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Determination of type A trichothecenes by high-performance liquid chromatography with coumarin-3-carbonyl chloride derivatisation and fluorescence detection

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Abstract

A method for the analysis of type A trichothecenes T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol by high-performance liquid chromatography with fluorescence detection using coumarin-3-carbonyl chloride has been developed. Different parameters concerning the analytical procedure such as stability of both the reagent and derivatised analytes, time and temperature of the derivatisation reaction, were studied and optimised. Three different clean-up procedures (solid-phase extraction with silica gel or C-18 cartridges, and liquid–liquid partition between toluene and dihydrogen phosphate buffer) were tested in order to remove the excess reagent peaks. The last procedure gave the best results when the buffer pH was 3–5.5, and is therefore recommended. Separations were performed on a stainless steel LiChrospher 100 C-18 reversed-phase column with pre-column of the same phase. The mobile phase was acetonitrile/water (65:35, v/v) containing 0.75% acetic acid at a flow-rate of 1.0 ml/min. The proposed method provides good separation between the four trichothecenes and good reproducibility (RSD of calibration standards <5%). The limits of detection of the studied trichothecenes at a signal-to-noise ratio of 3:1, with an injection volume of 20 μl were 10 ng/g sample for T-2 toxin and about 15 ng/g sample for the remaining mycotoxins. The calibration curve was linear between 10 and 2000 ng for the four trichothecenes assayed. The method was applied to the analysis of these mycotoxins in fungal cultures (corn and rice) of *Fusarium sporotrichioides,* and is also perfectly suitable for the quantification of type A trichothecenes in contaminated cereals.

Keywords: Derivatization, LC; Trichothecenes; Coumarin-3-carbonyl chloride; Mycotoxins

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 *Fusarium* toxins found naturally occurring 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.

Trichothecene mycotoxins are mould metabolites produced by various strains of *Fusarium* and other

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species of imperfect fungi [5–7]. These mycotoxins are characterized by a 12,13-epoxy-trichotec-9-ene ring system. Type A trichothecenes belong to the group that does not contain a carbonyl function at C8 conjugated with a double bond in C9-10 [8], which prevents these compounds from being detected by UV absorption.

The correlation between the presence of certain mycotoxins and adverse physiological effects demands for specific analytical methods for the mycotoxins in food in various commodities. Trichothecenes are only trace components in complex sample matrices and very sensitive and discriminating methods are needed. Enzyme-linked immunosorbent assay (ELISA) systems are ideal for the analysis of trace compounds within complex matrices whereas chromatographic methods are better suited to screening for a group of analogous compounds [9].

Analysis of trichothecenes by TLC lacks either sensitivity [10] or selectivity [11] and various solvent systems and spray reagents are required to separate and characterise the type A trichothecenes [12].

The use of supercritical fluid chromatography for trichothecene analysis has been also reported [13] but this technique has hardly been used.

Capillary GC requires extensive sample clean-up but provides high resolution, selectivity, sensitivity, and applicability to most trichothecene mycotoxins. Derivatisation (trimethylsilylation or fluoroacylation) to yield volatile compounds has been applied to trichothecenes [3,9,14–17]. The use of ECD provides considerable sensitivity to the analytical method for fluoroacyl derivatives [3,18–21].

HPLC detection of compounds without a chromophore is problematic. Alternative principles such as refractometry, photoreduction–fluorescence [22] or displacement chromatography with UV absorbers [23], impose severe limitations together with constraints on mobile phase selection.

A pre-column derivatisation method has been reported to form a chromophore with *p*-nitrobenzoyl chloride with an absorption maximum at 254 nm but some disadvantages are an interfering peak from the reagent and the great number of compounds absorbing at this wavelength [24]. A post-column derivatisation technique involves alkaline decomposition to form formaldehyde and a modified Hantzch reaction with methylacetoacetate and ammonium acetate [25]. This procedure is suitable for type B but not for type A trichothecenes.

Fluorescence detection permits high sensitivity often with good selectivity. Various hydroxyl derivatisation reagents have been proposed for HPLC with fluorescence detection [26–28]. The only commercially available reagent specifically developed for these compounds is 1-ethoxy-4-(dichloro-1,3,5-triazinyl) naphtalene, which is of low reactivity. 9-Fluorenylmethyl chloroformate has been applied to hydroxy compounds but gives low quantum yields and the potential for interference is high due to low excitation wavelength [29].

