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Fast methods for screening of trichothecenes in fungal cultures using gas chromatography–tandem mass spectrometry

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Abstract

The paper presents a fast method for trichothecene profiling and chemotaxonomic studies in species of *Fusarium*, *Stachybotrys*, *Trichoderma* and *Memnoniella*. Micro scale extracted crude *Fusarium* extracts were derivatised using pentafluoropropionic anhydride and analysed by gas chromatography with simultaneous full scan and tandem mass spectrometric detection. It was possible to monitor for up to four compounds simultaneous, making detection of acetyl T-2 toxin, T-2 toxin, HT-2 toxin, T-2 triol, T-2 tetraol, neosolaniol, iso-neosolaniol, scirpentriol, 4,15-diacetoxyscirpenol, 15-acetoxyscirpenol, 4-acetoxyscirpentriol, nivalenol, fusarenon-X, deoxynivalenol, 15-acetyl-deoxynivalenol and 3-acetyl-deoxynivalenol possible during a 23-min GC run. A slightly modified method could detect trichothecenes produced by *Stachybotrys*, *Memnoniella* and *Trichoderma*, by hydrolysing crude extracts prior to derivatisation with heptafluorobuturyl imidazole. All types of derivatised extracts could be reanalysed using negative ion chemical ionisation (NICI) GC–MS for molecular mass determination and verification purposes. A retention time index could be used for correction in retention time drifts between sequences and worked both in EI^+ and NICI mode.

Keywords: Trichothecenes; Satratoxin; Mycotoxin

1. Introduction

The trichothecenes (TR) are one of the most important groups of mycotoxins, and include more than 200 compounds, divided into the following four groups [1]: (i) type A trichothecenes (TA) are described as the trichothecenes not included in the following three groups; (ii) type B trichothecenes (TB) are characterised by the C_8 ketone group (Fig. 1) and are approximately only 10% as toxic as the type A trichothecenes [1]; (iii) type C trichothecenes are characterised by a ring from R^2 to the R^3 alcohol group (Fig. 1) and are generally named the macrocyclic trichothecenes. These are highly cytotoxic, and are produced by *Stachybotrys chartarum* and species of *Myrothecium* [2]; (iv) the very rare type D trichothecenes are characterised by a $C_{7,8}$ or $C_{9,10}$ epoxy group.

Analysis for TR is a complex task if specific detection of low levels of the toxins is needed. Usually analysis has employed four basic steps:

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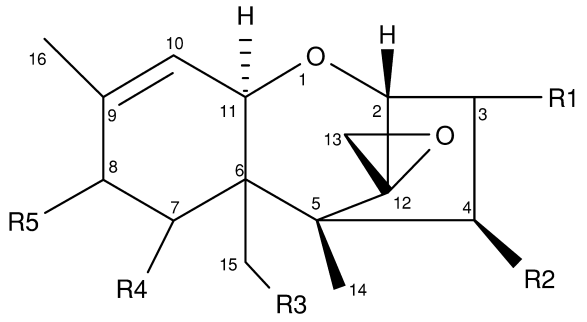

Fig. 1. The trichothecene skeleton.

extraction; solid-phase extraction (SPE) clean up; and a combined separation and detection step [3,4].

Generally, detection of TR has been performed by ELISA, thin-layer chromatography (TLC), liquid chromatography (LC) or gas chromatography (GC). LC combined with single UV or diode array detection (DAD) gives no specificity for TA all having end-absorption (λ_{max}<200 nm) and low specificity for TB having unspecific UV spectra with $\lambda_{max}\approx$225 nm [5], making mass spectrometry (MS) the only specific detection method for LC [6].

GC has been the most widely used method, but it requires that free alcohol groups are derivatised [4], although methods without derivatisation have been published [7]. Two groups of derivatives have been applied, the trimethylsilyl ethers, commonly used for TB and the perfluoro-esters which have been commonly used for TA [8–11]. The use of MS detection of the pentafluoropropionyl (PFP) or heptafluorobuturyl (HFB) esters gives the highest sensitivity if the MS have a mass range $>m/z$ 800 [4]. These derivatives can be formed by the imidazoles PFPI and HFBI, respectively, or the acid anhydrides PFPA and HFBA, respectively. The latter needs a nucleophilic catalyst as triethylamine, dimethylaminopyridine or imidazole [4]. Unfortunately TB reacts slowly with both types of reagents and can undergo isomerisation yielding more than one peak [9,10]. The fluorinated derivatives can be detected selectively to very low levels using negative ion chemical ionisation (NICI) MS or electron-capture detection (ECD), but also positive electron impact ionisation (EI^+) or positive chemical ionisation (PCI) have been used [12,13].

The type C TR can be detected directly by GC–MS as their trimethylsilyl ethers, but not as their thermally labile TFA, PFP or HFB esters [11].

The TR-producing fungal species are found within the genera *Fusarium*, *Stachybotrys*, *Memnoniella*, *Trichothecium*, *Trichoderma* and *Myrothecium*. In focus are *Fusarium* species as their toxins are of great economical importance for agriculture and food industry, whereas *Stachybotrys* growth in water-damaged buildings is becoming an economic problem for the building industry [14].

Deoxynivalenol (DON) is of major concern and is produced by *F. culmorum* and *F. graminearum* [15] often with smaller quantities of the acetylated derivatives, 3ADON or 15ADON [16–18]. A recently described species, *F. pseudograminearum* (formerly *F. graminearum* Group 1 [15,19]), is reported to produce DON and 3ADON [18].

