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Short Communication

Identification of mycotoxins in keratomycosis-derived *Fusarium* isolates by gas chromatography–mass spectrometry

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ABSTRACT

The production of mycotoxins from *Fusarium* species has been demonstrated in isolates cultured from patients suffering from keratomycosis. The method employed a combination of thin-layer chromatography directly performed on gel plugs taken from the growth medium, cartridge column chromatography, silylation and gas chromatography on a non-polar stationary phase capillary column linked to mass spectrometry. The sensitivities of detection obtained for a signal-to-noise ratio of 33:1, were 200 pg for single stage GC–MS and 20 pg using tandem GC–MS–MS. Two mycotoxins, diacetoxyscirpenol and T-2 toxin were identified in three cultures.

INTRODUCTION

Keratomycosis is an ulcerative fungal corneal infection and a number of *Fusarium* species have been isolated from patients with this condition. *Fusarium solani* and *F. oxysporum* have been reported as the commonest isolates from keratomycosis occurring in many tropical and sub-tropical areas of Asia, South America and Africa [1–4]. Infection by *Fusarium* species is frequently characterised by rapid corneal sloughing and marked visual loss and is difficult to treat [5]. Investigations of these infections have been restricted to identification of the *Fusarium* species present and there are no reports of the toxins that these fungi are known to produce, most commonly, when present in contaminated foodstuffs [6–8]. In humans and animals mycotoxins have been identified in blood, urine and faeces in cases where mycotoxicoses have occurred after ingestion of contaminated food [9–12]. We report here the first identification of *Fusarium* toxins in isolates taken from patients suffering from keratomycosis, employing thin-layer chromatography followed by gas chromatography combined with mass spectrometry and tandem mass spectrometry.

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EXPERIMENTAL

Fusarium cultures

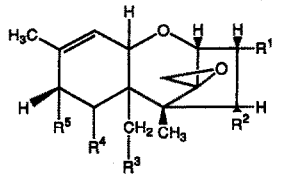
The *Fusarium* isolates were cultured from corneal scrapes of patients suffering from keratomycosis provided by Dr P.A. Thomas, Institute of Ophthalmology, St Joseph's Eye Hospital, Tiruchirapalli, India. The organisms were three point incubated on YES agar and grown at 25°C for 14–30 days. The YES agar contained yeast extract (Oxoid, Unipath, Basingstoke, UK) 20 g, sucrose (Sigma, Dorset, UK) 150 g, agar No. 1 (Oxoid, Unipath, Basingstoke, UK) 20 g and water 1 l, which was autoclaved at 121°C for 15 min. The three isolates cultured were Cu 90 (*F. solani*), Cu 57/88 (*F. solani*) and Cu 248 (*F. oxysporum*).

Thin-layer chromatography

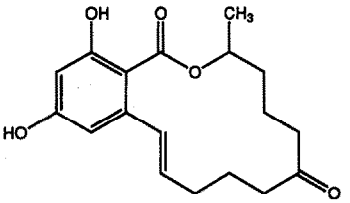
This was performed by the agar plug method [13] in which agar plugs were cut from the centre of a colony grown on YES agar with a flamed stainless steel tube (0.4 cm I.D.) and placed directly on a pre-coated silica-gel plate (BDH, Dagenham, Essex, UK). The plugs were removed after 30 s and the application areas were allowed to dry. The unknowns were run against a standard reference mixture of T-2 toxin (T-2), diacetoxyscirpenol (DAS), deoxynivalenol (DON), nivalenol (NIVA), fusarenone-X (FUS-X) and zearalenone (ZEA) (Fig. 1). All standards were obtained from Sigma (Dorset, UK). The TLC plates were developed in toluene–ethyl acetate–90% formic acid (5:4:1, v/v/v) and the toxins were detected by spraying firstly with 20% aluminium chloride in 60% aqueous ethanol followed by heating at 110°C for 20 min to produce fluorescent products (UV at 254 nm) for DON, NIVA and FUS-X. The plate was then sprayed with 20% aqueous sulphuric acid followed by a second heating at 110°C for 10 min to detect T-2 and DAS, again as fluorescent products; Underivatised ZEA fluoresces when irradiated with UV at 254 nm [14].

