LC-MS based analysis of secondary metabolites from Chaetomium and Stachybotrys growth in indoor environments

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LC-MS based analysis of secondary metabolites from *Chaetomium* and *Stachybotrys* growth in indoor environments

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PhD Thesis

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Eukaryotic Biotechnology

Preface

This PhD study was carried out at the section for Eukaryotic Biotechnology, Department of Systems Biology, Technical University of Denmark (DTU) in the period of 1st of March 2013 to 29th of March 2016. This work was supervised by DTU and financially supported by VILLUM Foundation.

First and foremost, I would like to thank my supervisors ***Birgitte Andersen*** and ***Kristian Fog Nielsen*** for their tremendous help and excellent supervision. I would like to express my deepest gratitude for their thoughtful guidance, fruitful scientific discussions and motivation as well as for all those laughs we shared during our field trips and meetings. You are the best "bosses" I will ever have. I would also like to thank my co-supervisor ***Geo Clausen*** for his assistance, advices and valuable inputs, especially when different perspective was most needed.

I cannot express enough thanks to my office mate and great friend ***Olivera Magdenoska.*** Thank you for being an awesome person, friend and someone I could always count on. I certainly had the best office mate ever, but I also made a friend for a lifetime. Special thanks goes to ***Christopher Phippen***, for always being there when needed. Without your moral support, valuable inputs and ceaseless readiness to help nothing would have been the same. I would also like to thank my other office mate and friend ***Sietske Grijseels***. Thank you for being there in the most troublesome times, you are one of the best people I know.

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Finally, I would like to thank all my friends and family for never giving up on me. You are the best! This journey would have never been possible without the unconditional love and endless support of my ***Parents***. Whatever I achieved and became is all because of you. Thank you for being the best people and best parents in the whole world!

Summary

Living and working in fungi-ridden buildings can be detrimental for occupants both in terms of the effects on their health and in an economic sense. This is especially true for hypersensitive, asthmatic and allergic individuals who often experience exacerbation of their conditions when present in wet, fungi contaminated indoor environment. Otherwise healthy people may also experience negative health effects, such as skin rashes, headaches, dizziness and chronic fatigue. During their growth on building materials, indoor fungi produce and release many different kinds of components. These components are suspected of causing adverse health effects, however that causality has yet to be documented. Fungi produce biomass in the form of mycelium and spores both of which contain an array of secondary metabolites and bioactive compounds. When fungal biomass dries up, whole viable spores, fragmented spores and mycelium parts containing secondary metabolites and bioactive compounds are released from the building material.

Not only is the variety of different fungal species present in the indoor environment large, the number of bioactive compounds they produce is also broad. This PhD study focused on fungal species characterized as so-called tertiary colonizers, namely *Stachybotrys* spp. and *Chaetomium* spp. Both *Stachybotrys* spp. and *Chaetomium* spp. require high water activity for optimal growth ($a_w \sim 0.98$), which, for the indoor environment, often translates into serious water ingress rather than a high level of condensation. Thus, presence of these species and/or their metabolites in indoor environment is a good indicator of water damage, whether old or new. Furthermore, secondary metabolites produced by *Stachybotrys* spp. and *Chaetomium* spp. are known mycotoxins, thereby increasing likelyhood of causing negative health impact. With this in mind, a prime goal of this PhD study was to develop and optimize methods for qualitative and semi-quantitative analysis of secondary metabolites and bioactive compounds produced by *Stachybotrys* spp. and *Chaetomium* spp.

The main analytical technique used for this purpose was liquid chromatography coupled to mass spectrometry. Utilizing advanced technology such as high resolution mass spectrometry enabled use of standardized MS/HRMS libraries and compound databases in metabolite profiling of *Stachybotrys* spp. and *Chaetomium* spp. Thus, the preliminary work performed in this PhD study was MS/HRMS library building, which contained all in-house available secondary metabolites (~1500). For metabolite profiling purposes, MS/HRMS library was supplemented with species- and genus-specific compound databases containing all secondary metabolites described in literature and not included in the library, as well as tentatively identified compounds. Metabolite profiling of *Stachybotrys* spp. and *Chaetomium* spp. was performed in pure agar cultures.

Thereafter, mapped secondary metabolites were screened for in extracts of artificially inoculated building materials and materials from naturally infected buildings.

Work performed on *Chaetomium* spp. showed that indoor strains have substantially different metabolite profiles in comparison to non-indoor reference strains. The study of *Chaetomium* spp. on artificially inoculated building materials revealed a preference for wood-based materials in these species. Commonly screened *Chaetomium* metabolites such as chaetoglobosins, were shown to be *C. globosum* specific, whilst cochliodones and chaetoglobin A were present in all *Chaetomium* contaminated indoor samples and thus are candidates for indoor specific *Chaetomium* biomarkers. Furthermore, this was the first time cochliodones were reported by indoor *Chaetomium* species. For the purpose of quantification a semi-quantative UHPLC-DAD-QTOFMS method was developed and semi-validated, enabling estimation of quantities for *Chaetomium* metabolites on different building materials. Finally, the aforementioned analytical tools were applied to the analysis of naturally contaminated building materials, where presence of all previously mapped metabolites was confirmed.

Work done on *Stachybotrys* spp showed no significant difference in metabolite profiles obtained *in vitro* and *in vivo*. Concurrently the study of *Stachybotrys* spp. on artificially inoculated building materials confirmed a preference of this genus for gypsum in comparison to wood-based materials. Metabolites belonging to macrocyclic trichothecene, atranone and spirocyclic drimane groups of compounds were found on both artificially and naturally infected building materials. Spirocyclic drimanes were shown to be good candidates for indoor specific *Stachybotrys* biomarkers, as they were both produced in high quantities by all indoor *Stachybotrys* species. The best ionizing *Stachybotrys* compounds were chosen and their detection was directly transferred to the UHPLC-QqQ instrument, thereby increasing the sensitivity of the analysis for the screened metabolites. The method was quantitative for the majority of the screened metabolites, enabling for estimation of the amounts found on the samples building materials. Finally, the method was used for analysis of dust samples collected in a water-damaged kindergarten. As a result, several macrocyclic trichothecens and spirocyclic drimanes were detected and quantified in the dust for the first time.

The analytical tools developed and optimized during this PhD study were successfully applied in analysis of secondary metabolites and bioactive compounds produced by *Stachybotrys* spp. and *Chaetomium* spp. This methodology represents a significant advance in the detection of fungal metabolites in indoor samples, including dust samples. As such, this PhD study could have an impact on our understanding of the relationship between mould exposure and sickness. Furthermore, the presented methodology is applicable to compounds produced by other commonly found indoor species.

Sammenfatning

At leve og arbejde i svampeinficerede bygninger kan være skadelig - både med hensyn til helbredet og økonomien. Dette gælder især for hypersensitive, astmatikere og allergiske personer, der ofte oplever forværring af deres tilstand, når de befinder sig i et vådt, svampeinficeret indeklima. Mennesker som ellers er sunde kan også opleve negative helbredspåvirkninger såsom udslæt, hovedpine, svimmelhed og kronisk træthed. Under deres vækst på byggematerialer producere og frigiver svampene mange forskellige komponenter. Disse komponenter er mistænkt for at forårsage negative helbreds påvirkninger, men deres årsagssammenhæng er endnu ikke blevet dokumenteret. Svampene producere biomasse i form af mycelium og sporer sammen med en række sekundær metabolitter og andre bioaktive stoffer. Når svampebiomassen tørrer ind bliver hele, levedygtige sporer, fragmenterede sporer og myceliedele, der indeholder sekundær metabolitter og bioaktive stoffer, frigivet fra byggematerialet.

