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Potentially harmful secondary metabolites produced by indoor *Chaetomium* species on artificially and naturally contaminated building materials

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Abstract

The presence of the fungal genus *Chaetomium* and its secondary metabolites in indoor environments is suspected to have a negative impact on human health and wellbeing. About 200 metabolites have been currently described from *Chaetomium* spp., but only the bioactive compound group, chaetoglobosins, have been screened for, and thus detected in buildings. In this study, we used a liquid chromatography-high resolution mass spectrometry approach to screen both artificially and naturally infected building materials for all the *Chaetomium* metabolites described in the literature. Pure agar cultures were also investigated in order to establish differences between metabolite production *in vitro* and on building materials as well as comparison to non-indoor reference strains. On building materials six different chaetoglobosins were detected in total concentrations of up to 950 mg/m^2 from *C. globosum* along with three different chaetoviridins/chaetomugilins in concentrations up to 200 mg/m^2. Indoor *Chaetomium* spp. preferred wood-based materials over gypsum, both in terms of growth rate and metabolite production. Cochliodones were detected for the first time on all building materials infected by both *C. globosum* and *C. elatum,* and are thus candidates as *Chaetomium* biomarkers. No sterigmatocystin was produced by *Chaetomium* spp. from indoor environment.

Practical implications

In this study we improve the risk assessment of water-damaged buildings contaminated with *Chaetomium* by providing a comprehensive picture of the secondary metabolites and bioactive compounds produced by the two most common indoor species within this genus. We also provide specific biomarkers for *C. globosum,* as well as a common biomarker for *C. globosum* and *C. elatum* for their identification. The presence of the common biomarker,

cochliodone , in e.g. dust can reveal hidden *Chaetomium* growth in a water-damaged building.

Introduction

Fungal growth indoors may have a negative health effect on many people, especially asthmatic and allergic people, who may experience exacerbation of their illness in the presence of moulds (WHO Regional Office for Europe, 2009). Otherwise healthy people may also experience negative health effects, such as skin rashes, headaches, dizziness and chronic fatigue (Täubel et al., 2011; Miller and McMullin, 2014). The causality between a specific fungal component(s) and a particular health effect has not yet been documented. It has been suggested that fungal cell wall components (e.g. β-glucan) and bioactive metabolites (e.g. macrocyclic trichothecens) produced by the indoor fungi could have a causal role (Rand and Miller, 2011; Brewer et al., 2013). There are several routes of exposure to fungal metabolites in mouldy buildings, the most important one being inhalation followed by dermal absorption (Beko et al., 2013). Only a few fungal metabolites have been toxicologically investigated and even fewer have been tested for inhalative toxicity. Evidences suggest that the most toxic effects are observed after inhalation in comparison to other routes of exposure as elimination by hydrolysis in the stomach, limited absorption in the intestines or removal in the liver are avoided (Craesia et al., 1987).

In Danish mould ridden buildings species of *Chaetomium* can be found on about 16 % of the samples and are associated with chipboard, linoleum and concrete (Andersen et al., 2011), and are also some of the most frequently found moulds (55-66 % of gypsum wallboard) in North American buildings (Flannigan and Miller, 2011; Miller and McMullin, 2014). Due to the morphology and growth characteristics of the fungus, the isolation frequency is

presumably underestimated, as the spores are produced in the asci within a perithecium (Samson et al., 2010). The perithecia are often produced in the dark in cracks and cavities (von Arx et al., 1986), for example in the interface between linoleum and concrete or between gypsum board and plywood (unpublished results). The mature ascospores do not become immediately airborne, but are extruded as a sticky mass onto perithecial hair and may be spread by insects and mites (von Arx et al., 1986). Because of this, even visible growth of *Chaetomium* can be difficult to detect using air or dust sampling techniques (Andersen et al., 2011). With *Chaetomium* spores being large (8-12 µm) and not readily airborne, deposition in the upper airways is unlikely (Nielsen et al., 1999), however, dried-up mycelium and spore fragments together with perithecium hair may be present in both the air and dust. These submicron-size fragments (0.3-1.3 µm) or micro-particles (Gorny et al., 2002; Kildesø et al., 2003; Madsen, 2012) are likely to be the vehicle of exposure (Green et al., 2006) in mouldy buildings.

More than 200 biological active metabolites are known from different species of *Chaetomium* (Zhang et al., 2012) and these include compounds like: chaetomugilins, cochliodinol, cochliodones and chaetoglobin A (Jerram et al., 1975; Ge et al., 2008; Phonkerd et al., 2008; Chen et al., 2012). More than 400 *Chaetomium* species have been described (www.indexfungorum.org), of which the most common species in the indoor environment are *C. globosum* and *C. elatum* (McGregor et al., 2008; Samson et al., 2010; Andersen et al., 2011, Wang et al., 2016). Both *C. globosum* and *C. elatum* have been reported to produce chaetoglobosins (Udagava et al., 1979; Thohinung et al., 2010), and chaetoglobosins A and C (Fig. 1) have been detected on building materials (Nielsen et al., 1999; Nielsen, 2003). The intravenous toxicity of these two metabolites was demonstrated in animal tests (Ohtsubo et al., 1978; Udagawa et al., 1979), but to our knowledge, no study of inhalative toxicity has

been published. Other *Chaetomium* species have been reported to produce the carcinogenic sterigmatocystin (Rank et al., 2011; Sekita et al., 1981a).

In order to identify relevant *Chaetomium* produced compounds for future exposures/health risks analyses, more comprehensive methods for their detection and possible quantification are required. The analysis of volatile biomarkers (MVOCs) produced by relevant indoor fungi is based on use of GC-MS techniques (Van Lanker et al., 2008; Polizzi et al., 2012). Several targeted multi-analyte screening methods based on liquid chromatography tandem mass spectrometry (LC-MS/MS) have been used for a variety of different metabolites in the indoor environment (Vishwanath et al., 2009; Polizzi et al., 2009). However, due to the lack of reference standards, these methods only targeted a few of the many described *Chaetomium* metabolites. Older literature (e.g. Nielsen et al., 1999) only used the less sensitive UV/Vis spectroscopy for detection with HPLC and could not identify many of the numerous metabolites detected. In recent papers (McMullin et al., 2013; McMullin et al., 2013) the authors analysed for biologically active *Chaetomium* metabolites from indoor environments in pure culture, but they did not investigate the occurrence of these metabolites on building materials or buildings.

The purpose of this study was therefore to map all secondary metabolites different *Chaetomium* species are able to produce in pure agar cultures and determine which of those can be produced on artificially inoculated building materials and materials from naturally infected buildings.

Materials and methods

Chemicals and standards

All solvents used in this study, including water for LC-MS analysis, were LC-MS grade; chemicals were analytical grade and were purchased from Sigma-Aldrich (Steinheim, Germany), if not stated otherwise. Water used in fungal work was purified on Milli-Q system (Millipore, Bedford, MA) and autoclaved.

Standards of secondary metabolites included in library have been collected over the past 30 years, either from commercial sources, as gifts from other research groups or purified from different in-house projects (Kildgård et al., 2014; Nielsen et al., 2011). This metabolite collection consists of approximately 1500 standards with 95% of them being of fungal origin and 5 % of bacterial origin. Standard stock solutions of chaetoviridin A (Adipogen AG, Liestal, CH), chaetoglobosin A (Enzo, Exeter, UK) and chaetoglobosin C (Sigma-Aldrich), used for external calibration and matrix effect evaluation, were prepared in methanol and kept at -20 °C.

