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Production of trichothecene mycotoxins on water damaged gypsum boards in Danish buildings

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Abstract

Water damaged gypsum boards heavily infested with *Stachybotrys chartarum* were found in a school and in a domestic residence in Copenhagen. Samples were extracted with dichloromethane and cleaned up on Sep-Pak® C_{18} modules. The trichothecenes were detected as the heptafluorobutyrlated derivatives by gas chromatography ion trap mass spectrometry with negative ion chemical ionisation. Extracts of samples from both locations yielded verrucarol after hydrolysis, strongly indicating the presence of toxic macrocyclic trichothecenes, probably satratoxin H and G. In addition the sample from the domestic residence yielded trichodermol.

Keywords: Satratoxins: Trichothecene; Trichodermol; Phycotoxin; *Stachybotrys chartarum*; Indoor environment; Sick building syndrome

1. Introduction

Stachybotrys chartarum (including synonyms *S. atra* and *S. alternans*) is the dominating fungus on water damaged gypsum boards, one of the most extensively used building materials in Denmark. Accordingly *S. chartarum* is one of the major fungal contaminants found in water damaged buildings in Denmark (Gravesen et al., 1994).

The trichothecenes produced by *S. chartarum* (Table 1) are six macrocyclic type C trichothecenes (Ueno, 1983), satratoxin F, G, and H, roridin E, verrucarin B and J, and four nonmacrocyclic compounds, trichoverrin A and B, and trichoverrol A and B (Eppley et al., 1977, Eppley et al., 1980; Harrach et al., 1981; Harrach et al., 1983; Bata et al., 1985; Jarvis et al., 1986; Croft et al., 1986).

The very toxic satratoxins H and G have been detected from *S. chartarum* growing on building materials (Johanning et al., 1996; Nikulin et al., 1994; Croft et al., 1986).

Recently, production of the immunosuppressive spirolactones and spirolactames have been detected from *S. chartarum*, *S. bisbyi* and *S. cylindrospora* (Roggo et al., 1996; Jarvis, 1991; Ayer and Miao, 1993; Johanning et al., 1996).

S. cylindrospora produces the trichothecenes trichodermol and trichodermin, also known from *Trichoderma* spp. and *Memnoniella echinata* (Jarvis et al., 1996; Godtfredsen and Vangedal, 1965; Corley et al., 1994; Ayer and Miao, 1993).

Hydrolysis of all trichothecenes produced by *S. chartarum* results in verrucarol whereas the trichothecenes produced by *S. cylindrospora* are hydrolysed to trichodermol (Jarvis et al., 1996; Ayer and Miao, 1993). By hydrolysing the different trichothecenes to verrucarol and trichodermol respectively, the total trichothecene content is detected, instead of the specific trichothecenes (Fig. 1). This is a convenient procedure as standards of the specific trichothecenes are not commercially available.

Trichothecene analysis has usually been performed by gas chromatography (GC) of various derivatives (Konstiainen and Rizzo, 1988; Krishnamurthy and Sarver, 1986; Krishnamurthy et al., 1987; Rosen et al., 1986). GC using mass spectrometric (MS) detection with negative chemical ionisation (NCI) of the heptafluorobuturylated (HFB) derivatives is considered the most sensitive method (Begley et al., 1986; Black et al., 1986; Black et al., 1987; Konstiainen and Rizzo, 1988; Scott et al., 1989).

High performance liquid chromatography with diode array detection (HPLC-DAD) has been less successful due to the poor excitation values and unspecific UV-spectra of these trichothecenes. However, for some macrocyclic trichothecenes, HPLC methods dependent on the retention time have been reported (Stack and

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Table 1
Trichothecene producing fungi found on building materials

Genera	Species	Toxin	Hydrolysis product	
Stachybotrys	*chartarum*	Roridin E	Verrucarol	Eppley and Bailey, 1973
Stachybotrys	*chartarum*	Satratoxin H	Verrucarol	Eppley et al., 1977
Stachybotrys	*chartarum*	Verrucarin J	Verrucarol	Eppley et al., 1977
Stachybotrys	*chartarum*	Satratoxin F	Verrucarol	Eppley et al., 1980
Stachybotrys	*chartarum*	Satratoxin G	Verrucarol	Eppley et al., 1980
Stachybotrys	*chartarum*	Trichoverrol A and B	Verrucarol	Jarvis et al., 1986
Stachybotrys	*chartarum*	Verrucarin B	Verrucarol	Croft et al., 1986
Stachybotrys	*chartarum*	Trichoverrin A and B	Verrucarol	Croft et al., 1986
Stachybotrys	*cylindrospora*	Trichodermol	Trichodermol	Ayer and Miao, 1993
Stachybotrys	*cylindrospora*	Trichodermin	Trichodermol	Ayer and Miao, 1993
Memnoniella	*echinata*	Trichodermin	Trichodermol	Jarvis et al., 1996
Trichoderma	*viride*	Trichodermin	Trichodermol	Godtfredsen and Vangedal, 1965
Trichoderma	*harzianum*	Harzianum A.	Trichodermol	Corley et al., 1994
Trichoderma	*harzianum*	Trichodermin	Trichodermol	Ichinoe and Kurata, 1983
Trichoderma	*longibrachiatum*	Trichodermin	Trichodermol	Ichinoe and Kurata, 1983