Extraction and derivatisation

The regions of the TLC plates developed from extracts of the *Fusarium* cultures, corresponding



Compound	R1	R2	R3	R4	R5
T-2	OH	OAc	OAc	H	i-C_4H_9COO
DAS	OH	OAc	OAc	H	H
DON	OH	H	OH	OH	=O
NIVA	OH	OH	OH	OH	=O
FUS-X	OH	OAc	OH	OH	=O



ZEA

Fig. 1. Structures of fusarium toxins discussed in text.

to the R_F values of the standards, were combined and added to a pre-washed (dichloromethane–methanol, 8:2, 5 ml) Bond Elut Silica cartridge column (Jones Chromatography, Mid Glamorgan, UK). The toxins were eluted with 5 ml dichloromethane–methanol (80:20, v/v). This eluate was evaporated to dryness on a rotary evaporator and dissolved in 100 μl acetonitrile. Trimethylsilyl ethers were prepared by adding 50 μl BSTFA + 1% TMCS (Pierce and Warriner, Cheshire, UK) and heating at 60°C overnight. The reagents were removed *in vacuo* and the residue dissolved in *n*-heptane containing 1% BSTFA.

GC–MS analysis

GC separations were carried out on a Restek Rtx_1 capillary column (30 m × 0.32 mm I.D., film thickness 0.25 μm; Thames Chromatogra-

phy, Maidenhead, UK) with a temperature programme of 150°C held for 2 min followed by a gradient to 300°C at 20°C/min. Samples were injected onto a temperature controlled Gerstel (Thames Chromatography) capillary injector. The column was installed in a Hewlett-Packard 5890 GC oven coupled directly to the source of a VG Analytical tandem hybrid mass spectrometer (Fisons Instruments, Manchester, UK). All spectra were obtained in positive electron ionization (EI) mode at 40 eV ionisation energy. Selected ion recording (SIR) was carried out by monitoring an appropriate number of ions in the time window of elution of the target toxin from the GC column. Tandem mass spectrometry data was obtained by multiple reaction monitoring (MRM) of the two ions corresponding to an appropriate characteristic decomposition of the derivatised toxin. The collision energy was 20 eV (laboratory frame of reference) and the collision gas was xenon introduced at such a pressure as to attenuate the parent ion beam intensity by 50%.

RESULTS AND DISCUSSION

Fusarium-derived toxin analysis has been performed by GC [15,16], HPLC [17,18], GC–MS [19–21] and LC–MS [22,23]. Limits of detection reported vary from 100 to 500 pg/μl. In this study we have developed a method for the rapid screening of growth medium for the presence of some of these toxins at high sensitivity and selectivity of detection. We chose to employ the trimethylsilyl ethers as the analytical derivatives on account of their ease of preparation; the spectra and GC retention times of these are summarised in Table I. In order to achieve both high sensitivity and confidence of detection we chose to employ selected ion recording using the first stage of the mass spectrometer and multiple reaction monitoring using both stages of the hybrid tandem instrument, at the ions shown in Table II. For authentic samples of both T-2 and DAS we have obtained limits of detection of 200 pg for SIR and 20 pg for MRM for a signal-to-noise ratio of 33:1. This improvement in detection limits is common in biological samples where there is ap-

TABLE I

EI MASS SPECTRAL DATA AND GC RETENTION TIME OF STANDARD TOXINS

Compound	Retention time (min)	Significant ions (m/z^a)
$DON(TMS)_3$	9:14	512(10.0), 497(4.7), 422(9.3), 326(6.0), 236(13.4), 207(12.7), 170(31.3), 103(20.0).
DAS(TMS)	9:38	378(56.6), 350(26.7), 290 (14.7), 255(9.3), 213(11.3), 175(31.4), 124(36.0), 106 (73.4).
$FUSX(TMS)_3$	9:43	570(6.0), 555(5.3), 480(12.0), 369(42.0), 251(10.6), 170 (47.3), 132(36.7), 117(35.0).
$NIVA(TMS)_4$	10:53	600(15.3), 585(42.0), 526(7.3), 483(27.3), 307(28.6), 207 (66.7), 117(26.6).
T-2(TMS)	11:38	436(18.6), 350(23.4), 290 (10.7), 173(17.3), 157(13.3), 122(42.0), 105(23.4).
$ZEA(TMS)_2$	12:12	462(32.0), 447(18.7), 333 (29.9), 305(21.9), 260(20.6), 151(46.0).

[a] Values in parentheses are relative intensities.