Fungal strains and cultivation

All 23 *Chaetomium* strains used in this study were from the IBT Culture Collection at the Department of Systems Biology, Technical University of Denmark (Table 1). For metabolite profiling and artificial inoculation on building material, two indoor and two *Chaetomium* reference strains were used: *C. globosum* (IBT 7029, reference; and IBT 41801, indoor), *C. elatum* (IBT 41944, indoor) and *C. virescens* (IBT 26237, reference). For metabolite profile comparison purposes, nine additional *Chaetomium* strains were used: four *C. globosum*, two *C. homopilatum*, two *C. longicolleum* and one *C. malaysiense* (Table 1). Furthermore, ten *Chaetomium* strains isolated from indoor samples were also inoculated on agar plates for

metabolite profiling, species identification and were consequently included in our IBT Culture Collection. Five different agar media were used for inoculation: Yeast Extract Sucrose agar (YES), Oatmeal agar (OAT), Malt Extract Agar (MEA), V8 juice agar (V8) and Potato Dextrose Agar (PDA) (Samson, et al. 2010). Once inoculated, all strains were incubated in darkness at 25 °C for 2 weeks. The identification of fungal strains and isolates to species level was performed using Ames (1963), von Arx et al. (1986) and Wang et al. (2014).

Artificially inoculated building materials

Five different types of building materials were used: chipboard, plywood, gypsum board (drywall/ plasterboard), masonite (hardboard/high-density fibreboard (HDF)) and medium density fibreboard (MDF). New, unblemished panels (90×240 cm) of each material were cut into sample blocks (13×18 cm) and each block was further divided in four equal oblong sections (13×4.5 cm) (Fig. 2). Each section represented one of four different conditions: 1) wallpaper adhesive + nonwoven woodchip wallpaper (wp1), 2) only wallpaper adhesive, 3) wallpaper adhesive + nonwoven wall-covering with pattern (wp2) and 4) non-treated material (no surface treatment), giving four inoculation sites per block. Wallpaper adhesive used was Pattex Direct Control Universal (Henkel, Düsseldorf, DE).

Each sample block was placed in a plastic box (22.5×17.5×4.5 cm) with a lid, sealed and sterilised using 1 x 40 kGy γ-irradiation (Sterigenics, Espergærde, Denmark). Spore suspensions of four *Chaetomium* strains (see Table 1) prepared from 10-day-old V8 cultures were used for streak inoculation of the 5 materials (20 samples in total). Boxes with sample blocks were weighed individually and streak-inoculated with one *Chaetomium* strain along the vertical lines in the middle of each individual segment (Fig.1). After inoculation 100 mL autoclaved, double-distilled water was added to each box. The blocks were left to absorb

water for 24 h after which any excess water was removed; the boxes were re-weighed and incubated at room temperature in darkness. The blocks were inspected and growth progress recorded once a week. Sampling for metabolite extraction was done four weeks after inoculation for *C. globosum* and *C. elatum,* while material inoculated with *C. virescens* was not analysed due to the absence of growth.

Naturally contaminated building materials

Ten samples, naturally contaminated with *Chaetomium* spp*.,* were analysed. Six building material samples (chipboard, gypsum board (3), concrete and OSB) originated from other research projects. The new clean materials had been submerged in sterile water for 24 hours and incubated at room temperature for 5 weeks, resulting in substantial fungal biomass produced. After it was determined (macro- and microscopically) that the fungal contaminant was *Chaetomium* spp*.,* the samples were donated to this project. Sampling for metabolite extraction was performed 6 months after first fungal growth was observed.

Four indoor samples were collected from water-damaged buildings (chipboard shelf, a gypsum board, a ceiling tile and cardboard). In order to verify *Chaetomium* contamination on the ten materials, tape preparations for phase contrast microscopy (200x, and 400x) were taken directly from the mould-infected area. This was done by gently pressing transparent adhesive tape to the infected surface and mounting it on a microscope slide in a drop of Shear's mounting fluid (Samson et al., 2010). Identification to species level of the contaminants from the naturally contaminated samples was performed by classic morphological methods (see "Fungal strains and cultivation") and secondary metabolite profiling. Sampling for metabolite extraction was performed immediately, either when the samples arrived at the lab or when discovered in the water-damaged building.

Metabolite extraction of building materials

Sampling from both artificially inoculated and naturally contaminated materials was done either by swabbing/scraping off fungal biomass from an area of approximately 1 cm^2 with a sterile Q-tip or by cutting pieces (1 cm^2) of infected material surface with a disposable scalpel, if scraping was not possible. Fungal biomass was placed in a 2 ml screw top vial, 1 mL of acetonitrile:water (75:25 v/v) mixture with 1% formic acid was added and extraction by sonication for 60 min was performed. The extract was transferred to a clean 2 ml vial, evaporated to dryness in a gentle stream of N_2, re-dissolved in 400 µL 1 % formic acid in acetonitrile: MilliQ water (75:25) mixture and centrifuged (15 min, 15000 g). The supernatant was directly used for chemical analysis. Samples received as a bulk material were only qualitatively analysed.

Metabolite extraction of pure cultures

The metabolite profiling was done on the 15-days-old MEA and PDA cultures using a micro-scale extraction method modified for *Chaetomium* metabolites (Samson, et al. 2010). Three agar plugs (6 mm ID) were cut across one colony from agar media and placed in a 2 ml screw top vial. 1.0 ml of extraction solvent, ethyl acetate/dichloromethane/methanol (3:2:1, vol/vol/vol) containing 1% formic acid, was added to each vial and the plugs were extracted by sonication for 60 min. The extracts were further treated as described previously in "Metabolite extraction of building materials".

UHPLC-DAD-QTOFMS analyses

Ultra-high performance liquid chromatography-diode array detection-quadrupole time of flight mass spectrometry (UHPLC-DAD-QTOFMS) was performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, California, USA) equipped with a

diode array detector scanning in range 200-640 nm and 20 times/s, using standard methodology, previously developed for all in house fungal extracts and published (Kildgård et al., 2014).

MS detection was performed on an Agilent 6550 QTOF MS equipped with Dual Jet Stream electrospray ion source, using hexakis-(2,2,3,3-tetrafluoropropoxy)phosphazene as lock mass. Other MS parameters, including information on automated data-dependant MS/HRMS (Auto-MS/HRMS) can be found in (Kildgård et al., 2014). All samples were analysed only in ESI^+ mode, due to the nature of analysed compounds.

Identification of secondary metabolites was performed using a combination of the following approaches: 1) direct search and matching of MS/HRMS data in MS/HRMS library containing all fungal secondary metabolites (~1500), (Kildgård et al., 2014), 2) aggressive dereplication of the full HRMS data where searching was performed using lists of possible known compounds that have been described in the literature but not available as standards and 3) UV/Vis detection of poorly ionizing compounds.

The MS/HRMS searching of all obtained spectra was done against our internal library containing 1500 compounds of which 95% are fungal secondary metabolites (Kildgård et al., 2014).