Eppley, 1980; Johanning et al., 1996; Frisvad and Thrane, 1987).

The scope of this work was to determine whether the very toxic macrocyclic trichothecenes are in fact produced in water damaged Danish buildings. This is the first report regarding detection of trichothecene mycotoxins of the trichodermol type on building materials and the first report of verrucarol type trichothecenes in Denmark.

2. Building description

2.1. The school

The building was from the 1970's and built on two levels, with a flat roof with roofing felt. The roof was drained through pipes hidden in the walls. These walls were made of double gypsum boards on each side of the wall frame, with the remaining space being filled with mineral wool. The material around a single pipe had been wetted for a substantial period before the leak was discovered. The inner side of the gypsum boards were found to be heavily infested with *S. chartarum* and the outer side slightly with *Penicillium chrysogenum*, *Aspergillus versicolor* and *Scopulariopsis* sp. At the time of sampling (August 1996) the materials were dry.

2.2. Domestic residence

The house, comprising two floors and a basement, was built in the 1940's in an area where the subsoil water is high. In the basement several rooms were used for residential purposes and a bathroom was also installed. The walls of these rooms were additionally insulated with mineral wool, a vapour barrier and gypsum boards.

Behind a large cupboard and a bed, both placed close to the wall, an area of approximately 800 cm^2 was blackened by growth of *S. chartarum*.

The water could have originated from two sources: incoming water from the ground as a result of insufficient drainage around the building and/or condensation of water as a result of insufficient ventilation in the basement combined with low room temperatures. At the time of sampling (November) measurements, using a capacitance instrument (Moisture Encounter, Tramex, UK.), showed a higher humidity in the lower parts of the outer walls than might have been expected.

3. Materials and methods

3.1. Fungal identification

Stachybotrys chartarum was identified directly on the samples by phase-contrast microscopy (200×, 400× and 1000×) of transparent adhesive tape pressed gently on the building material and stained with lacto-fuchsin (0.1 g acid fuchsin, Sigma F8129, in 100 g 90% lactic acid, Merck 366) (Samson et al., 1995).

S. chartarum was grown on oatmeal and V8 agar (Samson et al., 1995) for verification of identity.

Representative strains of *S. chartarum* have been deposited in the IBT Culture Collection at Department of Biotechnology, Technical University of Denmark as IBT 9300 (school) and IBT 9291 (domestic residence).

4. Sampling

During collection of samples in the two buildings all persons involved wore respiratory filters, retaining both volatile organic compound and fungal spores.