The oxygenated counterpart to DON, nivalenol (NIV), is also produced by *F. culmorum* and *F. graminearum* together with an acetylated derivative, fusarenon X (FX) [15,16,18]. The general opinion is that two chemotypes (DON/ADON and NIV/FX, respectively) of *F. culmorum* and *F. graminearum* exist [16,20,21]; however, some isolates have been reported to produce both DON and NIV [17,18]. A morphologically quite similar species, *F. crookwellense*, produces NIV and FX [16]. Other NIV- and FX-producers are *F. poae* [22,23], *F. equiseti* [23] and *F. kyushuense* [24].

T-2 toxin (T-2) is consistently produced by *F. sporotrichioides*, *F. acuminatum* ssp. *armeniacum* and an undescribed species ''powdery *F. poae*'' [15,25], but also individual strains of *F. sambucinum*, and *F. musarum* have been reported as T-2 producers [26,27]. T-2 producers do also produce HT-2, T-2 tetraol and neosolaniol (NEO) [25]. Diacetoxyscirpenol (DAS) and the related monoacetylated derivatives, 3MAS, 4MAS, and 15MAS as well as scirpentriol (SCR) are produced by *F. sambucinum*, *F. venenatum*, *F. poae*, ''powdery *F. poae*'' and *F. equiseti* [25,26,28]. However, as DAS biosynthetically is at a side branch of the T-2 toxin pathway [29], lower amounts of DAS have also been detected in many T-2 producers.

S. chartarum isolates can be divided into two groups: (i) those capable of producing the highly cytotoxic macrocyclic TR (yielding verrucarol upon hydrolysis) on most solid substrates; and (ii) those

producing atranones and often low quantities of the simple TA, trichodermol and its acetate, trichodermin [30]. The related species, *Memnoniella echinata*, is producing trichodermin [31]. *Trichoderma* spp. are often cited as TR producers but only a very few isolates actually producing TR are available in culture collections [32–34].

The objective of the present work was to develop a fast method for specific detection of TR in fungal cultures. The analytical procedure should be usable with the currently used micro-scale extraction procedure [35] and data files should be processed automatically if possible.

The present work demonstrates that TR from *Fusarium*, *Stachybotrys*, *Memnoniella* and *Trichoderma* can be detected by derivatisation of crude or hydrolysed extracts, which are analysed using GC with simultaneous MS and MS–MS detection.

2. Experimental

2.1. Chemicals

For retention time index [36], *n*-alkylbis(trifluoromethyl)phosphine sulphides (0.25 m*M* in hexane) $n=6, 8, \ldots, 20$ (RI 600, 800, . . . ,2000) were obtained from HNU-Nordion (Helsinki, Finland), and diluted to 1% in toluene. Analytical-grade acetone, toluene, NaOH, and $NaHCO_3$ were obtained from Merck (Darmstadt, Germany). Gradient-grade methanol (MeOH) and acetonitrile were obtained from Rathburn (Walkerburn, Scotland). Analytical-grade pentafluoropropionic anhydride (PFPA), heptafluorobuturylimidazole (HFBI) and imidazole were obtained from Sigma (St. Louis, MO, USA). 1,12-Dodecanediol was obtained from Fluka (Buchs, Switzerland). 1,10-Decanediol, 1,14-tetradecanediol and 1,16-hexadecandiol were obtained from Aldrich (Steinheim, Germany).

Source of standards: trichodermin was provided by Dr. Jytte Hansen, Løvens Kemiske Fabrik (Ballerup, Denmark). Acetyl T-2 toxin (AT-2), iso T-2 toxin (iso T-2), T-2 triol (T-2TR), T-2 tetraol (T-2TE), scirpentriol (SCR), 3-α-acetyl-diacetoxyscirpenol (3αDAS), 15-monoacetoxyscirpenol (15MAS), trichothecolone (TRO), neosolaniol (NEO), verrucarol (VER), 15-acetyl-deoxynivalenol (15ADON) and 3-acetyl-deoxynivalenol (3ADON) were obtained from Sigma. A mixture of 500 μg of each, DAS, T-2, HT-2 toxin (HT-2), NEO, NIV, FX and DON, were purchased from Romer Labs (Union, MO, USA).

All trichothecene standards were dissolved in acetonitrile at a final concentration of 0.05–1 μg/ml. Trichodermol (TRI) was derived by hydrolysing trichodermin. Alkane diols for internal standards were dissolved in MeOH at a final concentration of 5–10 μg/ml.

2.2. Sample preparation

Fungal cultures were identified by conventional methods [18,37,38] and are deposited in the IBT Culture Collection, BioCentrum-DTU, Technical University of Denmark, Kgs. Lyngby, Denmark. Several strains included in the present study were analysed in The European *Fusarium sambucinum* project [26,39]. For preparation of extracts, strains were cultivated on agar substrates for 14 days at 25°C [37].

Extracts were prepared by one of following two methods used for our LC–DAD metabolite profiling. These extracts have been stored at −20°C for later analysis:

(1) The method of Frisvad and Thrane [5] using 12 agar plates, three each of yeast extract sucrose agar (YES), Sigma yeast extract sucrose agar (SYES), oat meal agar (OAT) and potato sucrose agar (PSA). These were made in the years 1985 to 1995. Subsamples of 50 μl of the about 2 ml remaining extracts were used in this study.

(2) A modified version of the micro-extraction method Smedsgaard [35]. From colonies of *Fusarium* and *Trichoderma* on YES, SYES or PSA agar and *Stachybotrys* and *Memnoniella* on PSA agar 10 plugs of 6 mm diameter were extracted (approximately 3 cm^2 surface). Subsamples of 100 μl of the 300 μl remaining extracts of these were used.