Mangfoldigheden af forskellige svampearter i indeklimaet er så stor som antallet af bioaktive stoffer de producere. Dette PhD studium fokuserer på svampearter karakteriseret som såkaldte tertiære kolonisatorer, det vil sige *Stachybotrys* og *Chaetomium* spp. Både *Stachybotrys* og *Chaetomium* spp. kræver høj vandaktivitet for optimal vækst ($a_w \sim 0{,}98$), som under indeklimaomstændigheder ofte er ensbetydende med alvorlige vandskader snarere end kun højt niveau af kondensering. Derfor er tilstedeværelsen af disse arter og/eller deres metabolitter i indeklimaet en god indikator af en gammel eller ny vandskade. Endvidere er sekundær metabolitter produceret af *Stachybotrys* og *Chaetomium* spp. kendte mykotoksiner, hvorfor risikoen for at forårsage negativ indvirkning på helbredet forøges. Derfor var et af hovedformålene med dette PhD studium at udvikle og optimere metoder til kvalitativ og semikvantitativ analyse af sekundær metabolitter og andre bioaktive stoffer produceret af *Stachybotrys* og *Chaetomium* spp.

Den primære analytiske teknik anvendt til dette formål var væskekromatografi koblet til masse-spektrometri. Udnyttelse af avanceret teknologi såsom massespektrometri med høj resolution muliggjorde brugen af standardiseret MS/HRMS biblioteker og databaser over kemiske stoffer til metabolitprofilering af *Stachybotrys* og *Chaetomium* spp. Det præliminære arbejde i dette PhD studium var derfor at lave et MS/HRMS bibliotek, som indeholdte alle internt tilgængelige sekundær metabolitter (~1500). Til metabolitprofileringsformål, blev MS/HRMS biblioteket suppleret med databaser over arts- og slægtsspecifikke sekundære metabolitter beskrevet i litteraturen såvel som foreløbigt identificeret stoffer. Metabolitprofilering af *Stachybotrys* og *Chaetomium* spp. blev udført ved brug af agarkulturer. Derefter blev de fundne sekundære

metabolitter bestemt i ekstrakter af kunstigt inficeret byggematerialer og senere i materialer fra naturligt inficeret bygninger.

Resultaterne for *Chaetomium* spp. viste, at indeklimastammer har betydeligt forskellige metabolitprofiler i modsætning til ikke-indeklima reference stammer. Undersøgelse af *Chaetomium* spp. på kunstigt inficeret byggematerialer viste en præference for træbaseret materialer for disse arter. Almindeligt forekommende *Chaetomium* metabolitter, såsom chaetoglobosins, viste sig at være *C. globosum*-specifik, hvorimod cochliodones and chaetoglobin A var til stede i alle *Chaetomium*-forurenet indeklimaprøver og er derfor kandidater for indeklimaspecifikke *Chaetomium* biomarkører. Endvidere var dette første gang at cochliodones blev observeret i indeklima *Chaetomium* arter. Til kvantificeringsformål blev en semi-kvantitativ UHPLC-DAD-QTOFMS metode udviklet og semi-valideret, hvorved muliggjordes en estimering af mængden af *Chaetomium* metabolitter på byggematerialer. Afslutningsvis blev de beskrevet analytiske værktøjer anvendt til analyse af naturligt inficerede byggematerialer, hvor tilstedeværelsen af alle tidligere fundne metabolitter blev bekræftet.

Resultaterne for *Stachybotrys* spp. viste ingen signifikant forskel i metabolitprofiler fundet *in vitro* og *in vivo*. Undersøgelse af *Stachybotrys* spp. på kunstigt inficeret byggematerialer bekræftede tilstedeværelsen af denne slægt på gips i sammenligning med træbaseret materialer. Metabolitter tilhørende macrocyclic trichothecene-, atranone- og spirocyclic drimane-gruppen blev fundet på både kunstigt og naturligt inficeret byggematerialer. Spirocyclic drimaner viste sig at være gode kandidater til indeklimaspecifikke *Stachybotrys*-biomarkøre, da de både blev produceret af alle indeklima *Stachybotrys* arter såvel som i størst mængde. De bedste ioniserings stoffer fra *Stachybotrys* blev udvalgt og direkte overført til UHPLC-QqQ instrumentet hvorved følsomheden af analysen forøgedes af de screenede metabolitter. Metoden var kvantitativ for størstedelen af de screenede metabolitter, hvorved muliggjordes en estimering af mængderne der kan findes på byggematerialer. Afslutningsvis blev metoden anvendt til analyse af støvprøver indsamlet i en vandskadet børnehave. Som et resultat heraf blev flere macrocyclic trichothecener og spirocyclic drimaner for første gang detekteret og kvantificeret i støv.

De analytiske værktøjer, der er udviklet og optimeret under dette PhD studium, blev med succes anvendt til analysen af sekundære metabolitter og bioaktive stoffer produceret af *Stachybotrys* og *Chaetomium* spp. Denne metodologi repræsenterer et signifikant fremskridt i bestemmelsen af svampemetabolitter i indeklima prøver, heriblandt støvprøver. Som sådan kan dette PhD studium have en indflydelse på vores forståelse af forholdet mellem eksponering for skimmelsvamp og sygdom. Endvidere er den præsenteret metodologi anvendelig til stoffer produceret af andre almindeligt forekommende svampearter i indeklimaet.

List of publications

Paper 1 Birgitte Andersen, **Ina Došen**, Anna Malgorzata Lewinska, Kristian Fog Nielsen (2015) Pre-contamination of new gypsum wallboard with potentially harmful fungal species, *Indoor Air* (**Accepted**), doi: 10.1111/ina.12298

Paper 2 Sara Kildgaard., Maria Maansson, **Ina Došen,** Andreas Klitgaard, Jens C. Frisvad, Thomas Ostenfeld Larsen, Kristian Fog Nielsen, K (2014) Accurate dereplication of bioactive secondary metabolites from marine-derived fungi by UHPLC-DAD-QTOFMS and a MS/HRMS Library, *Mar. Drugs,* **12***,* 3681-3705.

Paper 3 **Ina Došen**, Kristian Fog Nielsen, Geo Clausen, Birgitte Andersen (2016) Potentially Harmful Secondary Metabolites Produced by Indoor *Chaetomium* species on Artificially and Naturally Contaminated Building Materials, *Indoor Air xxxxxx.* (**Accepted**) doi:10.1111/ina.12290

Paper 4 **Ina Došen**, Kristian Fog Nielsen, Christopher B. W. Phippen, Geo Clausen, Birgitte Andersen (2016) *Stachybotrys* secondary metabolites and mycotoxins on artificially and naturally contaminated building materials (**Manuscript in preparation**)

Paper 5 **Ina Došen**, Birgitte Andersen, Christopher B. W. Phippen, Geo Clausen, Kristian Fog Nielsen (2016) *Stachybotrys* mycotoxins: from culture extracts to dust samples, *Anal Bioanal Chem* (**Submitted**)

Poster communications

***Chaetomium* and *Stachybotrys* in water-damaged buildings**

Andersen, Birgitte; Lewinska, Anna Malgorzata; Nielsen, Jakob Blæsbjerg; Došen, Ina; Nielsen, Kristian Fog; Peuhkuri, Ruut Hannele; Rode, Carsten; Clausen, Geo; Thrane, Ulf.