Acylation is commonly used for labelling hydroxy compounds and permits a rapid, quantitative derivatisation. The use of anthracene-9-carbonyl chloride [29] or coumarin-3-carbonyl chloride [30] as derivatisation reagents of the hydroxyl function permits the fluorescence detection of trichothecenes, hydroxy-steroids and prostaglandins [27]. Coumarins as fluorophore possess a high molecular extinction coefficient and quantum yields.

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

2. Experimental

2.1. Culture preparation

Three strains of *Fusarium sporotrichioides* were used to inoculate cereal samples. These strains are held at the Department of Microbiology (Valencia University) and their references are FSp1, FSp3, and FSp6. They were maintained in Potato-Dextrose Agar (PDA). Cereal samples were purchased on the retail market and used without grinding. Two hundred grams of corn or rice grains were placed in 250-ml Erlenmeyer flasks with 90 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 PDA single-spore cultures and maintained at 26°C for 3 weeks. The cultures were dried at 50°C for 48 h and then finely ground with a laboratory mill [31].

2.2. Chemicals and reagents

Organic solvents were HPLC grade from Merck (Darmstadt, Germany). Chloroform, dichloromethane and toluene were dried over anhydrous sodium sulphate. Water was prepared with a Waters Milli-Q system (Waters Assoc., Milford, MA, USA).

Standards of T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS), and 4-dimethylaminopyridine (DMAP) were purchased from Sigma (St. Louis, MO, USA). Coumarin-3-carboxylic acid and thionyl chloride were from Aldrich Chemical Co. (Milwaukee, WI, USA).

2.3. Extraction

Finely ground cereal samples were thoroughly mixed. Aliquots (10 g) of the samples were blended in a high speed blender (Ultraturrax T25, Ika-Werk, Germany) with 50 ml of acetonitrile/water (84:16, v/v) for 5 min and filtered through Whatman No. 4 filter paper. Twenty five millilitres of the filtrate was defatted with hexane, trichothecenes extracted with dichloromethane (3×10 ml) and then concentrated to dryness under vacuum at 40°C. The residue was redissolved in 3.0 ml of chloroform/methanol (9:1, v/v) prior to clean-up procedure.

2.4. Sample clean-up

A 3-ml capacity Florisil Sep-Pak cartridge (Millipore Corp., Milford, MA) was preconditioned with 5 ml of methanol followed by 5 ml of chloroform/methanol (9:1, v/v). One and a half millilitres of the sample filtrate was passed through the cartridge. The trichothecenes were eluted with 20 ml of chloroform/methanol (9:1, v/v) and then concentrated to dryness under vacuum at 40°C. The residue was transferred to a vial with 2×0.5 ml of toluene and then evaporated to dryness under a slow stream of N_2 [32].

2.5. Synthesis of coumarin-3-carbonyl chloride

Coumarin-3-carboxylic acid was suspended in dry dichloromethane (30 ml) and refluxed while adding thionyl chloride (10 ml) dropwise for 40 min. Dichloromethane was added to prevent dryness during the reaction. Afterwards, the solvent was evaporated under vacuum at 40°C. The residue was dissolved in anhydrous chloroform and the solution was heated with careful addition of hexane until the solution turned cloudy. Pale yellow crystals of coumarin-3-carbonyl chloride were obtained on cooling. The solvent was evaporated at room temperature and the product was stored in amber bottle. The product was characterised by melting point (143°C, uncorrected), infrared spectrum and mass spectrometry, being in agreement with reference data [30]. Yields were 67–75%.

2.6. Preparation of standards

(A) Trichothecene standards. One milligram of each toxin was dissolved in 10 ml of acetonitrile to obtain a stock solution (100 μg/ml). These standard solutions were stored at −20°C and brought to room temperature before use. Standards of lower concentration were prepared by suitable dilution of the stock solutions.

(B) Coumarin-3-carbonyl chloride. A solution of 3.25 mg/ml was obtained by dissolving 65 mg in 20 ml with toluene.