2.2.1. Derivatisation of Fusarium extracts

Subsamples were transferred to 2-ml vials together with 100 μl internal standard solution (1,10-decanediol, 10 μg/ml in MeOH), evaporated to *absolute dryness* at 1 mbar, 1300 rpm, and 35°C in a rotational vacuum concentrator (RVC) from Christ

(Osterode, Germany) and derivatised using the method of Langseth and Rundberget [4]. Briefly, an aliquot was redissolved in 500 μl acetonitrile–toluene (15:85, v/v) containing 0.4 *M* imidazole and 100 μl PFPF, the vial was screw capped and heated to 60°C for 1 h. After the vial had cooled to room temperature, the toluene phase was washed with 1 ml 5% $NaHCO_3$ (5°C) and 1 ml water before it was transferred to an auto-sampler vial.

2.2.2. Hydrolysis and derivatisation of Stachybotrys, Memnoniella and Trichoderma extracts

Subsamples were transferred to a 2-ml vial together with 100 μl internal standard solution (1,12-dodecanediol, 10 μg/ml in MeOH), and evaporated to dryness in the RVC. An aliquot was redissolved in 200 μl 0.2 *M* NaOH in MeOH and placed at room temperature overnight. Samples were evaporated in the RVC, redissolved in 500 μl water and then extracted twice with 800 μl dichloromethane. The combined dichloromethane phases were evaporated to *absolute dryness* in a 2-ml vial. The aliquot was redissolved in 200 μl acetonitrile–toluene (1:4, v/v), 15 μl HFBI were added, the vial was screw-capped and heated to 70°C for 1 h. After the vial had cooled to room temperature, 500 μl water were added and the upper phase transferred to an auto-sampler vial.

2.3. Instrumentation

A GCQ (Finnigan Corporation, Austin TX, USA) integrated GC–MS^n system (ion trap with external ionisation) fitted with the high-temperature ion source was used.

Injection of 1.0 μl was performed by a Finnigan A200S autosampler, splitless (45 s, split 1:40) with hot needle, 2 s in a 4-mm I.D. Focusliner with glass wool (SGE, Ringwood, Australia) at 280°C on a 0.25-mm, 0.10-μm, 30-m HP-5 Trace column (Hewlett-Packard, Avondale, PA, USA). The syringe was washed with acetone 10 times before and 15 times after injection. GC program: 80°C for 1 min, 40°C/min to 160°C, then 4°C/min to 205°C, then 8°C/min to 240°C, then 40°C/min to 300°C holding 3 min. Helium of a purity of 99.999% (local supplier) was used as carrier gas at a constant linear gas velocity of 40 cm/s. Transfer line temperature was 275°C.

The GCQ was controlled from Xcalibur v. 1.2 (Finnigan) and operated in the electron impact mode (EI^+) 180°C. Trap settings: automatic gain control of 200 (arbitrary unit, range 1–300), and high mass adjustment set at automatic, multiplier voltage was set 200 V higher than the auto-tune value (gain of $3–3.5\times10^5$). The three lenses between the ion-volume and the trap were tuned to maximum response of the m/z 614 ion from the CF34 calibration gas. For MS–MS the parent ions were isolated $\pm m/z$ 0.5 (the narrower the band, the more fragmentation will be induced) and MS–MS was performed at a q value of 0.225 (arbitrary unit), with a CID voltage described in Tables 1 and 2.

An analysis was separated into nine segments (*Fusarium* extracts) as seen in Table 1 or two segments (*Stachybotrys*, *Trichoderma* and *Memnoniella* extracts) as seen in Table 2. Each segment consisted of repetitive: full scan (m/z 200–900) and 2–4 MS–MS scans for target compounds eluting during the segment (with a total scan time of 1 s), as seen in Tables 1 and 2, and illustrated in Figs. 2 and 3.

Samples were occasionally re-analysed by GC–NICI–MS for verification of molecular mass of unknown compounds. In this case the GCQ was operated in the NICI mode at 70–120°C using methane with a purity of 99.95% (local supplier) as reagent gas [40].

Before every major sequence (30–60 samples), the septum was changed and the column ''baked out'' at 320°C for 1 h, the ion volume in the MS changed, and the MS tuned. After three to four sequences the liner was replaced and 30 cm was cut off the column.

2.4. Data analysis

The individual components were detected in the extracted ion chromatogram of one of the most intense the daughter ions, as noted in bold in Tables 1 and 2. Ion ratio confirmation was performed using two to four ions, in a window ±20 s from the retention time of the reference compound, using the automated peak detection and integration part of the Xcalibur software.