Presented at: 10th International Mycological Congress, Bangkok, Thailand, 2014

Type: Poster – Annual report

Abbreviations

ACN	acetonitril
APCI	Atmospheric pressure chemical ionisation
C. globosum	*Chaetomium globosum*
C. elatum	*Chaetomium elatum*
C. virescens	*Chaetomium virescens*
C. longicolleum	*Chaetomium longicolleum*
C. malaysiense	*Chaetomium malaysiense*
C. homopillatum	Chaetomium homopillatum
DAD	Diode array detector
DCM	Dichloromethane
DG18	Dichloran 18 % glycerol agar
EI	Electron impact ionisation
EIC	Extracted ion chromatogram
ESI	Electro-spray ionisation
eV	Electron Volt
EtAc	Ethyl-acetate
FA	Formic acid
GC-MS	Gas chromatography mass spectrometry
HPLC	High pressure liquid chromatography
HR	High resolution
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
MEA	Malt extract agar
MeOH	Methanol
MRM	Multiple reaction monitoring
MS/HRMS	High resolution tandem mass spectrometry
MS/MS	Tandem mass spectrometry
MTR	Macrocyclic trichothecens
Q	Quadrupole
QqQ	Triple quadrupole
Q-TOF	Quadrupole time of flight
OAT	Oatmeal agar
PCDL	Personal compound database and library
RT	Retention time

PDA	Potato dextrose agar
PEI	Polyethyleneimine
S. chartarum	*Stachybotrys chartarum*
S. chlorohalonata	*Stachybotrys chlorohalonata*
S. nephrospora	*Stachybotrys nephrospora*
SSE	Signal supression and enhancement
TOF	Time of flight
UHPLC	Ultra high performance liquid chromatography
UV-Vis	Ultraviolet/visible light
V8	V8 juice agar
YES	Yeast extract sucrose agar

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1. Introduction

1.1. Introduction to the work performed in the thesis

The primary aim of this project was the establishment of an analytical methodology for the identification, detection and quantification of mycotoxins and other bioactive fungal secondary metabolites present in the indoor environment. Analytical methods established during this project were based on the use of liquid chromatography – mass spectrometry and covered everything from sample preparation to quantitative method validation. The most significant development in the analytical methodology was the identification of secondary metabolites utilizing MS/HRMS libraries and databases. The methodology was subsequently used in analysis of secondary metabolites produced by indoor species belonging to the genera of *Chaetomium* and *Stachybotrys*.

The presence of these two genera in indoor environment is often connected to water ingress as they require high water activity (minimum $a_w > 0.91$) for growth. During their growth on wet building materials, these species produce an array of secondary metabolites that may be responsible for the negative health effects some people experience when living or working in fungal contaminated buildings. With this in mind, the analytical methodology developed in this project was utilised in targeted approaches to identify as many secondary metabolites produced by *Chaetomium* spp. and *Stachybotrys* spp. as possible. Metabolite profiles of *Chaetomium* and *Stachybotrys* spp. were first obtained from pure agar cultures (*in vitro*). Subsequently, the same metabolites were screened for on artificially and naturally contaminated building materials (*in vivo*). Finally, dust, as the most common route of exposure to the bioactive fungal metabolites, was also evaluated for the presence of *Stachybotrys* metabolites. Characterisation of *Chaetomium* and *Stachybotrys* metabolites found on wet building materials and in dust using the presented methodology, could provide the necessary basis for toxicological and epidemiological studies. Furthermore, the presented methodology can be transferred to other fungal species commonly found in the indoor environment, thereby providing the knowledge for assessment of fungal secondary metabolites as causal factors for damp building related illnesses.

The work presented in this thesis is divided into two sections: i) introduction and ii) results and discussion. Publications resulting from this work have been arranged according to the project aim as presented in Fig. 1.

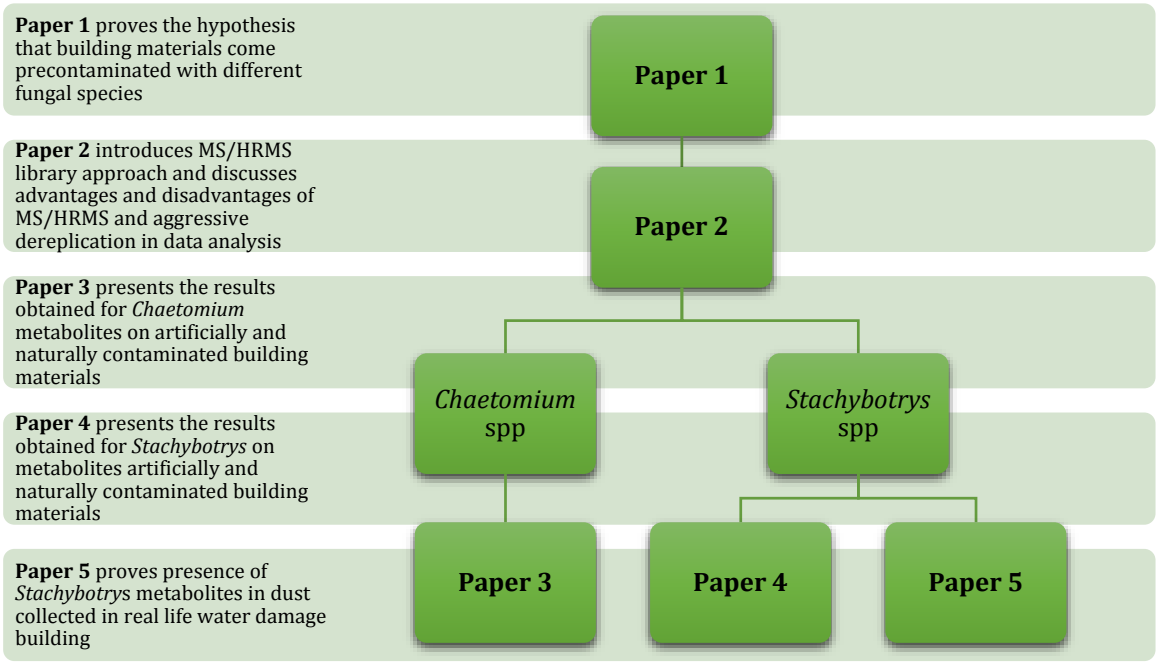


Fig. 1 The overview of the publications in this thesis according to the topics covered

The result section is divided in two subsections: i) methodology development (Section 2.1) and ii) *Chaetomium* and *Stachybotrys* metabolites in indoor environment (Section 2.2).

Section 2.1 discusses different aspects of the analytical methodology as well as challenges and results obtained during method development and optimisation.

Section 2.2 summarises and discusses separately the results obtained on *Chaetomium* and *Stachybotrys* metabolites from samples collected on artificially or naturally contaminated building materials as well as in dust.

Finally, future perspectives within the field of research as well as analytical methods are presented.

1.2. Indoor fungi

1.2.1. *Indoor associated fungal genera/species*

Various microorganisms among which are filamentous fungi are ubiquitous in the indoor environment (Hunter et al., 1988; Flannigan, 2011). The reported indoor mycobiota (fungal diversity) depends on several factors such as geographical position, climate, type of building materials, availability of water on the materials, as well as sampling and isolation procedures (Samson, 1985; Nielsen PhD thesis, 2002; Andersen et al. 2011).

Outdoor air is certainly important source of fungal spores and hyphal fragments in the indoor environment due to the natural ventilation and air exchange between outdoors and indoors (Sneller and Roby, 1979; Flannigan, 2011). Humans and their pets also contribute to contamination of indoor environment by bringing in fungal spores and fragments on clothes, shoes and skin from outdoors (Flannigan, 2011). On the other hand, literature reports little variation in mycobiota of indoor building materials in different parts of the world, suggesting widespread adoption of certain building materials (e.g. gypsum wallboard) and generally decreased air change rates in buildings (Flannigan and Miller, 2011). Only certain species present in indoor environment can grow on particular building material, and each species has its more and less preferred materials. Hence, combination a of given a_w and type of material will result in growth of one or few species on that material that are called associated fungi (Grant et al., 1989; Nielsen et al., 1999).