(C) DMAP. A solution of 6.5 mg/ml was prepared by dissolving 130 mg in 20 ml volumetric flask with toluene.

2.7. Derivatisation

All glassware was rinsed with toluene before use. Ten microlitres of DMAP solution was added to a 2.0-ml screw-cap amber vial containing evaporated samples or standards. Then, 10 μl of the coumarin reagent was added. The vial was closed and the mixture heated at 80°C for 20 min. The mixture was cooled in ice-water and then cleaned-up.

2.8. Purification of the reaction mixture

Three purification protocols were assayed to select the most suitable one. Two of them were based on solid-phase extraction (SPE) and the third consisted of a liquid–liquid partition.

(1) Silica gel cartridges (Waters) were preconditioned first with 5 ml of chloroform/methanol (7:3, v/v) and then with 5 ml of benzene. The cooled

reaction mixture was quantitatively transferred to the silica gel cartridge with benzene (5×1 ml). The benzene fractions were discarded; then a mixture of ethyl acetate/hexane (8:2, v/v, 5×2 ml) was added to the cartridge and discarded. The derivatised trichothecenes were eluted with a mixture of ethyl acetate/hexane (9:1, v/v, 2×7 ml). The eluate was collected and evaporated to dryness under a gentle stream of N_2 [30].

(2) The reaction mixture was evaporated to dryness. The residue was dissolved in 0.3 ml of acetonitrile, mixed with 0.7 ml of water and then transferred to a 3-ml Sep-Pak C-18 cartridge (Waters) previously preconditioned with 5 ml of methanol followed by 5 ml of acetonitrile/water (3:7, v/v). Excess reagent and by-products were eluted with 20 ml of acetonitrile/water (3:7, v/v). The derivatised toxins were recovered with 3 ml of acetonitrile. The eluate was collected and evaporated to dryness under a gentle flow of N_2.

(3) The cooled reaction mixture was evaporated to dryness and redissolved in 0.4 ml of toluene. After addition of 0.4 ml of 0.05 *M* dihydrogen phosphate buffer pH 5.5 and vigorous mixing, phases were allowed to separate. Three hundred ml of the upper organic phase was transferred to another vial and evaporated to dryness under a gentle flow of N_2.

The residue was dissolved with 100 μl (for the two SPE procedures) or 75 μl (for the third procedure) of acetonitrile/water (65:35, v/v) acidified with 0.75% of acetic acid (HPLC mobile phase). The solution was filtered through a 0.20-μm filter and 20 μl was injected into the liquid chromatograph.

2.9. HPLC analysis

The trichothecene derivatives were analysed by liquid chromatography. The HPLC system consisted of a Waters 600 pump and a Waters 474 scanning fluorescence detector. Signals were processed on a Digital Celebris 590 PC (Millennium software was used). 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 phase. The column was kept at room temperature. The mobile phase was acetonitrile/water (65:35, v/v) containing 0.75% of acetic acid at a flow-rate of 1.0 ml/min. The mobile phase was degassed by passing through a vacuum-degassing device (Waters). The excitation and emission wavelengths of the fluorometer were set at 292 and 425 nm, respectively, with slit widths of 18 nm.

3. Results and discussion

3.1. Stability of the solid reagent

For this study coumarin-3-carbonyl chloride was synthesised from time to time along a 4-months period, as described in Experimental. Solutions of each batch were prepared and used to derivatise known amounts of the four trichothecenes. The solid product remained stable at room temperature and in the dark for 2 months at least, as responses of recently prepared derivatives of T-2 toxin, HT-2 toxin NEO and DAS were the same during this time. In the light the stability of the solid reagent decreased more quickly. At 3 and 4 months from synthesis, the standards derivatised with the reagent showed responses that had decreased to 80% and below 20% of the initial values, respectively.