2.5. Quantitative analysis

The use of different quantities of a mixture of the

Table 1
MS–MS settings in the multiscan analysis for *Fusarium* trichothecenes

	M^{+} [b] (u)	Parent ion (m/z)	CID [c] (V)	MS–MS scan (m/z)	Retention index [d]	Scan time (min) [e]	Daughter ions m/z (%), ion used for peak detection in bold
C10diol-PFP_2(I.S.)	466	347	1.3	150–350	1067	3.0–4.3	**183(100)**, 165(70)
NIV-PFP_4	896	896	1.1	270–900	1279	4.3–5.5	**471**(100), 459 (41), 279(38), 307(38), 635(26), 896 (20)
Scirpentriol-PFP_3	720	705	1.1	200–710	1396	5.5–7.4	705 (50), **541**(100), 675(20), 377(15)
DON-PFP_3	734	734	1.1	200–735	1418	5.5–7.4	309(100), **473**(67), 281(35), 447(19), 637(14)
T-2 tetraol-PFP_4	882	719	1.3	200–720	1422	5.5–7.4	719(100), **555**(20), 691(10)
FX-PFP_3	792	792	1.1	275–796	1525	7.4–8.1	489(100), **695**(67), 307(33), 325(29), 471(24), 635(10)
Trichothecolone-PFP	410	410	1.1	200–415	1567	8.1–9.0	**353**(100), 368(80), 341(34)
4MAS-PFP_2 [a]	616	452	1.1	200–455	1584	8.1–9.0	229(100), **392**(75), 271(70), 377(60), 246(47)
15MAS-PFP_2	616	556	1.3	200–560	1591	8.1–9.0	**541**(100), 393(85), 556(80), 528(64)
15-Acetyl-DON-PFP_2	630	542	1.1	200–545	1634	9.0–11.0	**379**(100), 378(35), 527(20), 542(15)
3-Acetyl-DON-PFP_2	630	588	1.1	200–590	1640	9.0–11.0	**260**(100), 411(20), 424(18)
T-2 triol-PFP_3	820	719	1.3	200–720	1770	11.0–14.0	719(100), **555**(35)
Neosolaniol-PFP_2	674	451	1.1	200–455	1783	11.0–14.0	**392**(100), 228(20), 452(20)
Iso-neosolaniol-PFP_2 [a]	674	451	1.1	200–450	1799	11.0–14.0	**392**(100), 452(52), 228(35), 340(24)
DAS-PFP	512	452	1.1	200–454	1811	11.0–14.0	**229**(100), 424(72), 228(65), 364(65), 246(60), 289(58), 200(53), 437(52)
HT-2-PFP_2	716	572	1.1	200–575	1934	14.0–17.0	572(100), 377(35), 408(34), **524**(33)
3-α-Acetyl-DAS	408	348	1.1	150–450	2057	14.0–17.0	195(100), **229**(80), 247(78), 288(70)
Trichothecin	332	332	1.1	150–335	2000	14.0–17.0	**264**(100), 246(50), 177(50), 195(20)
T-2 toxin-PFP	612	451	1.1	200–456	2133	17.0–22.0	**391**(100), 227(20), 245(5)
Acetyl-T-2 toxin	508	406	1.1	200–410	2227	17.0–22.0	227(100), 226(90), **346**(84), 275(40)

[a] Standard not available.
[b] Calculated.
[c] Selected $\pm m/z$ 0.5, at a q value of 0.225.
[d] Calculated versus alkylphosphine standards, see text.
[e] On a new column, has to be adjusted slightly when the column has been ''baked out'' many times.

trichothecenes, DON, 3ADON, 15ADON, FX, NIV, AT-2, T-2, T-2TR, T-2TE, SCR, 15MAS and DAS, versus constant quantities of the C_{10}-, C_{12}-, C_{14}- and C_{16}-diol was tested (to a final concentration of approximately 5 μg/ml in derivatised extract of each), 11 different levels (including one blank) four times each. Samples were analysed using both EI^{+} and NICI.

2.6. Fungal extracts

Fungal extracts prepared previously and kept in the freezer were used almost from the start of the GC and MS-method development, as the choice of eluting speed from the GC and selection of parent ions also depend on interfering substances.

About 300 extracts from *F. venenatum*, *F. cul-*

Table 2
MS–MS settings in the multiscan analysis for verrucarol and trichodermol [a]

	M^{+} [a] (u)	Parent ion (m/z)	CID [b] (V)	MS–MS scan (m/z)	Retention index [c]	Scan time (min) [d]	Daughter ions m/z (%), ion used for peak detection in bold
C12diol-HFB_2(I.S.)	594	429	1.3	200–430	1340	3.0–6.4	**211**(100)
Trichodermol-HFB	446	446	1.1	200–450	1476	3.0–16.0	**431**(100), 403 (5), 446(20)
Verrucarol-HFB_2	658	444	1.1	200–450	1520	3.0–16.0	**429**(100), 444(60), 231(40), 215(20), 320(15), 416(15)

[a] Calculated.
[b] Selected $\pm m/z$ 0.5, at a q value of 0.225.
[c] Calculated versus alkylphosphine standards, see text.
[d] On a new column, has to be adjusted slightly when the column has been ''baked out'' many times.