TABLE II

IONS SELECTED FOR SIR AND MRM EXPERIMENTS OF STANDARD TOXINS

Compound	Ions selected for SIR (m/z)	Ion species	Ions selected for MRM (m/z)
$DON(TMS)_3$	512	M^+	512 → 422
	422	$M - Me_3SiOH$	
DAS(TMS)	378	$M - CH_3COOH$	378 → 350
	350	$378 - C_2H_4$	
$FUSX(TMS)_3$	570	M^+	570 → 555
	555	$M - CH_3$	
$NIVA(TMS)_4$	600	M^+	600 → 585
	585	$M - CH_3$	
T-2(TMS)	436	$M - C_4H_9COOH$	350 → 290
	350	$436 - C_2H_4$	
	290	$350 - CH_3COOH$	
$ZEA(TMS)_2$	462	M^+	462 → 447
	447	$M - CH_3$	

preciable chemical noise arising from the matrix. A combination of TLC with sample application by the agar plug technique, followed by a silica cartridge clean-up, provided sufficient purification for subsequent GC–MS analysis. This procedure avoided any lengthy solvent extraction of the growth medium.

The *Fusarium* isolates investigated here showed unambiguous evidence of the presence of DAS and T-2 toxins in all three isolates. The SIR and MRM data for these target analytes is shown in Figs. 2 to 5. While no rigorous quantitative estimation was made, we estimate that low picogram quantities are being extracted from each plug of medium.

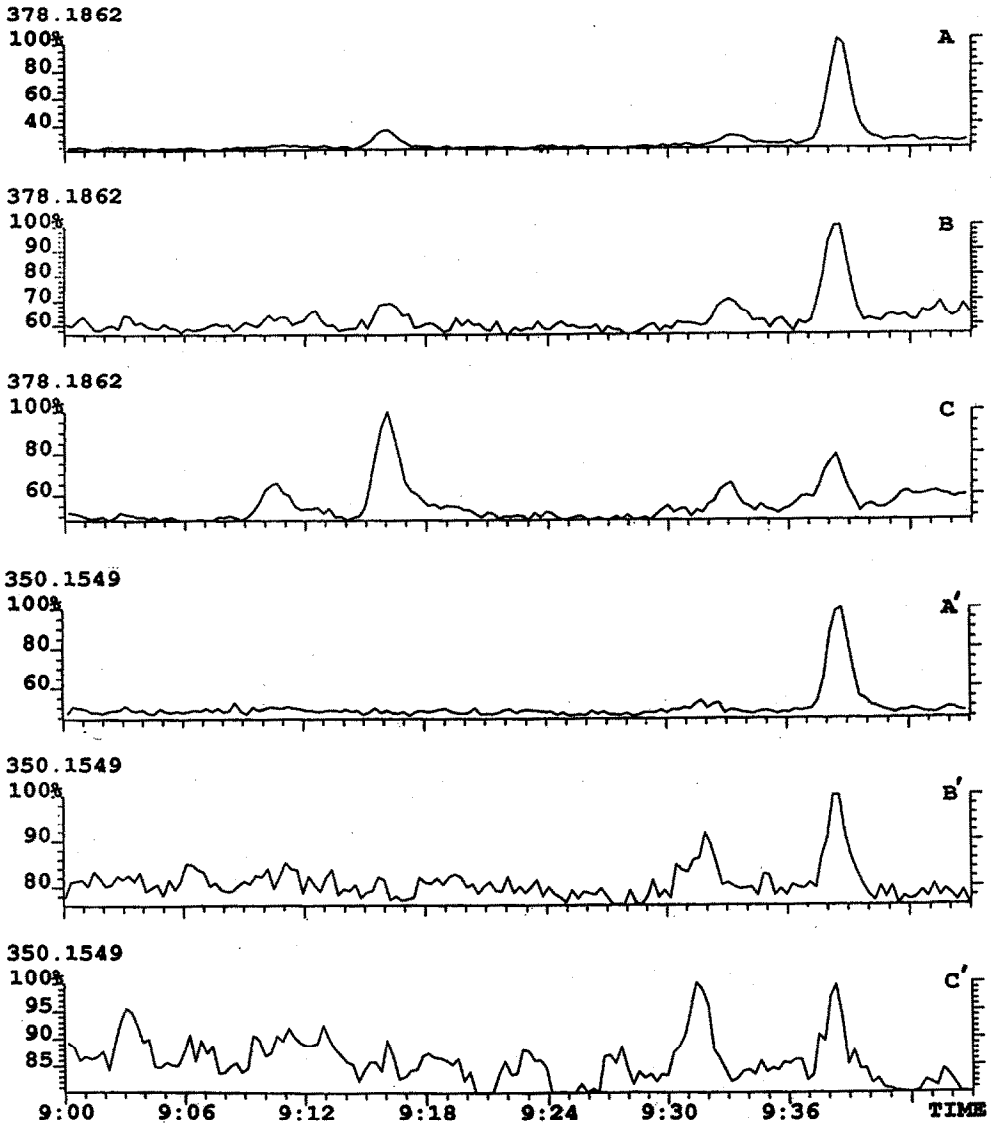


Fig. 2. SIR chromatogram plots for DAS(TMS) (m/z 378 and 350) for Cu 90 (A,A'), Cu 57/88 (B,B') and Cu 248 (C, C').