The search list used in the aggressive dereplication approach (Klitgård et al., 2014) was created by extracting Antibase2012 database for all compounds having *Chaetomium* spp. as a source as well as compounds described in the literature and not included in Antibase2012 (235 compounds of which 10 were available as reference standards). Adducts and common fragments included in this search function were: $[M+H]^+$, $[M+Na]^+$, $[M+H-H_2O]^+$ and $[M+NH_4]^+$. All analysed ions were treated as being single charged; the area cut-off was set to

10,000, and the mass spectrum was recorded below 10 % of the peak height in order to avoid overloading the detector (Kildgård et al., 2014).

For fast screening of larger number of samples and for semi-quantification purposes, MasHunter Quantitative Analysis for QTOF (version B.06.00) was used (Nielsen and Larsen, 2015). The method for screening included all *Chaetomium* compounds with known retention time selecting the most abundant ion.

Results and discussion

Fungal growth on artificially inoculated materials

The evaluation of fungal growth on five different materials, chipboard, plywood, gypsum board, masonite and MDF, is presented in Table 2. First sporadic hyphal growth of both strains of *C. globosum* was observed after two weeks. Once started, the growth and production of perithecia on plywood and chipboard was rapid, resulting in densely overgrown material after four weeks, while growth on gypsum was visually less dense. Both wood-based and gypsum-based materials proved to be good substrates for *Chaetomium* growth, but the wood-based materials supported the highest amount of biomass. Visually, growth rate was faster and denser in non-treated sections (no surface treatment) and in sections treated with adhesive compared to sections with wallpaper. *C. elatum* showed similar growth pattern to *C. globosum* on chipboard, but less dense growth on plywood and gypsum.

There was no visible growth after four weeks on either MDF or masonite by any of the four strains and microscopy revealed that none of the spores had germinated in any of four sections, even several months after inoculation. In the case of MDF, this could be explained by poor water absorption ability (5 % w/w) by the material (Table 2) providing unfavourable

conditions for *Chaetomium* growth. On the other hand, masonite also showed to be poor substrate for *Chaetomium*, despite the good absorption ability (17 % w/w) of the material. The reference strain, *C. virescens,* did not grow on any type of material used in this study, suggesting that its natural habitat is very different from indoor environments.

Comparing wallpapers type 1 (WP1) and type 2 (WP2), growth was more pronounced on WP1, while WP2 showed to be poorest in supporting the growth (Table 2). An inhomogeneous growth was observed on WP2 (non-woven wall covering with a polystyrene, pattern), regardless of inoculated strain or species, with the growth appearing only on surfaces in between pattern lines and never on the polystyrene pattern itself (Fig. 2). In addition to that, a recent research paper reports wallpapers with flatter or irregular surface structures to be advantageous in limiting mould germination and growth (Ryu and Moon, 2014).

Metabolite production

The metabolite production of *C. elatum* and *C. globosum* on artificially inoculated materials is presented in Table 3, while tables 4 and 5 show metabolites detected on naturally infected materials. All detected metabolites, regardless of sample type, belong to one of the following chemical groups: chaetoglobosins, chaetoviridins and chaetomugilins, cochliodones and chaetoglobosin A (Fig. 1).

Chaetoglobosins

Analysis of the *C. elatum* extracts from inoculated materials did not detect any of the chaetoglobosins (B, C, D, G, F or V) or prochaetoglobosins (III and III_{ed}), reported previously in pure cultures by Thohinung et al. (2010). On the other hand, chaetoglobosins

were detected on materials inoculated with two *C. globosum* strains (IBT 7029 and 41801). Four compounds with elemental composition ($C_{32}H_{36}N_2O_5$) were detected showing neither significant differences in their MS/MS spectra nor UV spectra. MS/HRMS library resulted in at least four peaks all identified as chaetoglobosin A (Fig. 3). Two of those were, by use of standards, unequivocally identified as chaetoglobosins A and C, while exact assigning of the remaining peaks to specific chaetoglobosins was not possible without standards. The same was observed with chaetoglobosins E and F. Due to their similar UV chromophores, distinguishing these compounds by UV/Vis was not possible, and identification based on their retention time compared to the logD values was too speculative. Hence, without the availability of reference standards, the only possibility left was to assign them as members of a specific group (Fig. 1). Since only very limited toxicological work has been conducted on these compounds abstaining from suggesting any differences should not pose a problem for current risk assessment.

On naturally contaminated materials chaetoglobosins were detected in all samples infected with *C. globosum* (with exception of cellular concrete) and in no samples infected with *C. elatum* (Table 4 and 5).

Chaetoviridins/chaetomugilins

This is another example of a metabolite group consisting of compounds with the same elemental composition and hence, due to the unavailability of standards, needed to be identified as members of specific group (Fig. 1). In extracts of artificially inoculated building materials, chaetoviridins and chaetomugilins were found only on building materials inoculated with *C. globosum* indoor strain IBT 41801. This strain produced chaetoviridins and chaetomugilins in all four sections on all materials, whilst for IBT 7029 no chaetoviridins or chaetomugilins were detected on any of the building materials (Table 3). The absence of

chaetoviridin A on materials inoculated with this strain was previously reported (Nielsen, et al. 1999) as an unidentified *Chaetomium* metabolite. On naturally infected materials, chaetoviridins and chaetomugilins were detected on all materials infected with *C. globosum* and none infected with other *Chaetomium* spp., including *C. elatum* (Table 4 and 5). Chaetoviridins and chaetomugilins were also not detected from any materials artificially inoculated with *C. elatum* (Table 3).

Cochliodones

C. globosum and *C. elatum* seemed to have a few metabolites in common, of which none have been previously linked to indoor strains or environments. UHPLC-DAD-QTOFMS analysis of artificially inoculated building material extracts of both *C. globosum* and *C. elatum* showed three common peaks with the consecutive retention times of 7.74, 9.53 and 11.39 minutes, sharing the same $[M+H]^+$ *m/z* of 639.2436 (Fig. 4). Aggressive dereplication matched all three peaks as the cochliodones A and B. Existing literature reports that cochliodones A and B are stereoisomers (Phonkerd et al., 2008). Comparison of fragmentation pattern of MS/MS spectra of all three peaks showed no significant difference among them. The peaks eluting at 9.5 and 11.4 min also showed splitting, which could be explained by reversible hemiacetal ring formation. Furthermore, rotation of the biaryl-structure around its axial stereocenter could easily provide three stereoisomers, which could explain presence of three peaks with *m/z* matching cochliodone A/B. Inconsistency in the available literature (several compounds with the same elemental composition, e.g. chaetospirone (Bitzer, 2005)) and limited knowledge of the biosynthetic pathways of these compounds, suggests that NMR data is necessary for their unequivocal identification. However, the presence of all proposed fragments (Supplementary information, Fig S1) in the

obtained spectra, strongly suggested the cochliodone structure as the correct structure. In the following text these compounds have been given the provisional names cochliodone 1 (7.74 min), cochliodone 2 (9.53 min) and cochliodone 3 (11.39 min). The cochliodones were first isolated by Phonkerd et al. (2008) from a soil derived strain of *C. cochliodes*. To the best of our knowledge, this is the first time that the cochliodones have been reported in *C. globosum* and *C. elatum.*

On artificially inoculated materials, *C. elatum* produced cochliodones 1, 2 and 3 on both the plywood and chipboard in all four sections. On gypsum cochliodones 1, 2 and 3 were found only on non-treated section (no surface treatment) and WP1 whilst the two other sections did not support enough biomass for extraction. *C. globosum* produced cochliodones 1, 2 and 3 on all materials in all sections. On naturally infected building materials, cochliodones were detected on all materials infected with either *C. elatum* or *C. globosum* (Table 4 and 5).