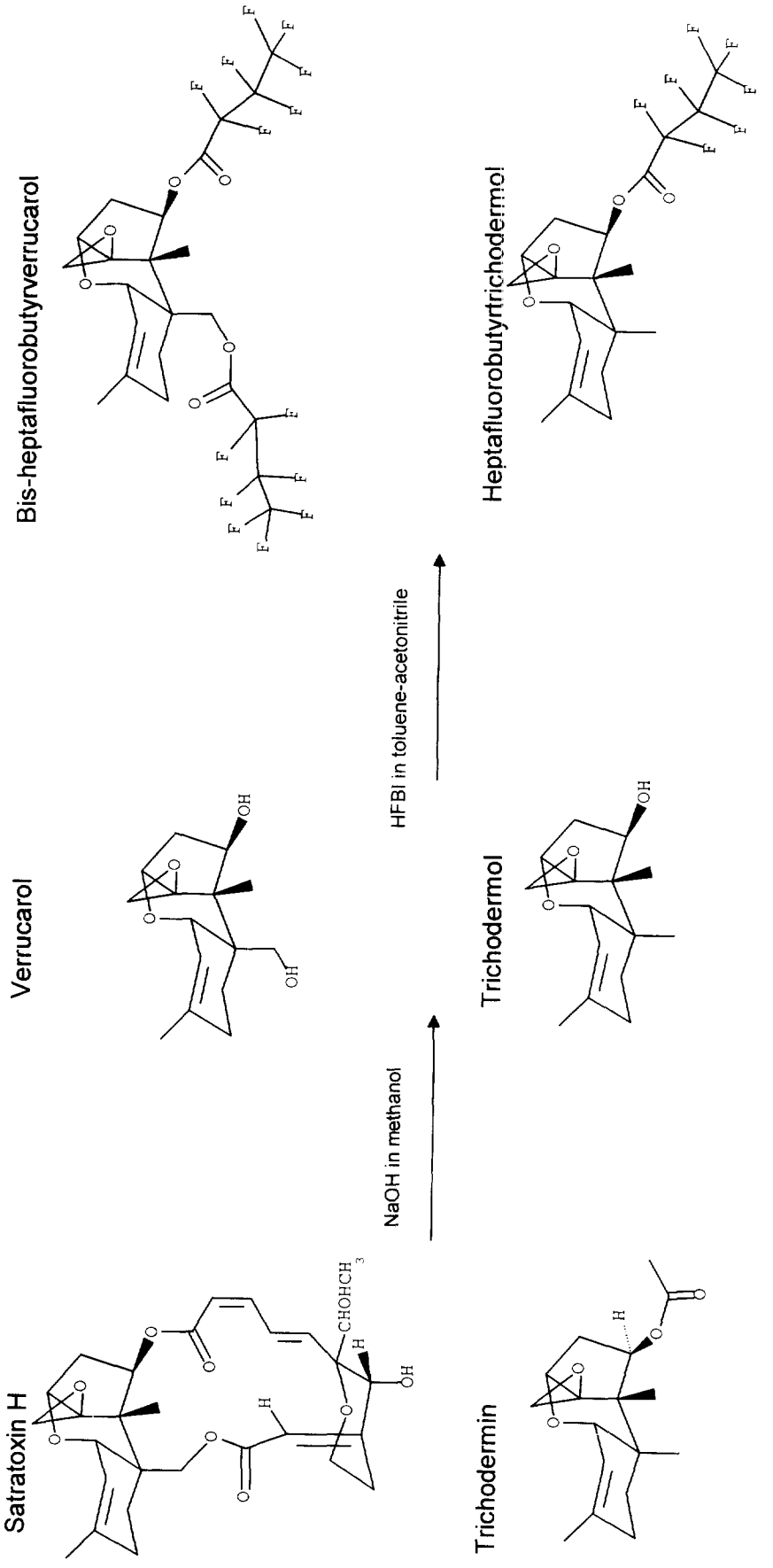

Fig. 1. Hydrolysis and HFB-derivatization of satratoxin H and trichodermin.

4.1. The school

Black fungal biomass from 0.1 m^2 was collected by scraping off the reverse side of the wall with a scalpel blade. Simultaneously, the material was collected onto a 0.45 μm filter by a sampling device (Dreborg et al., 1995) attached to a vacuum cleaner (sample A).

A piece of black insulation material, mineral fibre type (50 × 50 × 5 cm), sitting next to the heavily infected gypsum board was also transferred to the laboratory for extraction (sample B).

4.2. The domestic residence

In the basement of the domestic residence, 800 cm^2 of the outer cardboard layer of gypsum board infested with *S. chartarum* was removed with a scalpel and transferred to the laboratory for extraction (sample C).

5. Extraction

Samples were extracted with dichloromethane (Analytical grade), washed twice with water, and evaporated to dryness on a rotary evaporator *in vacuo* at 40°C. The brown oily residue was taken up in 2 × 5 mL dichloromethane, and stored at −80°C until analysis.

6. Sample clean up

Subsamples (2 mL) were evaporated *in vacuo* at 40°C and taken up in 2 mL methanol-water (1:1) and loaded onto a Sep-Pak® VAC 6 cc C_{18} (Waters, Wat0369051g) module and eluted with 9 mL methanol-water (7:3) using the modified method of Bata et al. (1985). The 11 mL methanol-water fraction was evaporated to dryness *in vacuo* at 5 mbar in a Chris Rotational Vacuum Concentrator (RVC).

7. Derivatization procedure for GC-MS analysis

Each aliquot was taken up in 250 μL 0.2 M NaOH in methanol and transferred to a 2 mL sample vial, and placed at 60°C for 60 min.