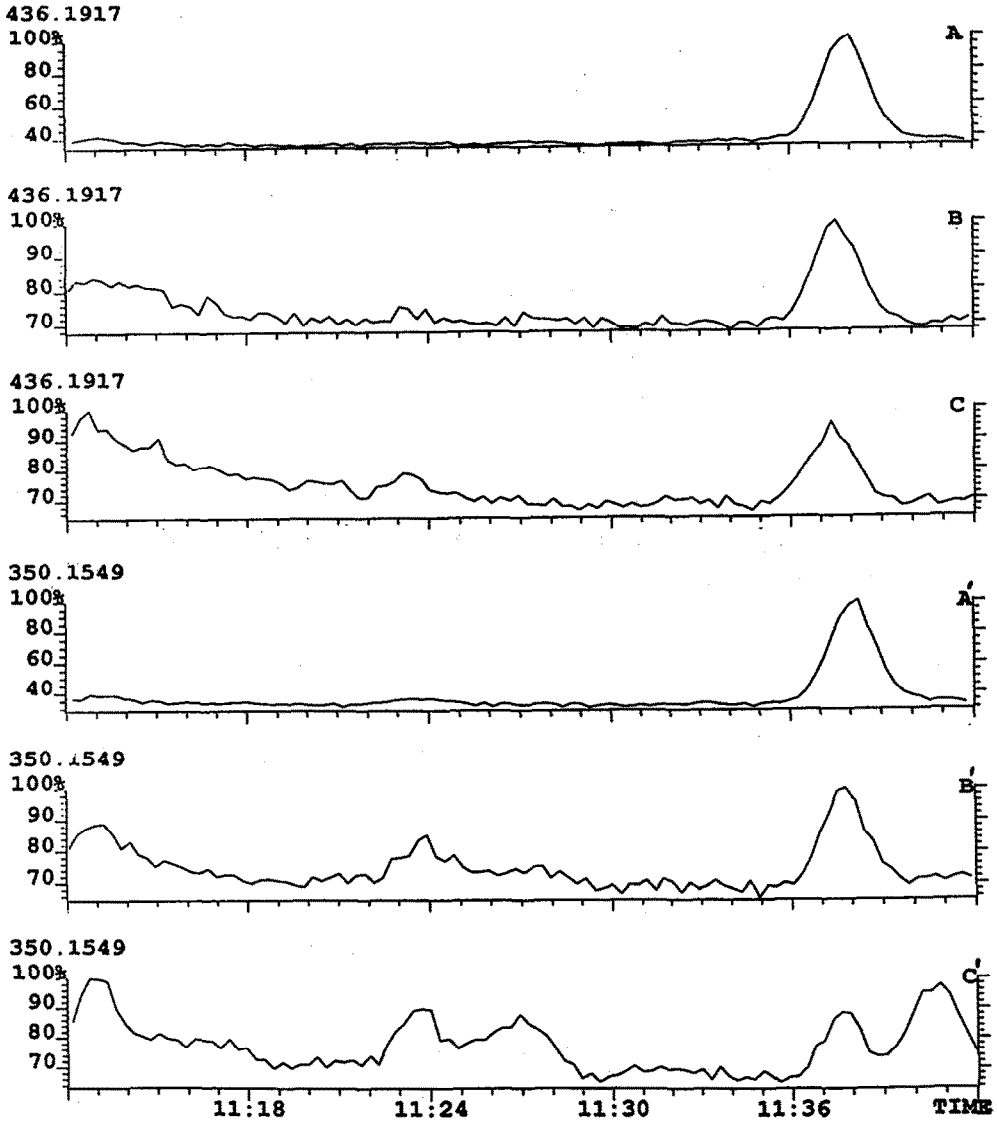


Fig. 3. SIR chromatogram plots for T-2(TMS) (*m/z* 436 and 350) for Cu 90 (A,A'), Cu 57/88 (B,B') and Cu 248 (C,C').