Chaetoglobin A

Additional metabolite was found to be common for *C. globosum* and *C. elatum*: chaetoglobin A, an azaphilone alkaloid dimer previously reported in *C. globosum* (Ge et al., 2008) (fragmentation pattern available in supplementary information, Fig S2). *C. elatum* produced chaetoglobin A in all four sections on chipboard, but none on plywood or gypsum, while *C. globosum* was able to produce it also on plywood. When it comes to natural contamination, chaetoglobin A was detected on different materials infected by both *C. globosum* (4 of 6) and *C. elatum* (1 of 1) (Tables 4 and 5).

Metabolite profiles of indoor and reference strains in pure cultures

Metabolite profiles of strains isolated from indoor samples collected during this study were used in species identification together with classical morphological methods. Morphologically *C. globosum* differ from *C. elatum* by having unbranched, coiled terminal hair, while *C. elatum* has straight dichotomously branched terminal hair. The results from chemical analyses are shown in Table 6. The results showed significant differences in metabolite profiles between reference and indoor *Chaetomium* strains (Fig. 5) except in the case of reference *C. globosum* strain (IBT 7029).

The chemical profiles of all 14 *C. globosum* strains were very similar. Several chaetoglobosins, chaetoviridins and chaetomugilins were identified in all analysed strains (Supplementary information, Table S1). There was no metabolite exclusively produced by any strain within this species. However, the *C. globosum* reference strain (IBT 7029) showed a lower production in comparison to the other strains.

Two indoor *C. elatum* strains had identical metabolite profiles. All compounds produced in pure cultures were also found on building materials inoculated by IBT 41944. The exception was cochliodinol, which was produced by both *C. elatum* and *C. globosum* in pure cultures but was not detected on any of the artificially inoculated building materials.

The six none-indoor reference *Chaetomium* species, *C. longicolleum* (2)*, C. homopilatum* (2), *C. malaysiense* (1) and *C. virescens* (1) were primarily screened for the presence of metabolites common to indoor species and not to obtain their full metabolite profiles. MS/HRMS library-matching resulted in positive hits for three important products of sterigmatocystin/aflatoxin biosynthetic pathways (Rank et al., 2011): sterigmatocystin, dihydrosterigmatocystin and 3-O-methyl sterigmatocystin. Sterigmatocystin was found in extracts of *C. virescens*, *C. malaysiense* and *C. longicolleum,* whilst 3-O-methyl

sterigmatocystin was found only in *C. virescens* extracts. The identity of these metabolites was confirmed by characteristic UV spectra and retention time (RT) (Fig. 5). *C. virescens, C. longicolleum* and *C. malaysiense* also produced several versicolorins (Suplementary information Fig. S3) (Kingston and Chen, 1976). Analysis of the two *C. homopilatum* strains indicated the presence of longirosterones (Supplementary information Table S1) based on accurate mass. Altogether six reference species showed no presence of any metabolites common with the 15 indoor strains. This shows that presence of sterigmatocystin in indoor environment can be linked only to *A. versicolor* and not to *C. globosum* and *C. elatum*.

Quantification of metabolites and bioactive compounds on building materials

Quantification of the major metabolite groups found on artificially inoculated building materials is presented in Table 6 (Results for individual metabolites are found in Supplementary information, Table S2). As expected from our previous study (Nielsen et al., 1999) based on HPLC-UV/Vis analyses, chaetoglobosins were found on materials in relatively high amounts, with the highest total amount (up to 95 µg/cm^2) being on chipboard and lowest on gypsum (up to 10 µg/cm^2). These concentrations were in accordance with what has previously been found for chaetoglobosin A (up to 13.8 µg/cm^2 on various substrates (Polizzi et al., 2009)). Same trend was seen with two other groups of metabolites: the highest amount of chaetoviridins (19 µg/cm^2) and cochliodones (21 µg/cm^2 for *C. globosum* and 5µg/cm^2 for *C. elatum*) were also found on non-treated (no surface treatment) chipboard.

Concentration estimates for individual metabolites (Supplementary information Table S2) pointed towards high concentration (up to 100 µg/cm^2) of three metabolites: chaetoglobosin A, chaetoviridin A and cochliodone 3. Other metabolites within each group were present in significantly lower amounts (the highest found concentration for other chaetoglobosins was

6.6 µg/cm^2, 1.7 µg/cm^2 for chaetoviridin C1 and C2 and 6.8 µg/cm^2 for cochliodone 2, all on chipboard). Using concentrations of these three metabolites to compare metabolite production between different artificially inoculated materials corroborated the findings on growth density. High metabolite production was found on chipboard and plywood compared to gypsum among materials, and high production on blank material (no surface treatment) and material treated with adhesive compared to surfaces with wallpapers.

Chaetomium species present in indoor environments

The use of pure culture metabolite profiles was explored in fungal contaminant identification in naturally infected samples. For *C. globosum,* identification to the species level could easily be performed based on secondary metabolite profile as all chaetoglobosins, chaetoviridins and chaetomugilins were exclusively produced by this species. In the case of the cellular concrete sample (Table 4), the contaminant was identified as *C. globosum*, however, species specific metabolites were not detected. This might be explained by the type of material, as concrete in our experiments, proved not to be very susceptible to fungal growth due to the low concentrations of nutrients, therefore biomass sampled might have not been sufficient.

The absence of metabolites characteristic for *C. globosum* necessitates morphological characteristics (e.g. the structure of terminal hair on the perichecia) to be combined with the chemical analyses. The water-damaged ceiling tile (Table 5) is a good example of old water damage, where no visible *Chaetomium* growth was present. However, scrapings of the tile onto V8 agar revealed the presence of viable *C. erectum* and subsequent chemical analysis showed presence of cochliodone 3, which was not detected from this species growing on any agar medium. Hence, these compounds were probably produced by one of the other common indoor *Chaetomium* spp. on the sampled material despite the absence of their viable spores. In cases like this, with the combination of old water damage, absence of any visible growth or

viable biomass and presence of several fungal species, detection of indoor contaminants is very challenging. Therefore, the presence of chemical biomarkers, such as the cochliodones, facilitates identification of indoor contaminants.

Previous papers (Vishwanath et al., 2009; Polizzi et al., 2009) base their identification of indoor *Chaetomium* spp. on metabolites only found in *C. globosum*. So far, *C. globosum* has been the most frequently found indoor species. The absence of common *Chaetomium* biomarker or markers for other species, however, prevents other species than *C. globosum* from being detected.

Whether these *Chaetomium* metabolites play a role in building dampness-related illness and/or discomfort remains to be determined. Certainly, effects of bioactive secondary metabolites should not only be studied individually. Additive and possible synergistic effects of fungal components and other microbial compounds present in the wet indoor environment also have to be taken into account. However, these combined effects cannot be assessed before effects of individual metabolites are known. Until more comprehensive exposure studies of the metabolites and their effects are conducted, no conclusions can be made on the relevance of fungal metabolites in buildings. Therefore, thorough studies on all frequent indoor contaminants and what they produce in buildings are needed.