The choice of sampling methods (e.g. air sampling, surface sampling, cultivation etc.) in the indoor environment is crucial in identification of indoor mycobiota, as no single method is capable of detecting all fungal species (Andersen et al., 2011). Furthermore, fungal diversity of indoor air, house dust and mouldy building materials can vary significantly when they are compared to each other (Andersen et al., 2011; Gravesen, 1972; Gravesen, 1978; Gravesen et al., 1999; Wickman et al., 1992). The choice of agar media for isolation is also important for identification of indoor present species, as the properties of particular agar media will influence which genera/species will grow and sporulate (Andersen et al., 2011). Several studies suggested that V8 juice agar supports good growth and sporulation of most indoor fungi (Andersen et al., 2011; Andersen and Nissen, 2000; Gravesen, 1972; Larsen and Gravesen, 1991; Samson et al., 2010). However, V8 has to be supplemented with other agar media in order to detect dust fungi such as *Wallemia* spp. and *Eurotium* spp. or in order to perform species identification of *Penicillium* spp. (Andersen et al., 2011). It should be noted that cultivation methods are only able to detect viable fungal spores. In order to obtain full representation of indoor mycobiota, cultivation methods should be supplemented with surface sampling using Scotch tape (Andersen

et al., 2011), swab sampling, as well as with molecular identification techniques (e.g. DNA sequencing, ribo-typing, PCR-based fingerprinting) and chemical analysis of fungal chemical biomarkers (proteins, *β*-glucans, MVOCs and secondary metabolites) (Samson et al., 2010). Moreover, certain species frequently found in indoor environment such as *P. italicum*, *P roqueforti*, *P. digitatum*, *P. glabrum*, *P. citrinum* cannot be associated with mouldy building materials, but rather with the presence of mouldy food and plants in the indoor environment (Andersen et al., 2011; Samson et al, 2010). Clearly, these species should not be considered as building material associated fungi and should be distinguished from true building materials contaminants such as *P. chrysogenum* or *P. brevicompactum*. Table 1 compiles most commonly reported indoor species.

Table 1 Fungal genera/species commonly reported as part of indoor mycobiota*

Genus	Species	Commonly found on	Minimum required a_w for growth
Wallemia	*sebi*[a]	Dust	0.69-0.75
Aspergillus	*ruber*[a,e]	Gypsum wallboard, dust	0.70-0.71
Aspergillus	*amstelodami*[a,d]	Insulation, wood, fabrics and textile, dust	0.71-0.76
Eurotium	*repens*[a]	Insulation, wood, dust	0.72-0.74
Aspergillus	*wentii*[a]	Gypsum wallboard	0.73-0.75
Aspergillus	*versicolor*[b]	Most materials, including dust	0.74-0-79
Aspergillus	*flavus*[b]	Insulation, gypsum wallboard, wood-based materials	0.78-0.80
Aspergillus	*sydowii*[b]	Insulation, gypsum wallboard, wood, ceiling tiles, textile	0.78-0.81
Penicillium	*brevicompactum*[b]	Wood-based materials	0.78-0.82
Penicillium	*chrysogenum*[b]	All materials	0.78-0.85
Penicillium	*expansum*[b]	Wood-based materials	0.82-0.85
Cladosporium	*sphaerospermum*[b]	Paint, wood, wallpapers, window sills, caulking	0-83-0.84
Cladosporium	*cladosporoides*[b]	Paint, wood, wallpapers, window sills, caulking	0.83-0.88
Cladosporium	*herbarum*[b]	Paint, wood, wallpapers, window sills, caulking	0.85-0.88
Alternaria	*alternata*[b]	Wood, ceiling tiles	0.85-0.88
Alternaria	*tenuissima*[b]	Wallpapers, gypsum wallboard	0.85-0.90
Aspergillus	*fumigatus*[b]	Insulation, gypsum wallboard, wood-based materials	0.85-0.94
Epicoccum	*nigrum*[b,f]	Wood	0.86-0.90
Aspergillus	*niger*[b,f]	Insulation, gypsum wallboard, wood-based materials	
Aspergillus	*ustus*[b,f]	Insulation, gypsum wallboard, wood	
Penicillium	*coryophylum*[b,f]	Most materials	
Penicillium	*crustosum*[b,f]	Gypsum wallboard, wood-based materials, textile	
Penicillium	*palitans*[b,f]	Most materials, but prefers wood-based	
Penicillium	*solitum*[b,f]	Insulation	
Ulocladium	*chartarum*[c]	Insulation, wood-based materials, ceiling tiles, textile	0.89
Trichoderma	*harzianum*[c]	Insulation, wood, gypsum wallboard, textile	0.90
Trichoderma	*viride*[c]	Gypsum wallboard, wood, textile	0.90
Verticillium	*lecanii*[c]	Wood-based materials, ceiling tiles, fabrics and textile	0.90
Stachybotrys	*chartarum*[c]	Gypsum wallboard, insulation, fabrics and textile	0.91-0.94
Stachybotrys	*chlorohalonata*[c]	Gypsum wallboard, insulation, fabrics and textile	0.91-0.94
Stachybotrys	*echinata*[c]	Gypsum wallboard, insulation, fabrics and textile	0.91-0.94
Neosartorya	*fischeri*[c]	Leather, paper	0.93
Chaetomium	*globosum*[c,f]	Wood-based and cellulose containing materials, insulation	
Chaetomium	*elatum*[c,f]	Wood-based and cellulose containing materials, textile	
Phoma	*glomerata*[c,f]	Paint, wood, wallpapers, caulking especially in bathroom	

*References: Nielsen, PhD thesis, 2002; Flannigan and Miller, 2011; Andersen et al., 2011; Samson et al., 2010; Northolt, PhD thesis, 1979; Prezant et al, 2008.

[a] Extremely xerophilic fungi minimum $a_w \leq 0.75$

[b] Moderately to slightly xerophilic fungi minimum a_w 0.75-0.89

[c] Hydrophilic fungi minimum $a_w \geq 0.90$

[d] Formerly *Eurotium amstelodami*

[e] Formerly *Eurotium rubrum*

[f] Minimum a_w required for growth not reporten in literature

1.2.2. *Factors influencing the growth of fungi in indoor environment*

As mention previously, the fungal growth in indoor environment depends on several factors of which the most important are water activity (a_w) and composition of the material (Flannigan and Miller, 2011). Regardless of the nutrients available for fungi on the given material, the primary requirement for fungal growth is the availability of the water in the material (Flannigan and Miller, 2011). The fraction of water in the material that is not chemically bound, and thereby is available for fungi to utilise it, is called water activity (a_w). Water activity, a_w is defined as the ratio of partial vapour pressure of water in the material (substrate) and partial vapour pressure of pure water at the same temperature and pressure. Water activity, a_w should not be confused with water content of the material. Different materials have different water absorption properties and different materials with the same water content often have different a_w values (Flannigan, 1993).

Every fungal species has different requirements for minimum a_w necessary for growth (Table 1). Whilst minimum a_w for growth can vary significantly for different fungal species (up to 0.3), literature reports that optimum a_w for most species is not that large and lies between 0.9-0.99 (Ayerst, 1969; Flannigan and Miller, 2011). Based on the minimum a_w required for their growth, fungi are categorized to i) extremely xerophilic, minimum $a_w < 0.75$; ii) moderately to slightly xerophilic, minimum a_w 0.75-0.89; and iii) hydrophilic, minimum $a_w \geq 0.90$ (Table 1, Lacey et al., 1980). It should be noted that all fungal species are generally hydrophilic i.e. water-loving, however some species are more tolerant to the conditions with lower a_w. Grant et al. (1989) classified indoor fungi based on field studies of a_w to: i) primary colonizers, capable of growing at low a_w (< 0.8), such as *Wallemia* spp., *Eurotium* spp., *Penicillium* spp.; ii) secondary colonizers, growing at moderate a_w (0.80-0-90), such as *Cladosporium* spp., *Aspergillus* spp.; and iii) tertiary colonizers, requiring $a_w > 0.9$, such as *Chaetomium* and *Stachybotrys* spp. (Grant et al., 1989; Flannigan, 1991; Nevalainen, 1993; Nielsen et al., 1999).

In the real-life buildings, the distribution of the water in the materials is never uniform and prone to constant fluctuations (Flannigan and Miller, 2011). Hence, different fungal species belonging to so-called primary, secondary and tertiary colonisers can be present on different parts of the same material, depending on the a_w of a particular part of the material, which was demonstrated by Grant et al. (1989). In cases where materials are thoroughly wetted and exhibit $a_w > 0.90$, the primary invaders will be species belonging to so-called tertiary colonisers such as *Chaetomium* and *Stachybotrys* spp. (Flannigan and Miller, 2011).