3.2. Stability of derivatisation reagent in solution

When the solution of coumarin-3-carbonyl chloride was kept at room temperature a rapid decrease in its derivatising capacity was observed. Seven days after the preparation of the solution, fluorescence signals were about 20% of the initial values (results not shown). Therefore, the solution was maintained at 4°C and used for reacting with a standard solution of the four mycotoxins studied. The results showed that the reagent solution is stable for at least 20 days since no significant differences with time ($P<0.05$) in detector responses were observed for the four trichothecene derivatives during this time. After 20 days a rapid decrease in detector responses was observed. Thirty days after its preparation the reagent solution provided signals that were about 40% of their original values. Thus, the reagent solution is reasonably stable for analytical purposes in solution at 4°C provided water is completely excluded. Acid chlorides have not been widely used in analytical derivatisation as reagents due to their susceptibility

to hydrolysis. Moreover, acids liberated by water present in the reaction mixture can decrease the concentration of the tertiary amine catalyst (DMAP, in this case) by ion pair formation. If the water content is kept low acid chlorides are highly reactive towards hydroxy functional groups providing short reaction time and low reaction temperature.

3.3. Stability of derivatives

Aliquots of standard solutions of the four trichothecenes were derivatised and stored in the dark at room temperature. They were injected into the liquid chromatograph at selected time intervals. All derivatives were stable during the first 12 h after reagent addition. Responses decreased slightly (<10%) at 24 h. After 48 h signals decreased drastically (about 50%) being practically undetectable after 4 days.

Thus, the procedure is completely compatible with the use of automatic injectors coupled to liquid chromatographs, which constitutes a great advantage for routine analysis of type A trichothecenes.

3.4. Time and temperature of reaction

The effect of both reaction temperature and reaction time was studied to optimise these parameters. At 0°C only 20% of the toxins were derivatised. At 20 and 50°C signals increased to reach 45 and 85%, respectively, of the highest values, which were obtained at 80°C. No significant increases were observed at temperatures above 80°C. Other authors have reported that this reaction proceeds to completion at room temperature [27]. This does not agree with our observations, were only 50–60% of the values obtained at 80°C were achieved at room temperature (22–26°C).

To study the effect of reaction time aliquots of the same standard solution of the four type A trichothecenes were derivatised by keeping the vials at 80°C for different time intervals. Results show that 15 min is enough for complete reaction. However, as there is no signal loss with excess reaction time a period of 20 min can be used routinely to ensure the best result.

The results obtained with the mycotoxins are very similar. This may be due to the fact that the reaction mechanism is the same for all four trichothecenes independent of the number of hydroxyl groups in their molecules.

3.5. Clean-up procedures

The purification procedure is one of the most critical steps of trichothecene analysis by HPLC using coumarin-3-carbonyl derivatisation and fluorescence detection. The excess reagent appears as a broad, highly fluorescent peak and a large time period is needed to clean the chromatographic system between two consecutive chromatographic runs. Therefore, a clean-up step for eliminating the excess reagent before chromatographic separation is required.

Three different purification procedures were contrasted to select the most suitable one. They were: (a) SPE using silica gel cartridges, (b) SPE using C-18 cartridges, and (c) partition between toluene and 0.05 *M* dihydrogen phosphate buffer. All of them are described in detail in the Experimental section. The results are shown in Table 1. The greatest response

Table 1
Influence of the clean-up procedure on the reproducibility of type A trichothecenes derivatised with coumarin-3-carbonyl chloride[a]

Clean-up method	Trichothecenes							
	T-2 toxin		HT-2 toxin		NEO		DAS	
	Area (%)	RSD (%)	Area (%)	RSD (%)	Area (%)	RSD(%)	Area (%)	RSD (%)
No clean-up	100	3.5	100	6.2	100	5.1	100	4.3
Silica cartridge clean-up	1.02	10.3	0.74	8.4	0.70	9.2	0.76	12.5
C-18 cartridge clean-up	17.63	16.5	18.58	19.2	16.08	11.6	17.72	14.3
Liquid–liquid partition	69.10	4.7	64.80	4.8	67.59	4.9	67.00	4.5

[a] Concentration of all toxins were 0.5 μg/20 μl. Data are the mean values and RSD of the areas (μV/s) from three replicates. Area values were normalised with respect to 100 (no clean-up).

for all analytes was achieved when no purification step was used. But this strategy implies excess reagent.

The silica gel cartridge clean-up procedure [30] produced very low responses for all four trichothecenes in comparison with the response of derivatives in the uncleaned solution making this procedure entirely unsuitable for analytical purposes. Moreover, the use of benzene is a disadvantage of this procedure. The low recovery values can be explained by the similar polarities of clean-up and elution solvents used in this procedure.