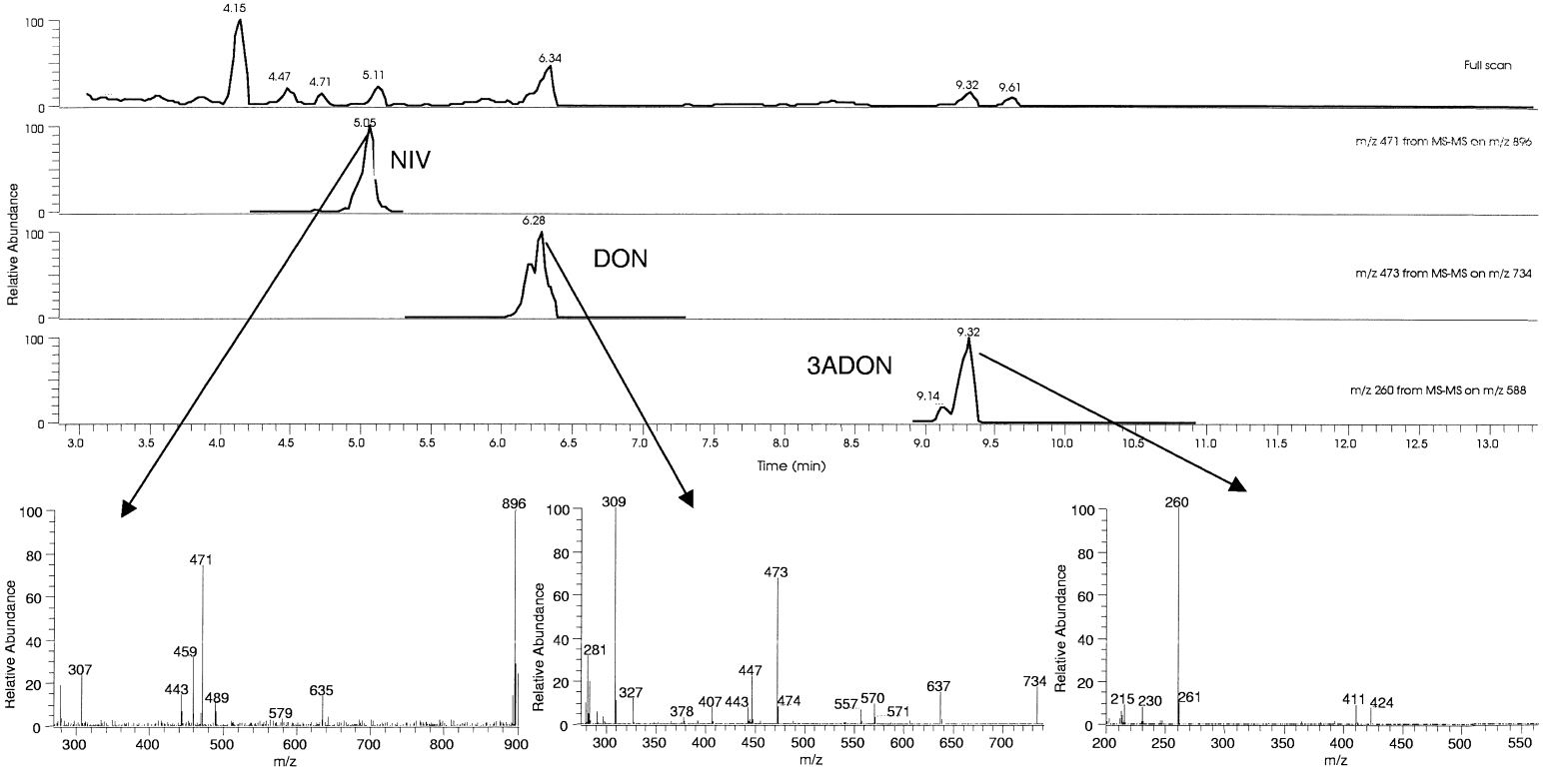

Fig. 2. Extract of *F. culmorum* IBT 9608 on YES agar. Column is partly overloaded as seen on the peak-shape.

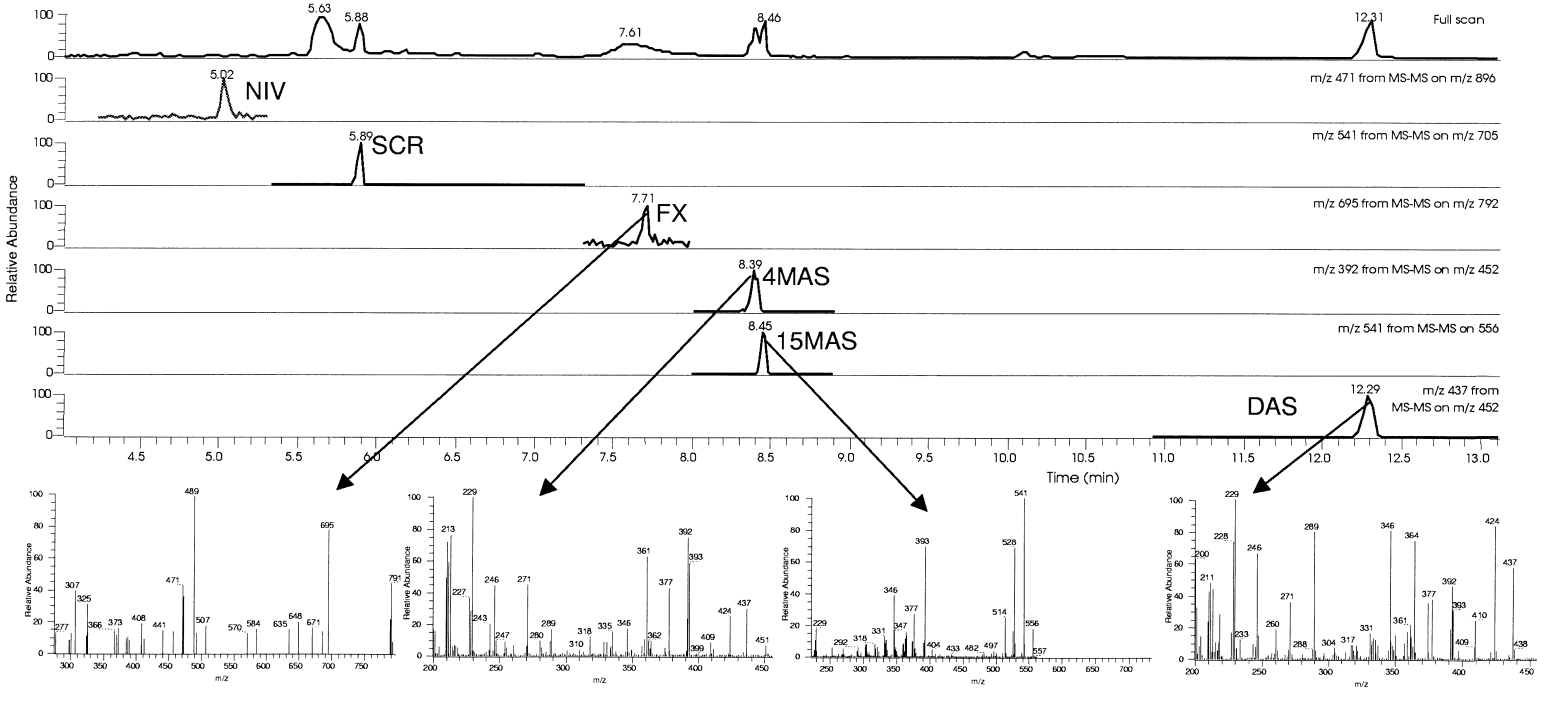

Fig. 3. Extract of *F. venenatum* BBA 64478 on PSA agar.

morum, *F. equiseti*, *F. graminearum*, *F. pseudograminearum*, *F. sambucinum* and *F. crookwellense* were analysed. All extracts have previously been analysed using LC–DAD.