There are many sources of water in the indoor environment. Condensation is one of the most common source of water in the indoor environment, due to the regular human activities such as

cooking, showering and laundering (Oxley and Gobert, 1983; Christian, 1993). Moreover, condensation can occur on inside surfaces of the external walls, especially in poorly insulated buildings, in cold-temperate climates and/or during winter. In these cases, condensation occurs when the warm moist air comes into the contact with the surface that is cooled to the dew point temperature (Flannigan and Miller, 2011). Condensation can also occur due to the thermal bridges that can create micro-climates with high a_w, such as in basements and under-floor crawl spaces as well as in roof spaces (Flannigan and Miller, 2011). The extreme cases include water ingress due to the flooding, melting snow, heavy rain, pipe leakage or dysfunctional building construction. (Andersen and Nissen, 2000; Andersen et al., 2011; Dales et al., 2008). In these cases, water ingress does not only cause damage to the building materials present, but it is also often followed by health complaints of the occupants of such buildings (WHO guidelines, Dampness and Mould, 2009).

The other important factor, besides the a_w, that influences which fungal species will grow on certain material is composition of that material. Most of the indoor fungi are saprotrophs (Flannigan and Miller, 2011), which means that they obtain their nutrients from decaying organic matter. They are able to degrade most of the natural and fabricated organic materials, especially if materials are hygroscopic (Samson et al., 2010; Gravesen et al., 1994; Nielsen, PhD thesis, 2002; Adan, PhD thesis; 1994). The most susceptible materials for fungal growth and biodeterioration are the ones containing natural organic polymers such as cellulose, hemicellulose and lignin, which can be broken down to simple sugars that are further utilised by fungi (Flannigan and Miller, 2011). Some fungal species such as *Chaetomium* and *Stachybotrys* spp. show strong or very strong cellulolytic activity (Siu, 1951). On the other hand, other fungal species such as *Eurotium* spp. as well as some *Aspergillus* and *Penicillium* spp., although not possessing cellulolytic activity are still able to contaminate indoor environment (Flannigan and Miller, 2011; Siu, 1951).

Materials predisposed for fungal growth primarily include wood including processed and modified wood materials such as OSB, chipboard, plywood and MDF, which are all, due to their composition highly susceptible to fungal growth (Nielsen, PhD thesis, 2002; Viitanen and Bjurman, 1995; Seifert and Frisvad, 2000). Another material of interest is gypsum wallboard as it is one of the most commonly used materials, both in Danmark (Nielsen, PhD thesis, 2002, Andersen et al., 2011) and worldwide (Flannigan and Miller, 2011). Gypsum wallboard is normally reinforced with the paper that makes it extremely prone to fungal contamination especially by cellulolytic *Stachybotrys* spp. (Miller et al., 2000; Gravesen et al., 1994; Andersen et al. 2011; Flannigan and Miller, 2011; Miller and McMullin, 2014). Moreover, all structural, finishing and furnishing materials such as wallpapers, insulation, cotton and cardboard are all

highly susceptible to fungal growth (Flannigan and Miller, 2011). Plastic materials and paints are also at risk of fungal contamination and decomposition as several species can utilise most plasticisers (*A. niger*, *Chaetomium* spp., *A. flavus*, *Aureobasidium pullulans*; Caneva et al., 2003) or water-based and/or acrylic paints (*Aureobasidium pullulans*, *Cladosporium* spp., *Alternaria* spp.; Nielsen, PhD thesis, 2002; Winters et al., 1975). Even completely inorganic materials such as glass, steel, brick and concrete can become a target of fungal contamination due to the adsorbed dust, organic debris and volatiles condensed on the surface of the materials creating the layer in which fungi grow and thrive (Flannigan and Miller, 2011).

Another factor that can potentially influence fungal growth on the building material is temperature. Most of the indoor fungi are mesophiles (Flannigan and Miller, 2011), which means they grow the best at moderate temperatures between 20 and 40 °C. Some of them are able to grow at temperatures as low as 0 °C (e.g. refrigerated food contaminants belonging to *Cladosporium* and *Penicillium* spp.) or as high as 55 °C (*Aspergillus fumigatus,* Flannigan and Miller, 2011). However, most of the indoor species have their optimum in the range 20-30 °C (Flannigan and Miller, 2011). Temperature change can also have indirect effect on fungal growth in indoor environment by influencing the change in minimum a_w at which particular species grow (Grant et al., 1989; Nielsen et al., 2004).

1.2.3. *Products of fungal growth present in the indoor environment*

During their growth on building materials, fungi produce and release a vast array of compounds. The presence of these fungal products in indoor environments is suspected to be, either individually or collectively, responsible for some of the adverse effects seen in occupants of damp buildings. The primary product of fungal growth on building materials is fungal biomass in form of mycelium and spores. Furthermore, fungi produce an array of volatile compounds (MVOCs) often causing the building to smell as the "old basement", as well as low-molecular-weight solid metabolites (fungal secondary metabolites) of which many possess some form of bioactivity and some are known to be toxic (mycotoxins) (Miller and McMullin, 2014).

Both fungal mycelium and spores contain various proteins and glycoproteins that are antigenic, whilst some of them are also allergenic (Ellis and Day, 2011). Literature reports have shown that fungal proteins are capable of causing IgE mediated allergic reaction (type I allergy) (Flannigan et al., 1991; Lacey, 1991; Gravesen et al., 1994) as well as non-IgE histamine release (Larsen et al., 1996). For many fungal species, which are commonly reported in the indoor environment IgE mediated allergy has been demonstrated (Simon-Nobbe et al., 2008). The

indoor associated fungal species most commonly connected with allergy occurrence are *Alternaria alternata*, *Epicoccum nigrum*, *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp. (Ellis and Day, 2011), however type I allergy has also been demonstrated for *Chaetomium* and *Stachybotrys* spp. (Simon-Nobbe et al., 2008). Some fungal species such as *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp. produce large amounts of small, dry spores that are easily released into the air (Andersen et al., 2011). Other species such as *Trichoderma* spp., *Ulocladium* spp., *Chaetomium* spp. and *Stachybotrys* spp. produce large spores, a small quantitiy of spores or spores in slime heads that are not readily airborne (Andersen et al., 2011). Aside from whole viable spores (3-30 μm) fragmented spores (1-5 μm) and mycelium parts (0.5-1 μm) from surface of the building materials (Gorny et al., 2002; Kildesø et al., 2003) are also released into the indoor environment when the fungal biomass dries out. Fragmented spores and mycelium parts are collectively called micro-particles (Gorny et al., 2002; Kildesø et al., 2003; Madsen 2012). It has been reported that damp buildings have higher percentage of both spores and microparticles (Cho et al., 2006; Foto et al., 2005; Green et al., 2011; Reponen et al., 2007). Micro-particles although not viable, contain secondary metabolites and are suggested to be the vehicle of exposure in the mouldy buildings (Green et al., 2006).

In addition to this, the fungal cell wall is built from (1→3)-β-D-glucan, a polysaccharide that is able to trigger an inflammatory reaction similar to endotoxin (Rylander, 1993; Rylander, 1996; Rand and Miller, 2011). It should be noted that cell walls of some fungal species such as *Wallemia sebi*, produce mixed-linkage glucans containing also (1→6)-β-linked side branches (Ooi and Liu, 1999; Schmid et al., 2001), which may cause different effects with comparison to (1→3)-β-D-glucan (Rand and Miller, 2011; Foto et al., 2006; Iossifova et al., 2008a).

All of the aforementioned products of fungal growth have been associated with occurrence of negative health effects in occupants of mouldy buildings. Table 2 gives an overview of all products of fungal growth in the indoor environment relevant from aspect of exposure and occurrence of negative health effects. Existing toxicological and epidemiological studies for *Chaetomium* and *Stachybotrys* metabolites are presented in section 1.4.