Conclusion

This is the first time, to our knowledge, that cochliodones and chaetoglobin A have been detected on different types of building materials. These compounds can be used as indoor *Chaetomium* biomarkers, since their presence was not found in any of the non-indoor strains screened. Regarding possible exposures in indoor environment, chaetoglobosin A, chaetoviridin A and cochliodone 3 seem to be of highest importance due to their high

quantities found on building materials. Whether one or more of the *Chaetomium* metabolites found in this study have toxicological significance and can act as causal factors for some of the reported health effects remains to be determined. The next step would be to investigate the presence of all these metabolites in settled dust as dust seems to accurately represent the diversity of the indoor microbiota in terms of presence of spores, micro-particles and metabolites. Settled dust is easy to sample and to become airborne by mechanical disruption (Täubel et al., 2011), which is relevant for the overall exposure assessment.

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Table 1. *Chaetomium* species and strains used in the study.

Genus	Species	IBT no.	Other no.	Origin
Chaetomium	*elatum*	41944[a]	BA Home A	Dust on curtain, DK
Chaetomium	*elatum*	42179[c]	BA Sample 3009	Cardboard from photo album, DK
Chaetomium	*erectum*	42278[c]	VKR 081 Vab	Dirt over front door, DK
Chaetomium	*globosum*	41766[b]	GR11BA 7b	Plywood wall construction, Greenland
Chaetomium	*globosum*	41800[b]	EK2-V8-Chae	Linoleum, DK
Chaetomium	*globosum*	41801[a]	EK1-V8-Chae	Carpet, DK
Chaetomium	*globosum*	41865[b]	MRO 068	Wooden skirting board, DK
Chaetomium	*globosum*	41904[b]	KFV-TH-CHI-V8	Dust on floor, DK
Chaetomium	*globosum*	42279[c]	VKR 057 V8	Indoor air, DK
Chaetomium	*globosum*	42296[c]	A-1	Gypsum board A, DK
Chaetomium	*globosum*	42298[c]	D-2	Gypsum board D, DK
Chaetomium	*globosum*	42299[c]	E-1	Gypsum board E, DK
Chaetomium	*globosum*	42300[c]	E-2	Gypsum board E, DK
Chaetomium	*globosum*	42301[c]	G-2	Gypsum board G, DK
Chaetomium	*globosum*	42302[c]	G-3	Gypsum board G, DK
Chaetomium	*globosum*	42303[c]	A-3	Gypsum board A, DK
Chaetomium	*globosum*	7029[a]	CBS 148.51	Stored cotton, USA
Chaetomium	*homopilatum*	41560[b]	CBS 337.68 = NHL 2259	Wood scobs, Japan
Chaetomium	*homopilatum*	41564[b]	CBS 167.61 = NHL 2260	Soil, Japan
Chaetomium	*longicolleum*	41566[b]	CBS 103.79	Dung, USA
Chaetomium	*longicolleum*	41567[b]	CBS 119.57	Soil, Madagascar
Chaetomium	*malaysiense*	41578[b]	CBS 669.82	Soil, Japan
Chaetomium	*virescens*	26237[a]	CBS 547.75	Wheat straw, India

[a] Strains used in artificial inoculation experiment.
[b] Strains used only for metabolite profiling.
[c] Strains isolated from naturally infected materials during this study.

Table 2. Growth evaluation of different *Chaetomium* spp. artificially inoculated on different building materials with two types of wallpaper (wp1 and 2) and adhesive, adhesive alone and blank with no treatment (see Fig. 1).

Materials		Water content (% w/w)	**Growth**			
			C. globosum IBT 7029	*C. globosum* IBT 41801	*C. elatum* IBT 41944	*C. virescens* IBT 26237
Chipboard	wp 1	20	+++	+++	+++	NG
	adhesive		++++	++++	++++	NG
	wp 2		++	+++	+++	NG
	blank		++++	++++	++++	NG
Plywood	wp 1	15	+++[a]	+++	++	NG
	adhesive		++++	+++	+	NG
	wp 2		++	++	+	NG
	blank		+++	+++	+	NG
Gypsum	wp 1	17	++	++	+	NG
	adhesive		++	+	NG	NG
	wp 2		+	+	NG	NG
	blank		++	++	++	NG
Masonite	wp 1	17	NG	NG	NG	NG
	adhesive		NG	NG	NG	NG
	wp 2		NG	NG	NG	NG
	blank		NG	NG	NG	NG
MDF	wp 1	5	NG	NG	NG	NG
	adhesive		NG	NG	NG	NG
	wp 2		NG	NG	NG	NG
	blank		NG	NG	NG	NG

[a] Growth evaluation: NG: no growth; +: 5 - 20 % of material covered; ++: 20 - 50 % covered; +++: 50 - 80 % covered; ++++: 80 - 100 % covered.

Table 3. Secondary metabolites found in artificially wetted building materials artificially contaminated with *Chaetomium globosum* (Cg) and *C. elatum* (Ce)

	Chipboard								**Plywood**								**Gypsum**							
	wp 1		adhesive		wp 2		blank		wp 1		adhesive		wp 2		blank		wp 1		adhesive		wp 2		blank	
Metabolite	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*	*Cg*	*Ce*
Cochliodone 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NA	+	NA	+	+
Cochliodone 2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NA	+	NA	+	+
Cochliodone 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NA	+	NA	+	+
Chaetoglobin A	+	+	+	+	+	+	+	+	+/-	-	+	-	+/-	-	+	-	-	-	+/-	NA	+/-	NA	+/-	-
Chaetoglobosin A	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	NA	+	NA	+	-
Chaetoglobosin C	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+/-	-	+/-	NA	+/-	NA	+/-	-
Chaetoglobosin E/F	+/-	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+/-	-	+/-	NA	+/-	NA	+/-	-
Chaetoglobosin G/D/ U/V	-	-	-	-	+/-	-	+	-	+	-	+/-	-	+/-	-	+	-	+/-	-	-	NA	+/-	NA	+/-	-
Chaetoviridin A/ Chaetomugilin C	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	NA	+/-	NA	+/-	-
Chaetoviridin C/ Chaetomugilin D/S	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	NA	+/-	NA	+/-	-
Prochaetoglobosin I	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	+/-	-	-	NA	-	NA	-	-
Prochaetoglobosin II	-	-	+/-	-	-	-	-	-	-	-	-	-	-	-	-	-	+/-	-	-	NA	-	NA	-	-
Prochaetoglobosin III/III_{ed}	+	-	+	-	+	-	+/-	-	+	-	+	-	+	-	+	-	-	-	-	NA	-	NA	-	-
Prochaetoglobosin IV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	NA	-	NA	-	-

Four sections on each type of material: wp1- nonwoven woodchip wallpaper + wallpaper adhesive; wallpaper adhesive; wp2 – nonwoven wallcovering with pattern + wallpaper adhesive; blank – non-treated material.
+/-: compound produced only by one strain of the two strains of *C. globosum* (IBT 7029 or IBT 41801);
NA: not analysed, insufficient growth observed in these sections

Table 4. Secondary metabolites found in artificially wetted building materials naturally contaminated of *Chaetomium* spp.