Samples were evaporated at 5 mbar in a RVC and taken up in 800 μL water. The water was extracted three times with 800 μL dichloromethane and the combined dichloromethane phases were evaporated to dryness at 5 mbar. The aliquot was taken up in 250 μL toluene-acetonitrile (9:1), and 10 μL heptafluorobutyrimidazole (Sigma H9903) was added, and the mixture was heated at 90°C for 60 min. After cooling, 800 μL hexane was added and the solution washed twice with 800 μL water before evaporating to dryness with N_2-flow. Samples were taken up in 50 μL toluene and transferred to a 500 μL sample vial, ready for GC-MS analysis.

8. GC-MS analysis

A Finnigan GCQ® integrated GC-MS system, ion-trap with external ionisation, was used for the analysis. Separation was performed on a 0.18 mm, 0.18 μm, 10 m J & W polydimethyl siloxane column (DB1 121–1012) with splitless injection of 1.0 μL at 260°C (split 45 sec., 45 cm^3/min), on a 2 m 0.18 mm deactivated silica precolumn. The temperature program was 80°C for 1 min; 12 C/min to 140 C; (holding 1 min) 4°C/min to 185°C; and then a 16°C/min to 250°C (holding 3 min) pressure programming was used for holding a constant linear gas velocity of 45 cm/sec. The transfer line temperature was 260°C.

The mass spectrometer was operated in negative ion mode using methane as reagent gas with a pressure of 1 bar; the ion-source temperature was 110°C giving a maximum s/n ratio, but a high fragmentation ratio. The scan mode was from 35 amu to 1000 amu.

9. Results and discussion

The *Stachybotrys* trichothecenes mostly elutes in the first 2–4 mL of the C_{18} mini-column clean up procedure.

When derivatisization of the alcohol groups of roridin A was tried, no bis-heptafluorobuturyle-roridin A was detected. However some HFB_2-verrucarol was seen as a result of transesterification.

On-column injection was investigated but did not seem more sensitive than splitless injection, making the latter the preferred method, as splitless injection is more reliable and robust. Deterioration of the column was seen using both injection methods; the column could be used for approx. 100–200 injections before replacement (detection limit increased 10–50 times).

Fragmentation patterns were better at 60°C, but extensive tailing was seen as a result of sample condensing in the ion-source. Mass shifting was a problem when using high methane pressures (1 bar), especially for HFB_2-verrucarol, where 25–75% of the 638 amu ions was detected as 637 amu ions.

The detection limit of GC-MS detection was 10 pg verrucarol, 1 μL injected and s/n of 3 for the 3 ions, 638, 302 and 213. The method detection limit was 0.02–0.2 μg satratoxin, depending on the quantities of interfering compounds and how much the sample was concentrated. In the samples, HFB-trichodermol and HFB_2-verrucarol could be recognised in the total ion current chromatogram (Fig. 2). The standard deviation of the method is approx. 5% based on an 8-point calibration curve of verrucarol $R^2 = 0.9987$, hydrolysed roridin A $R^2 = 0.8912$, and several analyses of the same extracts. Sometimes low