Table 2 Products of fungal growth in the indoor environment relevant for exposure and occurence of negative health effects*

Fungal agent	Short description	Activity
Viable spores	Unicellular or multicellular units of sexual reproduction, with approximate size of 3-30 μm	Possibly allergenic, antigenic and carriers of secondary metabolites/mycotoxins
Proteins	Products of fungal cells with versatile functions, with molecular weight range of 10-70 kDa	Antigenic and allergenic; capable of causing IgE mediated allergic response (type I allergy)
(1→3)-β-D-glucan	Structural component of fungal cell wall	Non-allergic, inflammatory effect
Micro-particles	Non-viable micron to sub-micron size (0.5-5 μm) particles containing fragmented spores and mycelium	Possibly allergenic, antigenic and carriers of secondary metabolites/mycotoxins
MVOC	Highly volatile organic low-molecular weight compounds produced by growing fungi as part of their primary and secondary metabolism	Responsible for mouldy odour in the buildings, no epidemiological evidence connecting them to negative health effect
Fungal secondary metabolites /mycotoxins	Low-molecular-weight non-volatile products of secondary metabolism with various chemical structures. Airborne attached to the spores and micro-particles	Most of them are bioactive, whilst some are toxigenic.

*References: Nielsen et al., 1999; Rand and Miller, 2011; Miller and McMullin 2014; Ellis and Day 2011; Nevalainen et al., 2015; Täubel et al., 2011

1.3. Bioactive metabolites from hydrophilic fungi

As previously stated, an excess of water in the indoor environment will lead to high a_w of building materials due to absorption and consequently a prevalence of species such as *Chaetomium* and *Stachybotrys* spp. (Andersen and Nissen, 2000; Flannigan and Miller, 2011). A damp indoor environment and the corresponding presence of fungi can lead to negative health effects not only in asthmatic and hypersensitive individuals but also in otherwise healthy people (WHO Regional Office for Europe, 2009; Miller and McMullin, 2014). *Chaetomium* and *Stachybotrys* spp. are frequently found on damp building materials both in Denmark and worldwide (Andersen et al., 2011; Miller and McMullin, 2014). *S. chartarum senso lato* is one of the most studied organisms in indoor environment due to the production of toxic metabolites and their connection to the occurrence of negative health effects (Johanning et al., 1996; Croft et al., 1986). With respect to indoor *Chaetomium* metabolite production, existing studies have mostly been focused on metabolites produced by *C. globosum* (Fogle et al., 2007; McMullin et al., 2013a; McMullin et al., 2013b). The presence of *Chaetomium* and *Stachybotrys* metabolites have been reported on building materials on several occasions (Nielsen et al., 1999; Fogle et al., 2007; Täubel et al., 2011; Vishwanath et al., 2009). It can be expected that secondary metabolites

produced on the building materials will remain in the indoor environment long after the contamination occurs due to their non-volatile and often heat-stabile nature (Nevalainen et al., 2015). Hence, the presence of *Chaetomium* and *Stachybotrys* metabolites in indoor environment is relevant not only for exposure/health effect studies but can also serve as a good indicator of past water damage and possible need for remediation. This section provides short overview of *Chaetomium* and *Stachybotrys* bioactive metabolites found on damp building materials.

1.3.1. Chaetomium *bioactive metabolites*

Currently there are more than 200 known bioactive metabolites produced by different *Chaetomium* spp. (Zhang et al., 2012). The most commonly found 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). Metabolites produced by frequently found indoor *Chaetomium* species include chaetoglobosins, chlorinated and non-chlorinated azaphilones (chaetoviridins and chaetomugilins) and chaetomin (Miller and McMullin, 2014) (Fig. 2). So far, chaetoglobosin A and C (Nielsen et al., 1999; Fogle et al., 2007; Täubel at al., 2011) as well as chaetomin have been reported on building materials (Vishwanath et al., 2009). Nielsen et al., (1999) also reported unknown compound eluting at 21.4 min, with a UV spectrum corresponding to that of chaetoviridin. All these metabolites were reported to have some kind of bioactivity. Chaetoglobosins A and C interact with mammalian cells by binding to actin (Fogle et al., 2007), and have been shown to be lethal to various cell lines (Alberts et al., 2002; Umeda et al., 1975). Their intravenous toxicity has been demonstrated in animal tests (Ohtsubo et al., 1978; Udagawa et al., 1979), however no study of inhalative toxicity has yet been published. Azaphilones (chaetoviridins and chaetomugilins) have been reported to have cytotoxic, antimicrobial and antifungal activity (Zhang et al., 2012; Yamada et al., 2012; Li et al., 2013; McMullin et al., 2013b; Xu et al., 2014). Antibiotic, antifungal and antiviral activities were also reported for chaetomin (Cole and Cox, 1981; Kumar et al., 2013). Some *Chaetomium* spp. were reported to produce acutely toxic and carcinogenic sterigmatocystin (Rank et al., 2011; Sekita et al., 1981a), however these species has never been found indoors. Throughout this PhD project, we investigated production of above-mentioned as well as all other *Chaetomium* bioactive metabolites so far described in literature, on damp building materials.