We believe that this is the first report of the production of these toxins in ocular *Fusarium* isolates taken from infected patients and are proceeding to characterise a larger number of *Fusarium* isolates, details of which will be reported elsewhere.

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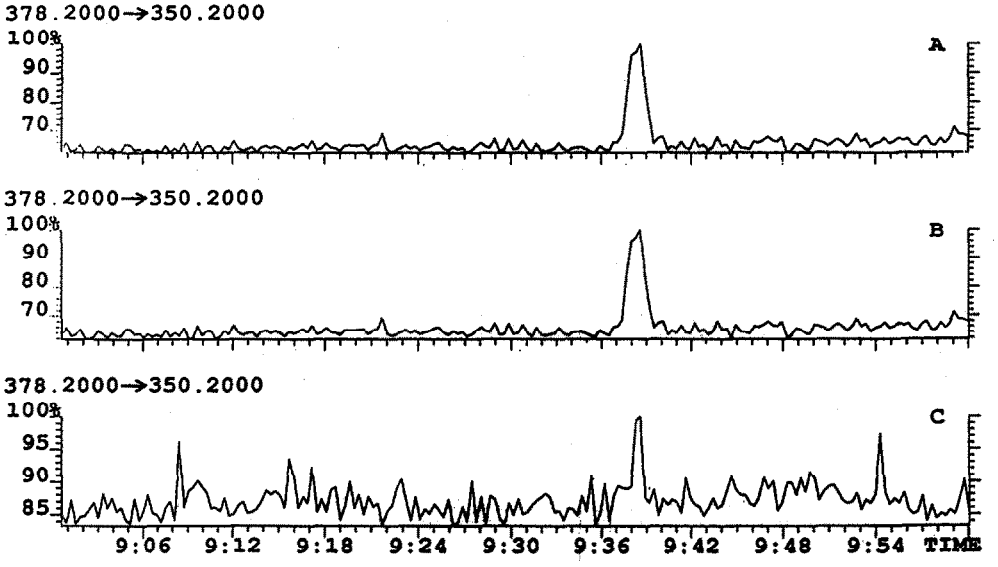

Fig. 4. MRM chromatogram plots for DAS(TMS) (378 → 350) for Cu 90 (A), Cu 57/88 (B) and Cu 248 (C).

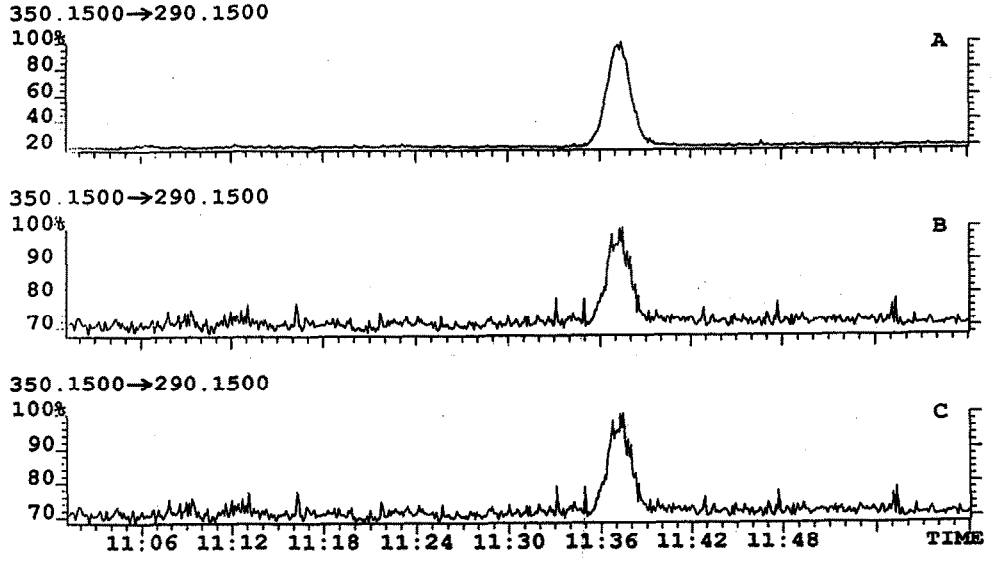

Fig. 5. MRM chromatogram plots for T-2(TMS) (350 → 290) for Cu 90 (A), Cu 57/88 (B) and Cu 248 (C).

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