Sample type (number of samples)	Identified metabolites	Species isolated
Cellular concrete (4)	cochliodones 1, 2 and 3, chaetoglobin A	*C. globosum*
Chipboard (3)	**chaetoglobosin A**, chaetoglobosin C, chaetoglobosin G/D/U/V, chaetoglobosin E/F, prochaetoglobosin I, prochaetoglobosin II, chaetoviridin C/chaetomugilin D/S, **chaetoviridin A/chaetomugilin C**, **cochliodones 1, 2 and 3**	*C. globosum*
Gypsum board 1 (1)[a]	**cochliodones 1, 2 and 3**	*Chaetomium* sp.[b]
Gypsum board 2 (4)	**chaetoglobosin A**, chaetoglobosin C, chaetoglobosin G/D/U/V, chaetoglobosin E/F, chaetoviridin C/chaetomugilin D/S, **chaetoviridin A/chaetomugilin C**, **cochliodones 1**, **2 and 3**, chaetoglobin A	*C. globosum*
Gypsum board 3 (7)	**chaetoglobosin A**, chaetoglobosin C, chaetoglobosin G/D/U/V, chaetoglobosin E/F, chaetoviridin C/chaetomugilin D/S, **chaetoviridin A/chaetomugilin C**, **cochliodones 1, 2 and 3**, chaetoglobin A	*C. globosum*[c]
OSB (4)	**chaetoglobosin A**, chaetoglobosin C, chaetoglobosin G/D/U/V, chaetoglobosin E/F, chaetoviridin C/chaetomugilin D/S, **chaetoviridin A/chaetomugilin C**, cochliodones 1, 2 and 3, chaetoglobin A	*C. globosum*

[a] Sampled and analyzed on both face and reverse cardboard.
[b] The fungus was identified using tape preparation directly on material, but not viable for species identification.
[c] Each sample was contaminated with different *C. globosum* strain, 7 strains isolated in total.
Metabolites in bold are found in the highest amounts.

Table 5. Secondary metabolites found in water-damaged building materials naturally contaminated with *Chaetomium* spp.

Sample type (number of analyses pr sample)	Identified metabolites	Species on material
Cardboard pages (photo album) (5)	Cochliodone 1, cochliodone 2, **cochliodone 3**, chaetoglobin A	*C. elatum*
Ceiling tile (3)	**Cochliodone 3**	*C. erectum*
Chipboard (3)	Cochliodone 1, cochliodone 2, **cochliodone 3**, chaetoglobin A	*Chaetomium* sp.[a]
Gypsum board (3)	Cochliodone 1, cochliodone 2, **cochliodone 3**, **chaetoglobosin A,** chaetoglobosin C, chaetoglobosin G/D/U/V, chaetoglobosin E/F, chaetoviridin C/ chaetomugilin D/S, **chaetoviridin A/chaetomugilin C**, chaetoviridin E, chaetoviridin H	*C. globosum*

[a] The fungus was identified using tape preparation directly on material, but not viable for species identification.
Metabolites in bold are found in the highest amounts.

Table 6. Estimation of concentration range (µg/cm$^{2)}$ for four major metabolite groups found on artificially inoculated building materials

		Concentration range (µg/cm^2)							
		Chaetoglobosins		Chaetoviridins		Cochliodones		Chaetoglobin A	
Materials		*C. globosum*	*C. elatum*	*C. globosum*	*C. elatum*	*C. globosum*	*C. elatum*	*C. globosum*	*C. elatum*
CHIPBOARD	wp type 1	0.33-2.88	NA	0.64-2.75	NA	0.09-4.36	0.32-4.81	0.97	1.26
	adhesive	1.02-95.43	NA	1.44-1.77	NA	0.04-4.46	0.07-3.72	2.33-2.33	2.97
	wp type 2	1.07-6.52	NA	0.47-8.49	NA	0.02-3.68	0.86-2.65	2.02-6.99	2.17
	blank	0.69-81.72	NA	1.59-19.19	NA	1.9-21.04	0.03-2.62	3-3.19	2.54
PLYWOOD	wp type 1	0.2-10.64	NA	0.19-9.32	NA	1.44-7.05	0.07-3.5	NA	NA
	adhesive	0.67-39.84	NA	0.74-13.38	NA	0.21-11.74	0.04-2.99	0.11-0.25	NA
	wp type 2	0.78-36.32	NA	0.36-14.11	NA	0.02-14.57	0.55-1.88	NA	NA
	blank	1.15-62.4	NA	1.26-15.26	NA	0.2-12.89	0.62-2.03	0.02	NA
GYPSUM	wp type 1	2.32-2.64	NA	0.1-6	NA	0.09-4.75	0.05-1.68	NA	NA
	adhesive	6-9.92	NA	0.2-8.85	NA	0.07-6.75	NA	NA	NA
	wp type 2	2.24-2.56	NA	0.29-3.88	NA	0.34-3.46	NA	NA	NA
	blank	2.24-2.72	NA	0.54-0.55	NA	0.71-4.01	0.19-2.85	NA	NA