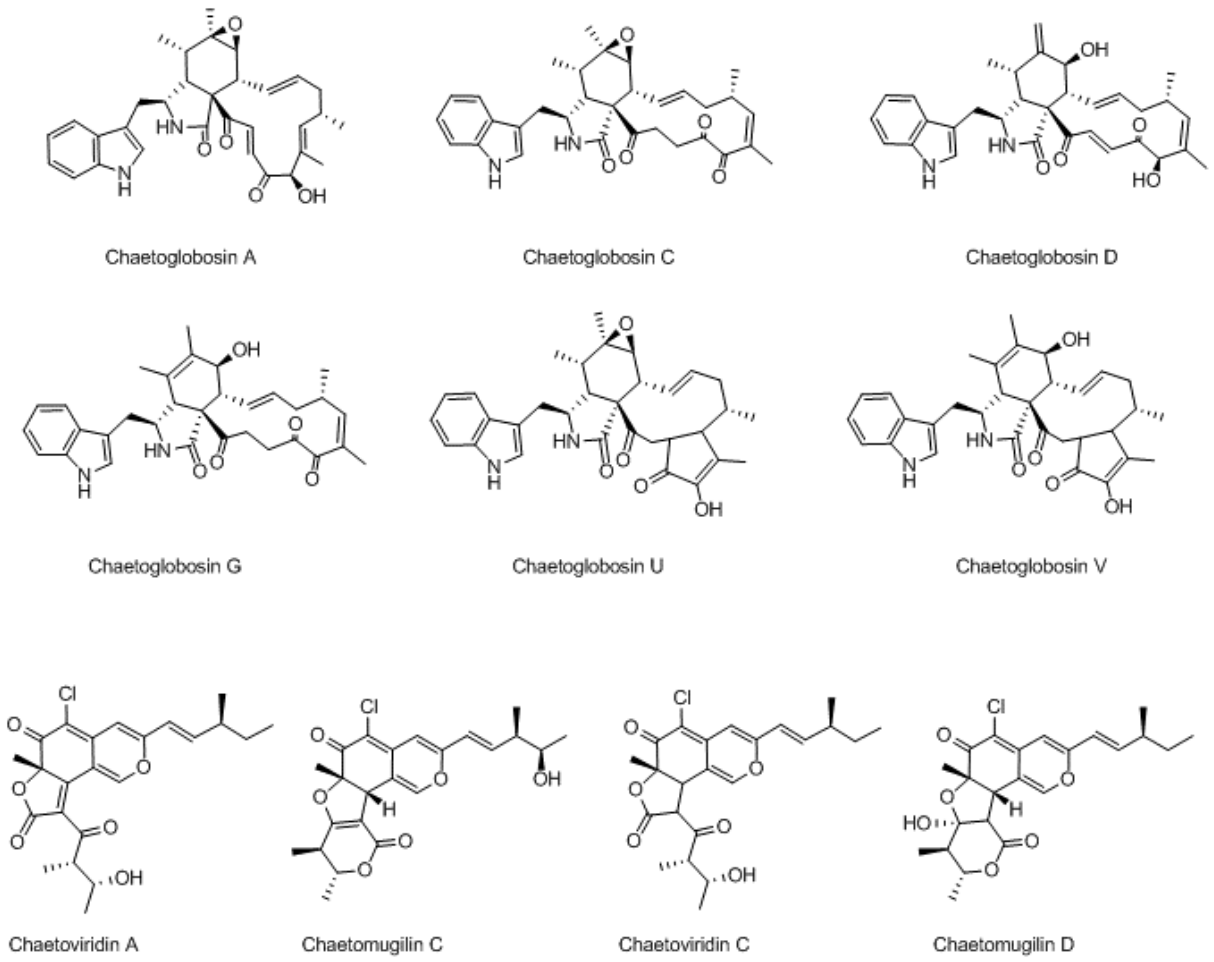


Fig. 2 Chaetoglobosins and azaphilones produced by *C. globosum*

1.3.2. Stachybotrys *bioactive metabolites*

At the time of writing there are approximately 140 bioactive *Stachybotrys* compounds reported in the literature (Antibase, Laatsch 2012; Hinkley and Jarvis, 2000; Nielsen, 2002; Li et al., 2013; Ma et al., 2013; Wu et al., 2014). *S. chartarum* and *S. chlorohalonata* are most frequently found species in the indoor environment (Andersen et al., 2011; Flannigan and Miller, 2011; Miller and McMullin, 2014). *S. chartarum* exists in two chemotypes S and A, differing only in the nature of the metabolites they produce (Andersen et al., 2002; Andersen et al., 2003). *S. chartarum* chemotype S produces macrocyclic thrichothecens (MTR) which are some of the most cytotoxic compounds currently known (Nielsen PhD thesis, 2002; Ueno, 1983; Feinberg and McLaughlin, 1989). They are known to be amongst most potent inhibitors of protein synthesis in eukaryotes (Feinberg and McLaughlin, 1998; Wannemacher and Wiener, 1997) and are believed to be responsible for severe mycotoxicoses in both farm animals and humans (Flannigan and Miller,

1994; Forgacs, 1962; Jarvis et al., 1986). Besides high cytotoxicity, MTR are also capable of inducing inflammation (Nielsen, 2003). Compounds belonging to MTR group are satratoxins and roridins. So far, satratoxins G and H have been reported on damp building material (Nielsen et al., 2003; Täubel et al., 2011; Vishwanath 2009).

Both *S. chartarum* chemotype A and *S. chlorohalonata* produce atranones and dolabellanes (Andersen et al. 2002; Andersen et al. 2003). Dolabellanes show low cytotoxicity (Rodriguez et al., 1998; Iwashima et al., 2002), whilst atranones do not possess cytotoxic activity but do show inflammatory effect and inhibition of protein synthesis (Andersen et al., 2002). Regardless of chemotype, toxigenic strains produce the simple trichothecens trichodermin and trichodermol (Miller and McMullin, 2014) which are interestingly non-cytotoxic although they are precursors in biosynthesis of MTR (Nielsen PhD thesis, 2002; Bamburg, 1976). The most dominant metabolites produced by both chemotypes of *S. chartarum* as well as by *S. chlorohalonata* are spirocyclic drimanes (Miller and McMullin, 2014; Hinkley and Jarvis, 2000; Andersen et al., 2002; Andersen et al., 2003). Current analytical methods analyse for only one spirocyclic drimane, stachybotrylactam, which has been reported on damp building materials by Täubel et al. (2011) and Vishwanath et al. (2009). Spirocyclic drimanes represent large group of bioactive compounds showing broad range of biological activities including enzyme inhibition, disruption of the complement system, inhibition of TNF-a liberation, cytotoxicity and neurotoxicity and stimulation of plasminogen, fibrinolysis and thrombolysis (Hasumi et al., 1998; Kaneto et al., 1994; Kohyama et al., 1997; Nozawa et al., 1997; Nielsen, 2003). One of the aims of this PhD project was to investigate the presence of as many of the *Stachybotrys* metabolites, which have currently been described in the literature, in the indoor environment. Representatives of metabolites belonging to different chemical groups are shown in Fig. 3.

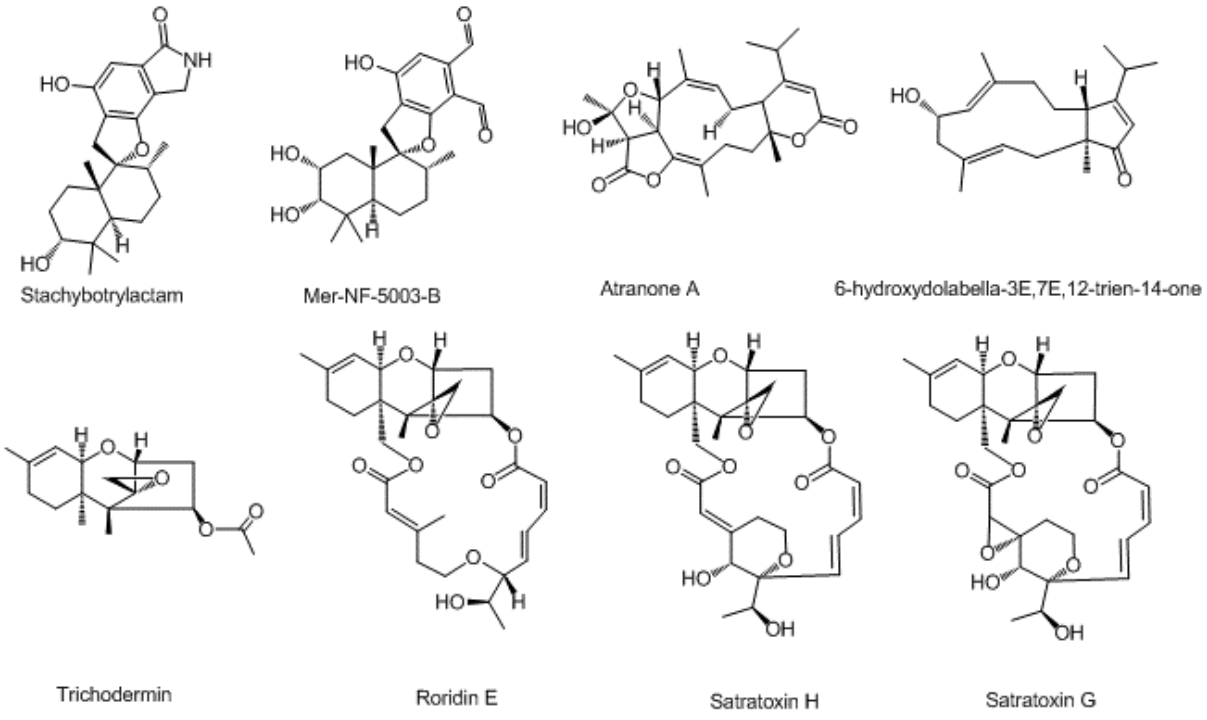


Fig 3. *Stachybotrys* metabolites produced by species found in damp indoor environment

1.4. Health issues related to indoor dampness

The association between presence of moulds and dampness in buildings and occurrence of negative health effects in occupants of these buildings has been recognized since 2005 (Miller and McMullin, 2014; NAS 2004; NIOSH 2012; WHO 2009). Several epidemiological studies in Europe, Canada and USA demonstrated that exposures to fungi in damp indoor environment increase the risk of for asthma, respiratory symptoms, hypersensitivity pneumonitis, rhinosinusitis, bronchitis as well as other respiratory infections (Mendell et al., 2011; Park and Cox-Ganser, 2011; Qansah et al., 2012; Jaakola et al., 2013; Miller and McMullin, 2014). All these negative health effects can be divided in two groups: i) fungi-related allergic and asthmatic symptoms; and ii) fungi-related non-allergenic symptoms (Rand and Miller, 2011). The latter group of symptoms include: lower respiratory tract symptoms, cough, increased susceptibility to lower respiratory tract infections, headaches, fatigue and memory problems, peripheral appendage numbness and other nervous system effects (Rand and Miller, 2011). As this PhD study was conducted with the aim of investigating mycotoxins and bioactive metabolites produced by *Chaetomium* and *Stachybotrys* spp., symptoms of fungi-related allergy and asthma are only briefly mentioned in section 1.3 in connection to possible causal factors of these. Hence,

this section will only focus on reported mycotoxicoses in damp indoor environments contaminated with *Chaetomium* and *Stachybotrys* spp. as well as on a short overview of thus far conducted toxicological studies on their metabolites.