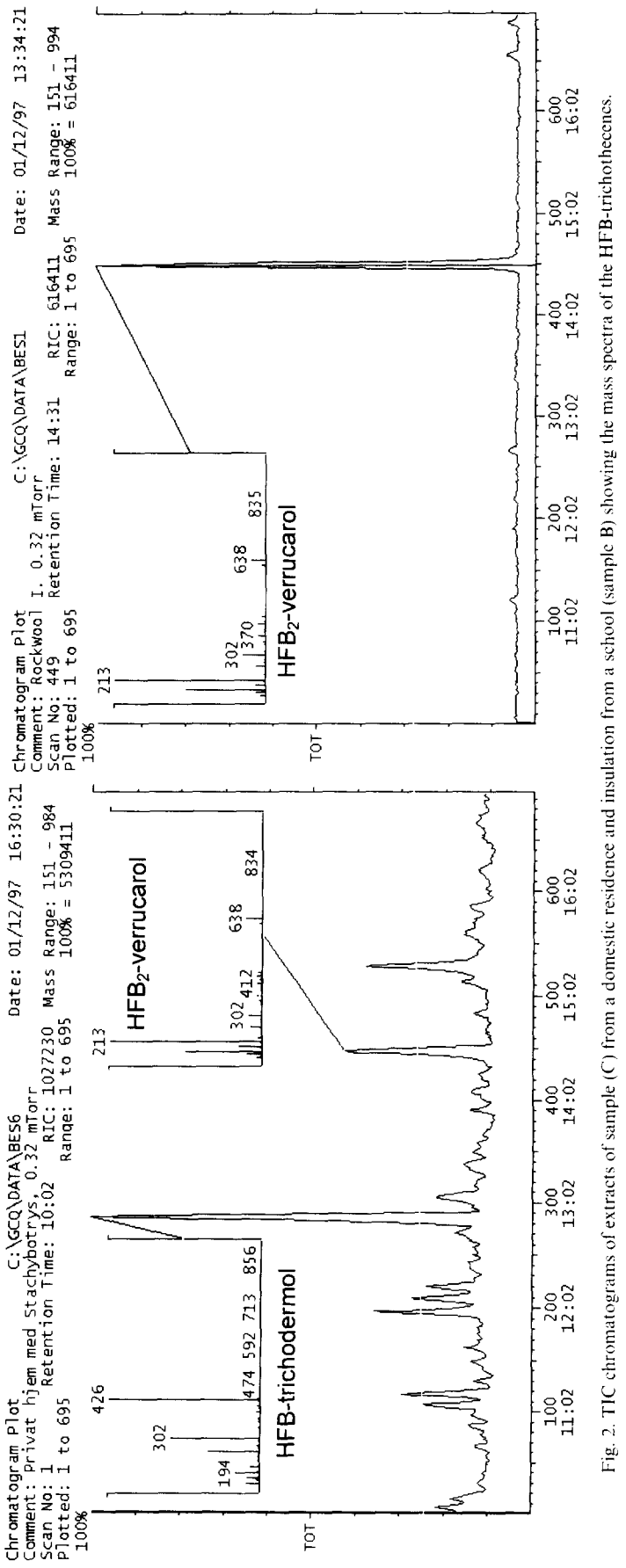

Fig. 2. TIC chromatograms of extracts of sample (C) from a domestic residence and insulation from a school (sample B) showing the mass spectra of the HFB-trichothecenes.

values were seen, probably due to NaOH residue or water reacting with HFBI or decomposition of HFB_2-verrucarol. Important samples should therefore be analysed twice.

In the three hydrolysed samples, verrucarol (Table 2) was detected, strongly indicating the presence of macrocyclic trichothecenes, as *S. chartarum* is known to produce these metabolites on building materials (Table 1). The detected amount of verrucarol has been interpreted to originate from satratoxins (Croft et al., 1986; Nikulin et al., 1994; Johanning et al., 1996).

HPLC-DAD analysis of concentrated cleaned-up samples using the method of Smedsgaard (1997) showed several small peaks eluting close to standards of roridin A, T-2 toxin and trichodermin (results not shown). These peaks had UV-maxima around 250–260 nm as in many of the macrocyclic trichothecenes from *S. chartarum*, indicating the presence of macrocyclic trichothecenes (Rodricks and Eppley, 1974).

The trichodermol (Table 1) was probably produced by *S. chartarum* or *S. cylindrospora*, as less potent toxigenic isolates of *S. chartarum* (IBT 14915 and CBS 413.95 = *S. atra* #29, in Nikulin et al., 1994) have been found to produce low quantities of both trichodermol and verrucarol-type trichothecenes (unpublished results, in preparation). More samples are under investigation.

The taxonomy of *Stachybotrys* and related genera has been reviewed by several authors (Jong and Davis, 1976; Domsch et al., 1980), but a thorough taxonomic revision of this genus is needed, especially because of the variable toxigenicity of the indoor isolates.

10. Conclusion

The CG-MS method with negative ion chemical ionisation is a very efficient technique since molecules containing no strongly electronegative groups are not detected, thereby yielding an extra specificity. The method is also 10 times or more sensitive than the usual methods, i.e. GC with MS-EI or electron capture detection of the trimethylsilylated compounds. It was observed that hydrolysis of the macrocyclic trichothecene roridin A to verrucarol was quantitative (results not shown).

Results show that trichothecenes, probably the macrocyclic forms, are produced by *S. chartarum* in Danish buildings on common building materials, verifying American, Swedish and Finnish findings (Johanning et al., 1996; Sorenson et al., 1987; Must et al., 1996; Nikulin et al., 1994). This finding is very important for the ongoing public debate in Denmark, where many water damaged buildings have been recognised.

For the first time, trichothecenes of the trichodermol type have been detected on building materials, most probably produced by *Stachybotrys chartarum*.

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Table 2
Trichothecenes in extracts from water damaged Danish buildings

Sample place	Sample	Area of growth (cm^2)	Verrucarol (nmol)	Satratoxin H[1] (μg)	Trichodermol
School	A	600–1000	2.2	1.2	No
School	B	2500	37	20	Trace
Domestic residence	C	800–1000	27	14	Yes

[1] Calculated as satratoxin H. All samples has been analysed at least twice.

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