The C-18 cartridge clean-up procedure yielded responses that were about 20% of the responses obtained with no clean-up procedure. RSD values were higher than 15% for ten replicates.

The liquid–liquid partition procedure allowed the recovery of about 70% of the analytes and a lower dispersion for all the mycotoxins assayed among the different replicates (RSD$<$5%). The excess reagent was eliminated thus avoiding the need to wash the chromatographic system after each analysis. Other advantages of the procedure are that it is less time-consuming and less expensive. Higher levels of trichothecene recovery can be reached by repeating the extraction but more time and sample handling are also needed.

Therefore, of the three procedures tested the last one is the most suitable. Under these conditions the calibration curve proved to be linear for mycotoxin amounts in the range from 10 to 2000 ng. Statistical coefficients for all four regression lines are given in Table 2. The detection limits in cereal cultures (at signal-to-noise ratios of 3:1) were 10 ng/g of sample for T-2 toxin and about 15 ng/g of sample for the remaining toxins.

3.6. Influence of the buffer pH on purification by liquid–liquid partition

Ideally, an analytical reagent used for making fluorescent derivatives should be nonfluorescent or easily removable from the reaction mixture. The reagent used in this study is strongly fluorescent but can be hydrolysed easily. If the reaction mixture is washed with a buffer solution, the corresponding fluorescent acid is formed. By selecting the appropriate pH, the acid can be extracted into the aqueous phase.

With those premises in mind different pH values ranging from 3.0 to 7.0 were studied for the 0.05 *M* dihydrogen phosphate buffer solution used for liquid–liquid extraction to find the best value. Peak areas were practically unchanged for all four analytes in the pH range 3.0–5.5, but decreased when the pH of the buffer was higher than 5.5. When pH was 7.0 signals decreased and became 20% of the values obtained in the 3.0–5.5 pH range. Thus, the pH of the washing dihydrogen phosphate buffer solution has a remarkable influence on the stability of derivatised trichothecenes.

3.7. Application to rice and corn cultures

The proposed analytical method was tested for its suitability to determine T-2 toxin, HT-2 toxin, DAS and NEO in different rice and corn cultures of *Fusarium sporotrichioides*, a mould proved to produce these four mycotoxins.

Fig. 1 displays a representative chromatogram obtained from the analysis of a rice sample inoculated with *F. sporotrichioides* strain FSp3. There is no peak interfering with any of the four tricho-

Table 2
Linearity of the fluorometer response to coumarin-3-carbonyl derivatives of type A trichothecenes (n=7)

Toxin	Intercept		Slope		Correlation coefficient	Standard error of estimate
	Estimation	Standard error	Estimation	Standard error		
Neosolaniol	−19 658	63 589	15 646	501	0.996633	134 793
Diacetoxyscirpenol	−35 698	61 247	14 835	483	0.996758	129 797
T-2 toxin	−52 316	15 698	16 897	636	0.996127	170 942
HT-2 toxin	−16 329	16 487	16 040	332	0.997964	89 283

