 693.
[33] P.M. Scott, T. Delgado, D.B. Prelusky, H.L. Trenholm, J.D. Miller, J. Environ. Sci. Health B29 (1994) 989.
[34] P.M. Scott, G.A. Lawrence, J. Food Protect. 58 (1995) 1379.
[35] N.W. Trucksess, M.E. Stack, S. Allen, N. Barrion, J. AOAC Int. 78 (1995) 705.
[36] E.W. Sydenham, P.G. Thiel, R. Vleggaar, J. AOAC Int. 79 (1996) 1365.
[37] R. Schumacher, R. Krska, M. Grasserbauer, W. Edinger, H. Lew, Fresenius J. Anal. Chem. 360 (1998) 241.
[38] G.M. Ware, Y. Zhao, S.S. Kuan, A.S. Carman, J. AOAC Int. 82 (1999) 90.
[39] E.W. Sydenham, P.G. Thiel, Food Addit. Contam. 4 (1987) 277.
[40] H. Cohen, B. Botin-Muma, J. Chromatogr. 595 (1992) 143.
[41] R. Maycok, D. Utley, J. Chromatogr. 347 (1985) 429.
[42] G.W. Stratton, A.R. Robinson, H.C. Smith, L. Kittilsen, M. Barbour, Arch. Environ. Contam. Toxicol. 24 (1993) 399.
[43] R. Schmidt, E. Ziegenhagen, K. Dose, J. Chromatogr. 212 (1981) 370.