In addition about 150 extracts of *Trichoderma* and 150 extracts from *Stachybotrys* and *Memnoniella* were analysed. These included about 75 extracts from *S. chartarum* isolates, which previously had been cleaned up using polyethylene imine silica and analysed by LC–DAD for the presence of macrocyclic TR [41].

3. Results and discussion

3.1. Derivatisation

3.1.1. Fusarium trichothecenes

The PFP derivatives were preferred over HFB, as NIV-HFB_4 has a mass of 1096 u, 96 u more the upper range of the GCQ (similar problem for T-2TE), and the HFB derivatisation also seems to damage the column more than the PFP derivatisation.

The method of Langseth et al. [23] was the only method capable of derivatising both TA and TB to one single product each, although very concentrated extracts of TB producing fusaria occasionally yielded a small artefact eluting 5–10 s prior to the metabolite as illustrated in Fig. 2, where it is clear for 3ADON, the split in the DON peak is probably an ion-trap problem. The method also works using heptafluorobutyric anhydride (Sigma) if the HFB derivatives should be preferred (results not shown) as also noted by others [25].

Using the method of Black et al. [9] with HFBA or PFPA in the acetonitrile–toluene containing 2 mg/ml of 4-dimethylaminopyridine (Sigma), it was impossible to fully derivatise the TB even though the time and temperature was optimised. Several products of the TB with the correct molecular mass were detected (3–4 for NIV, three for FX and DON). The addition of HFBI or pentafluoropropionic imidazole (PFPI) (Pierce, Rockford, IL, USA), respectively, to the derivatisation mixture did not overcome these problems.

The method of Nielsen et al. [40] using HFBI or PFPI in toluene–acetonitrile gave full derivatisation of the TA to one single product each, except for NEO, but was incapable of fully derivatising the TB.

3.1.2. Stachybotrys trichothecenes

We tried to pass the hydrolysed sample through a strong cat ion-exchanger (Fluka, 02497), packed in syringes for neutralisation of the NaOH [42], which was satisfactory for standards; however, with fungal extracts huge quantities of interfering peaks and irreproducible derivatisation were observed, hence the liquid–liquid extraction seems to be a powerful clean-up step. When the dry hydrolysed samples are being redissolved it is very important that the water is added before the dichloromethane, as it yields cleaner samples.

Using the method of Black et al. [9] only HFB_3-TRI was detected, and about 20–50% of the VER was derivatised to HFB_4-VER due to the rearrangement to apotrichothecene (10,13-cyclotrichothecane) [43]. This phenomena was also partly observed when using HFBA and the method of Langseth et al. [23], making the use of an imidazole based reagent necessary although it is more unstable and expensive, and less robust. The HFB derivatives of TRI and VER yielded lower detection limits than with PFP (~5×), making HFBI our choice.

3.2. Gas chromatography

The retention time of the target components decreases slightly from sequence to sequence due to column cutting as the film is distilled off due to the rough treatment, consequently the timing of the scan segments has to be adjusted slightly. The use of a column with thicker film, 0.25 μm, RX5 (Restek, Bellefonte, PA, USA), gave a two to six times higher background signal and did not give sharper peaks, making the HP-5 our choice.

The use of acetone as cleaning solvent for the syringe did not give *carry-over* in contrast to toluene, MeOH or dichloromethane which gave a *carry-over* of about 0.1–0.01‰ even if the syringe was cleaned 15 times before and after injection.

3.3. Mass spectrometry

The EI^+ full scan spectra showed concentration dependency, which is a typical ion trap problem even

on the GCQ where the ions are generated outside the trap. This combined with the sometimes high background signal due to co-eluting compounds made automatic processing impossible, due to both false-negatives and -positives. However, in the multi-scan mode, it was possible to confirm a target compound manually.

Tuning of the lenses (1 and 3) proved to be the most important tuning parameter, and in the previous software versions (Xcalibur ver. 1.0 and 1.1) they had to be tuned manually to maximum sensitivity of the m/z 614 ion, usually giving a 10-V higher potential of the transmission lenses than the auto-tune value. This gave a two to 20 times higher sensitivity for the high mass ions, especially in the full scan mode as well as increased spectral reproducibility.

3.3.1. EI^+ MS–MS

MS–MS generally provided lower detection limits due to longer ion-times (time the gate lens is open) especially in ''dirty'' extracts. The MS–MS spectra were more reproducible than the full scan spectra, and made automatic processing (AP) of the datafiles possible.

Generally the MS–MS of the PFP derivatives spectra contained two to 10 specific ions, giving a precise detection, except for T-2TR and T-2TE which had identical spectra only showing sequential loss of pentafluoropropionic acid, which is not very specific. This resulted in a few apparent false-positives (in samples with no other member of the T-2 toxin family).

HFB-trichodermol (Table 2) was very unusual, showing only two ions, m/z 446 (M^+, 20%) and 431 (100%), even when trying an extended scan range (m/z 10–700).

Detection limits at S/N 5 for the 1 μl injected samples using the most abundant ion in the MS–MS spectrum, was for AT-2, 3αDAS 70–120 pg, HFB_2-VER 20 pg, for the PFP derivatives of NIV, DON and FX about 15–30 pg, TRI about 10 pg, and for 3ADON, 15MAS, DAS, NEO, HT-2, T-2 about 30–70 pg. On pure standards such as these, full scan MS can give significant lower detection limits (~5–10×, on a new column), as 10–70% of the parent ions are lost during the isolation process of MS–MS.