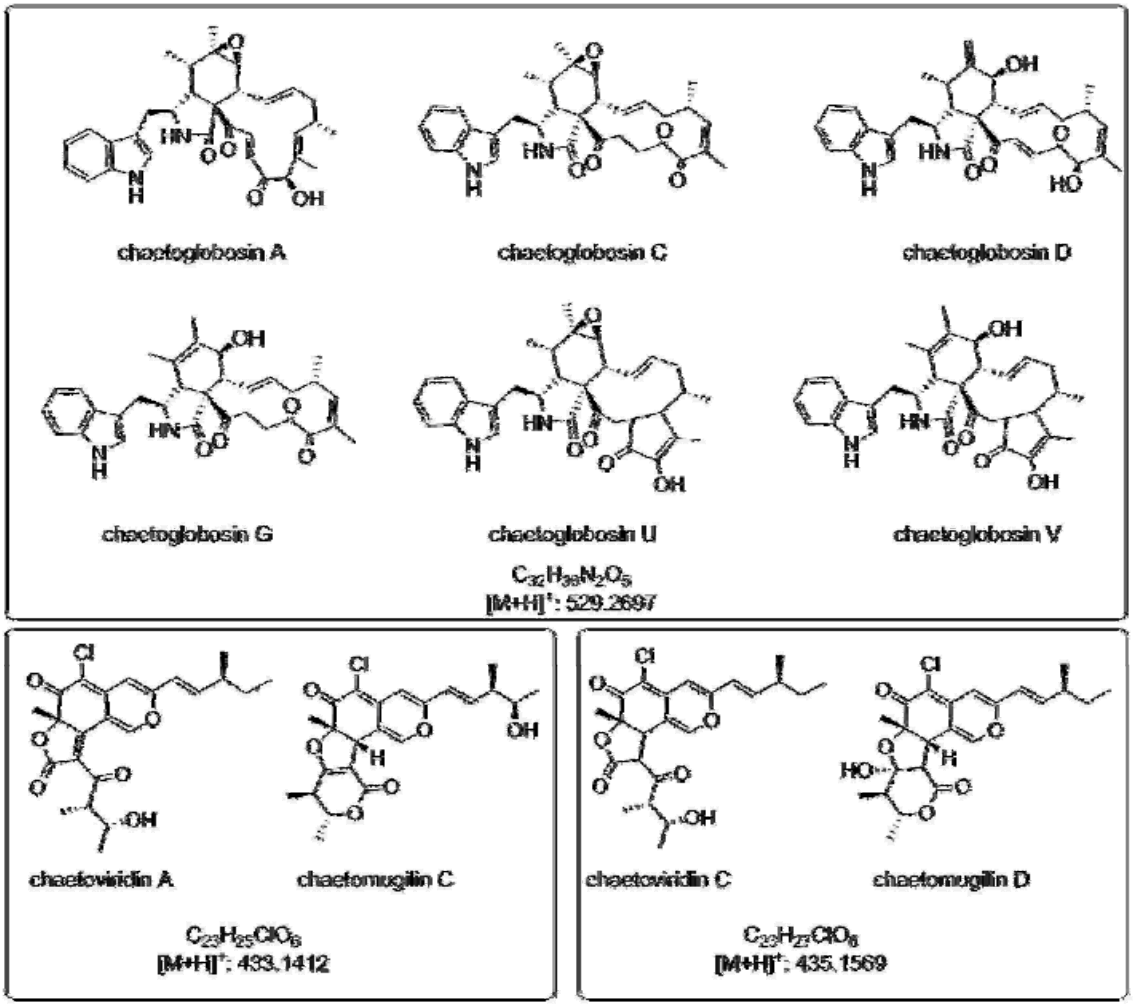
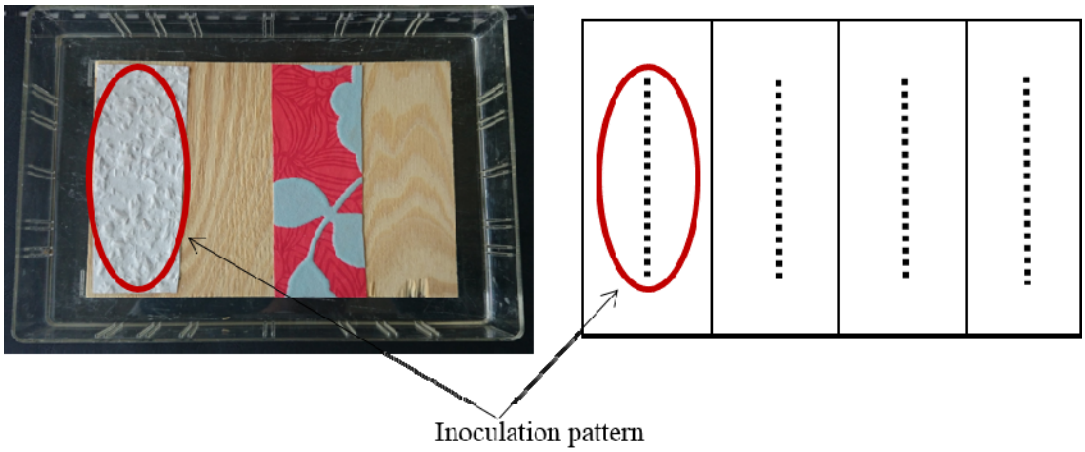
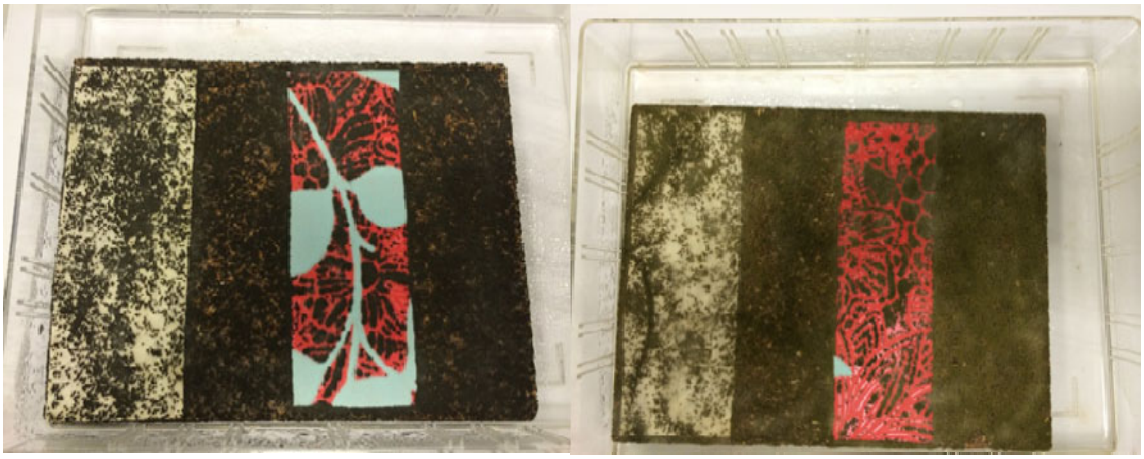


Fig. 1 Chemical structures of chaetoglobosins, chaetoviridins and chaetomugilins with the same elemental composition found in *C. globosum*



Sections from left to right: wallpaper adhesive + nonwoven woodchip wallpaper (wp1), wallpaper adhesive, wallpaper adhesive + nonwoven wall-covering with pattern (wp2), non-treated material



From left to right: *C. elatum* and *C. globosum* on chipboard showing characteristic absence of fungal growth on pattern of wallpaper type two (red wallpaper with blue leaves)

Fig. 2 Artificially inoculated building blocks with inoculation pattern and specific growth in different sections

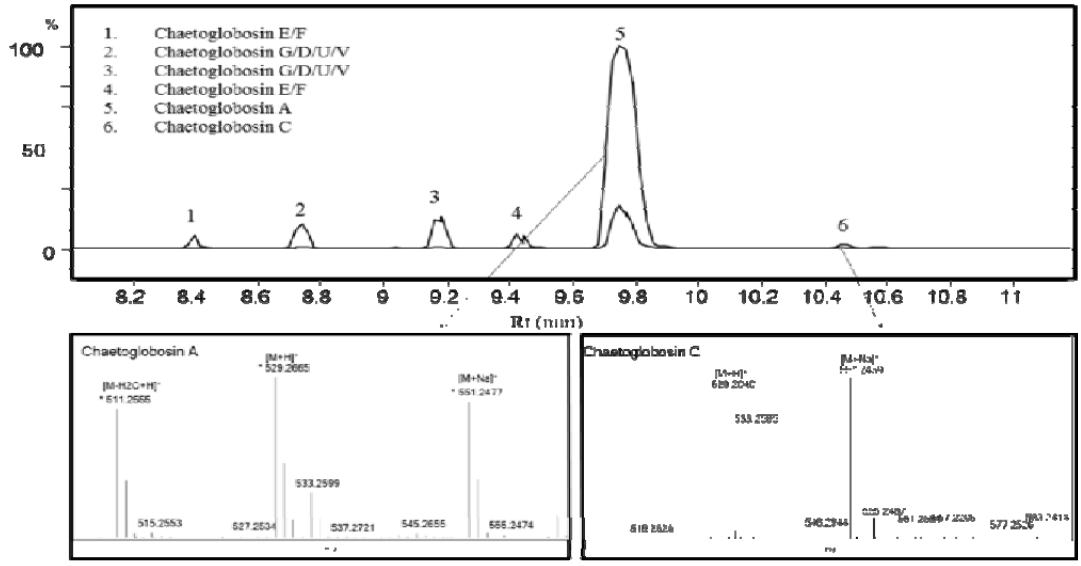


Fig. 3 Combined extracted ion chromatograms of m/z 529.2697 corresponding to pseudomolecular ion of chaetoglobosin A, C, G, D, U and/or V resulting in four peaks: 2, 3, 5 and 6; and of m/z 531.2853 corresponding to pseudomolecular ion of chaetoglobosin E and F (peaks 1 and 4). MS spectra of identified chaetoglobosin A (peak 5) and C (peak 6) were presented below