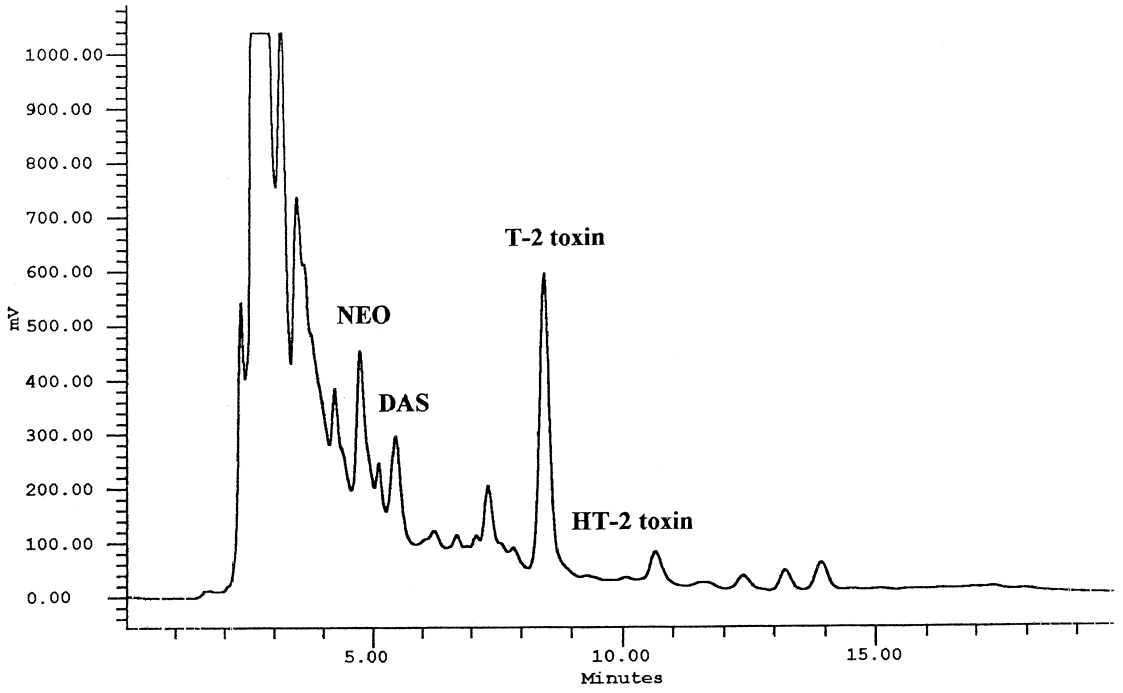

Fig. 1. Liquid chromatogram of the extract from a rice sample with a moisture level of 35% and inoculated with *F. sporotrichioides* strain FSp3 and incubated for 3 weeks at 26°C. Clean-up procedure: toluene/0.05 *M* dihydrogen phosphate buffer pH 5.5 partition. HPLC conditions: reversed-phase chromatography on LiChrospher 100 C-18 (250×4 mm, 5 μm). The mobile phase was CH_3CN/water (65:35, v/v) containing 0.75% acetic acid at 1.0 ml/min. Injected volume: 20 μl. Fluorometer wavelengths: excitation (292 nm) and emission (425 nm).

thecenes studied and the attained resolution is good. This also occurred with the other samples of contaminated rice and corn. Table 3 lists the levels of the four trichothecenes found in the samples of inoculated cereals.

The method proposed here 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 other alternative, published methods for type A trichothecenes that use GC-ECD [18,19], and needs lower analysis time as in only 12 min DAS, NEO, T-2 and HT-2 toxin can be eluted, detected and quantified; other related trichothecenes may require some minutes more [30]. However, a derivatisation step is also necessary as usual for GC determinations. The proposed method is much more sensitive than other HPLC methods that use UV detection for these trichothecenes [24,33,34] and it is perfectly suitable for the determination of type A trichothecenes in contaminated cereals. The stability of the derivatives is sufficient to allow the usage of automatic injectors. Although coumarin-3-carbonyl chloride is not commercially available at this time and must be synthesised in the laboratory, preparation is not difficult and the solid reagent can be used for 2 months at least.

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Table 3
Levels of type A trichothecenes found in corn and rice cultures of three *F. sporotrichiodes* strains[a]

F. sporotrichioides strain	Substrate	Moisture level (%)	Trichothecenes (μg/g)			
			T-2 Toxin	HT-2 Toxin	DAS	NEO
FSp1	Corn	35	0.221	0.120	0.039	0.040
	Corn	45	0.030	0.120	0.055	0.031
	Corn	55	0.039	0.058	0.030	tr
	Rice	35	2.44	0.065	tr	tr
	Rice	45	0.132	0.126	0.114	0.042
	Rice	55	0.290	0.106	tr	0.030
FSp3	Corn	35	1.31	0.047	0.286	0.073
	Corn	45	0.671	0.089	0.059	0.063
	Corn	55	2.12	0.086	0.883	0.097
	Rice	35	1.85	0.045	0.254	0.321
	Rice	45	3.31	0.049	0.088	tr
	Rice	55	2.78	0.096	0.033	tr
FSp6	Corn	45	0.030	0.120	0.055	0.031
	Corn	55	0.039	0.058	0.030	tr

[a] Data are mean values of measurements made in triplicate. tr, trace (<0.030 mg/g).

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