Using the C_{10}–C_{16} diols as internal standards, by integrating m/z 347, 375, 403 or 431 (most abundant ion, due to loss of CF_3CF_2H), respectively, in the full chromatogram generally improved the linear standard deviation (SD) from 30–40 to 15–20%. The C_{12} and C_{14} diols gave lower SD then the C_{10} and C_{16} diols, although this was not statistically significant. Such ''poor'' quantitation is fully acceptable for our purpose where specificity is the main target.

The *n*-alkylbis(trifluoromethyl)phosphine spectra were dominated by four prominent ions: $[M-69]^+$ (loss of CF_3) always being the largest, $[M-34]^+$ (presumable loss of H_2S), then $[M-101]^+$ and $[M-103]^+$.

3.3.2. NICI analysis

The high pressure of methane, which was necessary to obtain a high sensitivity and a high m/z ion, gave a poor mass resolution of approximately m/z 0.5–1 (10% valley). This made automatic processing very difficult, as both full scan and MS–MS spectra became irreproducible.

HFB derivatives of DAS and T-2 gave ca. 25% more ions than the PFP derivatives, due to the higher number of fluorine atoms in the molecule increasing the electron capturing ability.

Fragmentation changed towards smaller ions by increasing ion volume temperature, and 90°C was a compromise. If only type B trichothecenes were analysed, ion-volume could be held at 70°C, whereas 120°C is needed for DAS, T-2, iso-T-2 and HT-2, due to condensation of these in the ion source. The very small (1 cm^3) ion volume made the peaks two to three times wider than in EI^+ analysis.

Detection limits at S/N 5 for the 1 μl injected samples using the most abundant ion in the MS spectrum, was for HFB_2-VER 5–20 pg, for the PFP derivatives of NIV, DON and FX about 10–30 pg, for 3ADON and 15MAS 20–50 pg, and for DAS, NEO, HT-2, T-2 about 50–120 pg. Quantitation yielded an SD of 40–60% using external and the different internal standards.

The only ion detected in the *n*-alkylbis(trifluoromethyl)phosphine spectra were $[M-69]^-$ ion, due to loss of one of the CF_3 groups.

NICI MS–MS was also tried and gave higher specificity, but much poorer detection limits (10 times or higher) were seen, even though less CID

voltage (0.7–0.9 V) was applied and a wider ion isolation was used ($m/z \pm 1$).

3.4. Trichothecene profiles in fungal extracts

3.4.1. Fusarium

Each isolate has often been grown on more than one agar substrate, which has resulted in more than one extract per isolate. The results have been summarised for each isolate showing a qualitative profile of trichothecenes in Table 3. In general it should be noted that all extracts originally were made for a broad screening by LC–DAD covering all metabolites. This means that those isolates where no trichothecenes were detected are not necessarily unable to produce these metabolites. The lack of detection could be due to poor growth conditions, unsatisfactory extraction procedure or storage conditions. It was not the intention in the present work to cover the variability in metabolite production among isolates of the same species. On the other hand, in an attempt to test for false-positive detection of trichothecenes, 23 extracts of *F. arthrosporioides*, *F. avenaceum*, *F. flocciferum*, *F. proliferatum*, *F. solani*, and *F. torulosum* all regarded as unable to produce trichothecenes were included. No trichothecenes were detected in any case (data not shown).

3.4.2. Fusarium crookwellense

The only trichothecene detected from this species was FX, which is in accordance with literature [16,44]. The presence of other reported trichothecenes [16,45] could not be verified due to lack of standards.

3.4.3. Fusarium culmorum

This observed trichothecene patterns fall into well-known groups (chemotypes); a group producing DON and 3ADON and a group producing NIV and FX [23]. A single isolate, IBT 9608, which has been marked as atypical by Langseth et al. [23] (as isolate 94/1098-6) did produce all four trichothecenes (see Fig. 2). This co-production of DON and NIV type metabolites is rare, but has been observed in a few European isolates of *F. culmorum* (H. Hestbjerg and K.F. Nielsen, unpublished results).

3.4.4. Fusarium equiseti

These isolates produced NIV, FX, SCR, 15MAS and DAS in various amounts, which is in accordance with other reports [23].

3.4.5. Fusarium graminearum

This species could be divided into two three groups, following Miller et al. [16]: NIV/FX producers, DON/3ADON producers, and DON/15ADON producers. In addition a few isolates produced traces of DON, DON/15ADON, or DON/3ADON together with NIV/FX. The co-production of DON- and NIV-related trichothecenes has been reported from a limited number of strains [17,46,47].

3.4.6. Fusarium pseudograminearum

The three isolates of this newly described species included in this study did produce DON and 3ADON as reported elsewhere [18]. In one of the extracts a trace amount of FX was detected; however, further examinations to clarify any co-production of DON- and NIV-related trichothecenes by *F. pseudograminearum* are needed.

3.4.7. Fusarium sambucinum

These isolates produced SCR and mono- and diacetylated derivatives thereof in various amounts, which is in accordance with other reports [26,48].

3.4.8. Fusarium venenatum

This species produces huge quantities of DAS, SCR, 15MAS and/or a component tentatively identified as 4MAS. The software sometimes failed to detect DAS, 15MAS and ''4MAS'' due to 1–3 min wide peaks, which were not detected by the peak-finder working best in a 30–50 s window. This overloading also changes the retention time of other compounds by up to 10 s. 4MAS was only found in extracts containing DAS and SCR. It eluted 15 s before 15MAS both as PFP and HFB derivatives, and showed a $[M]^-$ of m/z 616 (PFP) and m/z 716 (HBF) indicating that the compound had two free alcohol groups.