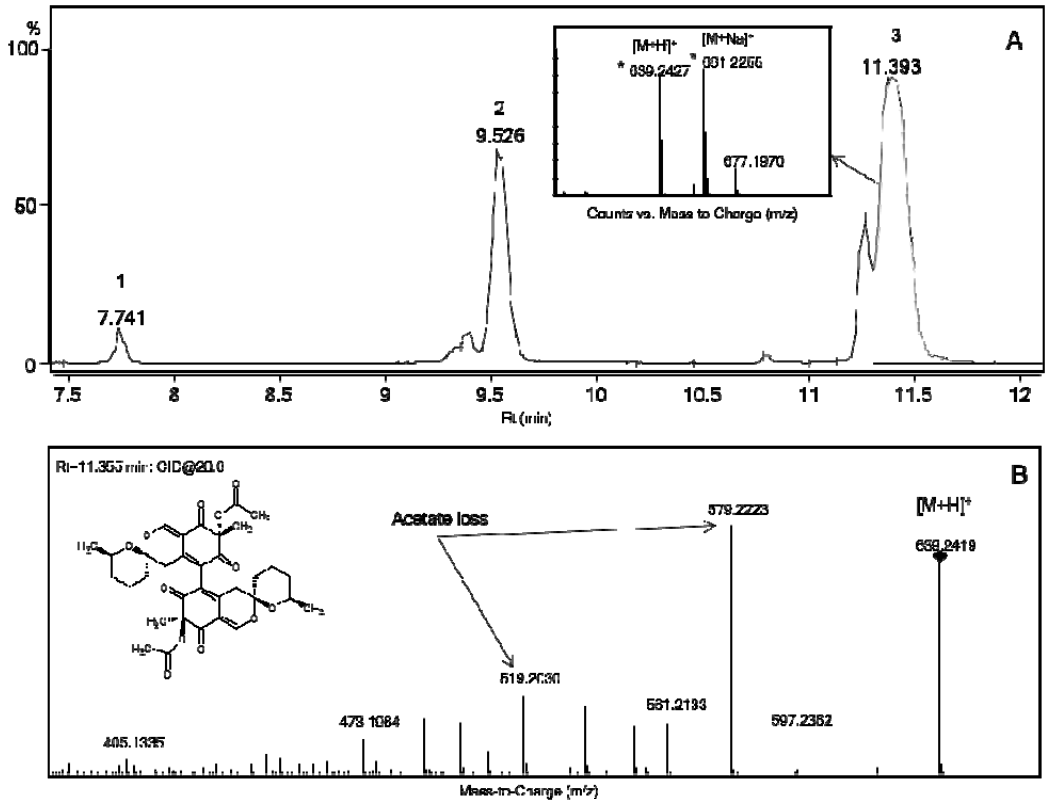


Fig. 4 Chromatogram (A) of three peaks identified as 1 – cochliodone 1 (Rt 7.74 min), 2 – cochliodone 2 (Rt 9.52 min) and 3 – cochliodone 3 (Rt 11.39 min) together with MS/MS spectrum of cochliodone 3 at 20 eV (B)

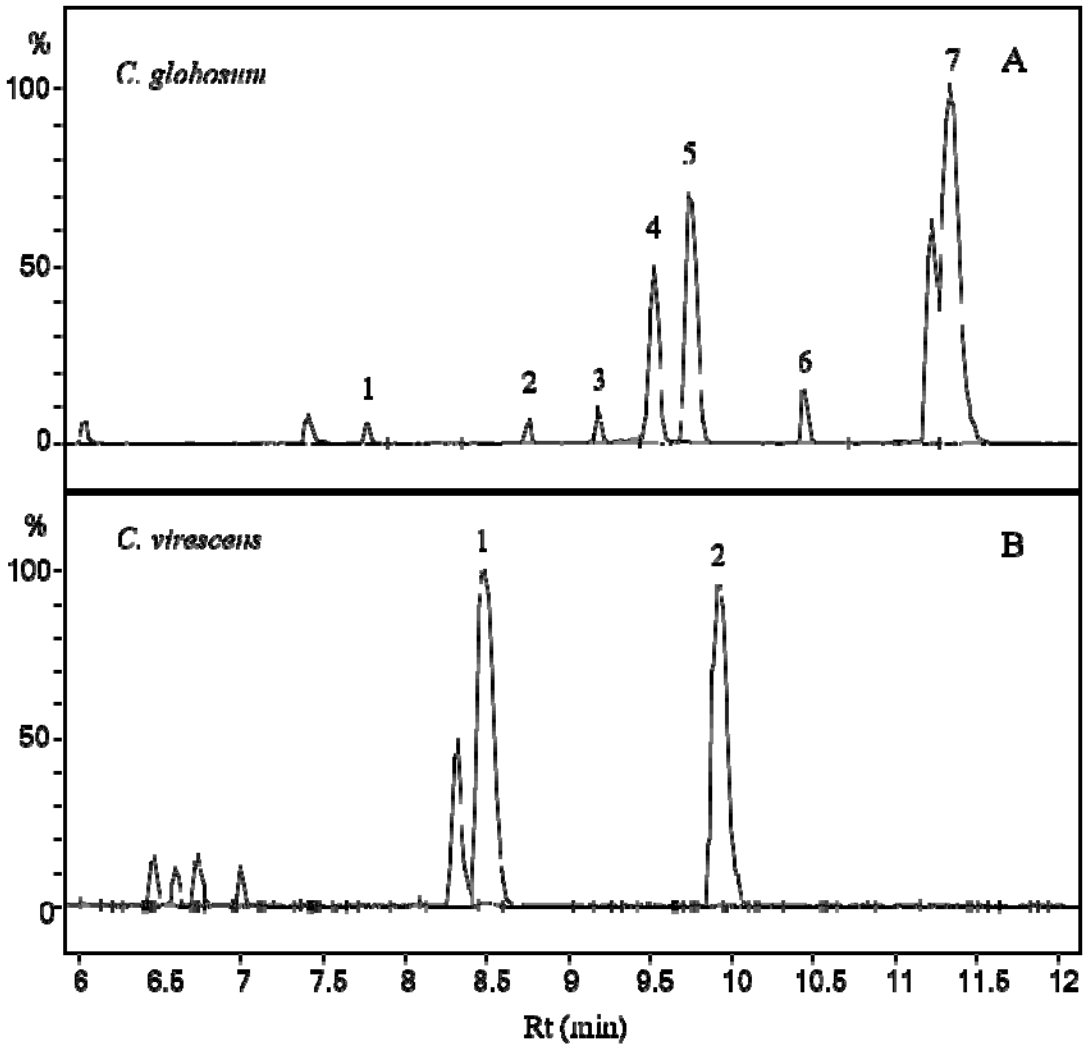


Fig. 5 Comparison of combined extracted ion chromatograms (6-12 min) of indoor (*C. globosum*, IBT 41801) and reference strain (*C. virescens*, IBT 26237) MEA extracts: A: *C. globosum* 1 – cochliodone 1, 2 – chaetoglobosin G/D/U/V, 3 – chaetoglobosin G/D/U/V, 4 – cochliodone 2, 5 – chaetoglobosin A, 6 – chaetoglobosin C, 7 – cochliodone 3; B: *C. virescens* 1 – 3-O-methylsterigmatocystin, 2 – sterigmatocystin