In some extracts low quantities of NIV and FX were detected (see Fig. 3). As this not been reported previously, an extract of *F. venenatum* BBA 64478 grown on PSA was loaded onto a 0.5-g C_{18} (end-capped) SPE cartridge (Supelco), activated with 10

Table 3
Trichothecenes detected in extracts of *Fusarium* cultures

Species (no. isolates examined)	Trichothecene profile[a]	No. isolates	Selected isolates in IBT Culture Collection[b]
F. crookwellense (6)	FX	3	1361, 2201, FRC-R-7162
	nd	3	
F. culmorum (19)	NIV, FX	1	9245
	FX	5	2873, 8096, 8097, 8099, FRL-F4305
	DON, 3ADON	8	1505, 2241, 2316, 8984, 9553
	NIV, FX, DON, 3ADON	1	9608
	nd	4	
F. equiseti (17)	NIV, FX, SCR, 4MAS, 15MAS, DAS	2	9567, 9570
	NIV, FX, SCR, 15MAS, DAS	2	9568, 9607
	NIV, FX, SCR, 15MAS	1	9566
	NIV, FX, SCR	1	9571
	NIV, FX, 15MAS, DAS	1	9095
	NIV, SCR, 4MAS	1	9579
	NIV	2	9076, 9513
	DAS	3	1001, 2800, FRL-F4366
	nd	4	
F. graminearum (33)	NIV, FX, DON, 15ADON	1	FRC-R-6791
	NIV, FX, DON	1	ITEM 639
	NIV, FX	4	1915, ITEM 1348, FRL-F7420, FRL-F7441
	FX	1	9203
	DON, 3ADON	8	1508, 1953, 2987, 8041, 9319
	DON, 15ADON	7	9314, FRC-R-7295, FRL-F6965, BBA 62065, NRRL 6267
	DON	2	1952, MRC 6302
	nd	9	
F. pseudograminearum (3)	DON, 3ADON	3	FRL-F5126, FRL-F5373, FRL-SU176
F. sambucinum (25)	SCR, 4MAS, 15MAS, DAS, NEO, T-2	4	1807, 8159, BBA 64995
	SCR, 4MAS, 15MAS, DAS	18	1743, 2364, 2747, BBA 64996,
	SCR, 4MAS, DAS	1	1733
	SCR, DAS, NEO, HT-2	1	BBA 62433
	nd	1	
F. venenatum (13)	NIV, FX, SCR, 4MAS, 15MAS, DAS	5	1337, 2203, 9476, BBA 64478, BBA 64757
	NIV, SCR, 4MAS, 15MAS, DAS	5	1335, 2204, 2205, BBA 64537
	SCR, 4MAS, 15MAS, DAS	2	BBA 65030, BBA 65031
	nd	1	

[a] nd, none detected, others see text.

[b] IBT Culture Collection, at BioCentrum-DTU (senior author's address), unless otherwise indicated: BBA, Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany; FRC, Fusarium Research Center, Pennsylvania State University, University Park, PA, USA; FRL, Fusarium Research Laboratory, University of Sydney, NSW Australia; ITEM, Istituto Tossine e Micotossine da Parassiti Vegetali, Bari, Italy; MRC, Programme on Mycotoxins and Experimental Carcinogenesis, Medical Research Council, Tygerberg, South Africa; NRRL, ARS Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL, USA.

ml MeOH and 10 ml water. The cartridge was then eluted with 10 ml of: water, 10% MeOH, 20% MeOH, 40% MeOH and MeOH. Using both EI^+ and NICI it was shown that the first fraction contained NIV, the second, NIV, FX and SCR, the third FX, SCR, 4MAS and 15MAS, the fourth 15MAS, 4MAS and DAS, and the last traces of 15MAS and DAS.

3.4.9. Stachybotrys

VER (originating from roridins, verrucarins and satratoxins) was detected in about 35% of all *S. chartarum* isolates tested (approximately 150) whereas the precursor, TRI, was detected in about 75% of all isolates, including 90% of the isolates yielding VER. Complete data on most of these isolates as well as LC–DAD results from the unhydrolysed extracts can be found in Andersen et al. [41].

All extracts from *S. dichroa* IBT 9772 (=CBS 182.80), IBT 9773 (=CBS 526.50) and IBT 9774 (=IHEM 17452) contained VER (originating from verrucarin J and roridin E, determined by LC–DAD) and TRI. *S. oenanthes* IBT 9473 (=CBS 252.76) produced TRI, whereas this was not detected in *S. oenanthes* IBT 9777 (=IHEM 17454). In none of the one to three isolates available of *S. parvispora*, *S. theobromae*, *S. bisbyi*, *S. albipes*, and *S. cylindrospora* were VER or TRI detected, although isolates of the latter species have been reported to produce trichodermin [49].

3.4.10. Memnoniella echinata

Isolates of *Memnoniella echinata* IBT 14914, IBT 9459 (=CBS 343.50), IBT 9782 (=NRRL 2373) produced varying quantities of trichodermin, confirming results of Jarvis et al. [30].

3.4.11. Trichoderma spp.

Out of 150 different isolates only one isolate, IBT 9471 (=ATCC 90237), produced detectable quantities of TRI, which may have partly originated from its production of harzianum A [34].

4. Conclusion

Trichothecenes in crude *Fusarium* spp. culture extracts and crude hydrolysed *Stachybotrys*, *Trichoderma* and *Memnoniella* extracts could be automatically detected in derivatised extracts by simultaneous GC–MS and GC–MS–MS analysis.

About 60 extracts, originating from small (<1 cm^2) pieces of culture can be analysed per day and with slight modifications different derivatisation and cleanup techniques can be used to verify controversial findings. Derivatised samples could also be reanalysed using GC–NICI–MS for verification and molecular mass determination.

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