1.4.1. *Reported cases of mycotoxicoses in damp indoor environment*

So far, cases of negative health effects occurring in the damp indoor environment have only been linked to presence of *Stachybotrys* spp. and a causal role of *Chaetomium* metabolites in the occurrence of mycotoxycoses is yet to be documented. Clearly, the damp indoor environment mycobiota does not consist of one but rather several different species, therefore effects on human health is unlikely to be attributed to the presence of single a species or a single product of fungal growth. However, several reports of health issues in dwellings contaminated with *S. chartarum* throughout last 30 years led to more extensive research of *Stachybotrys* spp. in indoor environment whereas in comparison there has been relatively little research into *Chaetomium* spp..

Historically, *Stachybotrys* have been shown to cause severe toxicoses in farm animals fed with contaminated hay (Jarvis et al., 1986; Forgacs and Carl, 1962; Szathmary, 1983; Le Bars et al., 1977; Tantaoui-Elaraki et al., 1994; Pepepljnjak, 1983). First isolation of *S. chartarum* spp. from indoor environment dates back to 19th century, when the fungus was collected from a wallpaper in home in Prague (Miller et al., 2003). Croft et al. (1986) reported first case of a causal link between human disease and a contaminated indoor environment. In this case, the occupants of the dwelling contaminated by *S. chartarum* reported range of symptoms including headaches, sore throat, diarrhoea and fatigue, which all disappeared after the house was cleaned. Other cases included the case of workers from New York working with *S. chartarum* contaminated cardboard and suffered from acute response (Johanning et al., 1993; Johanning et al.,1995; Johanning et al., 1996). Moreover, *S. chartaru*m has been associated with six separate cases of infant idiopathic pulmonary hemosiderosis (Miller et al., 2003). Idiopathic pulmonary hemosiderosis is a rare lung disease characterised by alveolar capillary bleeding and accumulation of haemosiderin in the lungs. This condition is very rare in infants with general prevalence of 0.2-1.2 per 10^6 children per year in the Western world (Dearborn, 1999; Nielsen PhD thesis, 2002). First case was reported in Cleveland, Ohio (Dearborn, 1999) and other cases have been reported in Texas (Elidemir et al., 1999), Kansas City (Flappan et al., 1999), Belgium (cited in Nielsen et al., 2002) and Quebec (cited in Miller et al., 2003). All these case have been associated with presence of *S. chartarum* in indoor environment.

1.4.2. *Toxicological studies on mycotoxins*

As mentioned previously, toxic effects of *Chaetomium* metabolites in humans have not been documented yet. The only two metabolites, for which intravenous toxicity has been demonstrated in animal tests are chaetoglobosin A and C (Ohtsubo et al., 1978; Udagawa et al., 1979). No data on inhalative toxicity currently exists either for these or any other *Chaetomium* metabolite. When it comes to *Stachybotrys* metabolites, studies demonstrating effects of exposure to pure MTR showed that they affect a range of cell functions and biochemical pathways (Rand and Miller, 2011). These effects include depressed macrophage activity (Sorenson et al., 1987; Plasencia and Rosenstein, 1990), inflammatory reaction (Routsalainen et al., 1998), apopotosis (Okumura et al., 1999; Yang et al., 2000), membrane damage (Peltola et al., 1999) and cytotoxicity (Nielsen et al., 2001). Flemming et al. (2002) showed that the effects of *Stachybotrys* spores on rodents were both dose and time dependant. There appears to be a difference in inflammatory response of rodent lungs when exposed to *Stachybotrys* spores and pure satratoxins, suggesting that only exposure to spores stimulates inflammatory tissue reaction, whilst the same effect was not observed upon exposure to pure satratoxin (Rand et al., 2002). *In vitro* studies on rodent macrophages (Nielsen et al., 2001; Huttunen et al., 2004) and boar spermatozoa (Peltola et al., 2002) showed low cytotoxicity for the atranones. Literature reports show that spirocyclic drimanes possess immunosuppressant properties (Kaise et al., 1979) in addition to being endothelin receptor agonists (Roggo et al., 1995), protease inhibitors (Kaneto et al., 1994), cholesterol esterase inhibitors (Sakai et al., 1995), inositol monophosphatase inhibitors (Stefanelli et al., 1996) and also showing cytotoxic properties (Hinkley et al., 1999).

The effects of *Stachybotrys* metabolites in the indoor environment cannot be considered in isolation and the effects of metabolites from other fungal species possibly present must be taken into account. So far a number of in vitro studies have pointed to either inflammatory or cytotoxic effects of spores/metabolites of the following *Aspergillus* species found indoors: *A. versicolor*, *A. fumigatus* and *A. niger* as well as some *Penicillium* spp. (Rand and Miller, 2011; NAS 2004; Rand et al., 2005; Rand et al., 2006).

1.5. Analytical tools used in analysis of secondary metabolites

During this PhD project, two different methods for analysis were developed and utilised. During this development, several aspects of the analytical method were optimised, starting with sampling, sample clean-up, LC-MS separation and analysis as well as identification and quantification of *Chaetomium* and *Stachybotrys* metabolites. This section provides a short overview of the methodology and techniques used throughout this PhD study.

1.5.1. *Sample preparation*

Sampling and sample clean-up are crucial and often the most time-consuming steps in analysis of mycotoxins and other bioactive secondary metabolites (Nielsen, PhD thesis, 2002). This is especially true when analysis is performed directly on materials collected from a water damaged indoor environment. In these cases particular difficulties arise due to the wide variety of different materials found indoors as well as the amount of interfering compounds found in them. Cleanliness of the sample greatly determines the quality of the analysis as whole, as cleaner samples give higher reliability of the obtained data, due to the absence of interfering compounds. When sampling is performed in the indoor environment the variation in type of samples can be significant, as the sample is dependent on the type of building materials on which fungal growth occurs, sampling method, characteristics of fungal species among others. All of these factors can influence the reproducibility and reliability of the analytical method.

Methods of sampling performed in the indoor environment include: i) scraping off fungal biomass from different surfaces (Nielsen et al., 1999; Polizzi et al., 2009), ii) cutting off mould infected wallpapers and other mould-infected surface materials (Vishwanath et al., 2009; Polizzi et al., 2009; Bloom et al., 2007), iii) collecting the fungal biomass with sterile swabs swiped across contaminated surfaces (Peitzsch et al., 2012), iv) dust sampling using vacuum cleaner (Vishwanath et al., 2009; Täubel et al., 2011; Kirjavinen et al., 2015), sterile swabs (Peitzsch et al., 2012), air filters (Gottschalk et al., 2008). Fungi produce an array of chemically diverse compounds with diverse chemical properties and as such no single extraction procedure can provide complete extraction of all metabolites. In addition to that, in cases where samples contain fungal biomass that cannot be physically separated from background material on which it grows (e.g. wallpaper) or is mixed with (e.g. dried fungal biomass in dust) the resulting extract is expected to contain the mixture of fungal metabolites and compounds extracted from background material. Depending on the nature of these compounds, interference can be

encountered to a greater or larger extent with further analysis of target analytes. Nielsen (PhD thesis, 2002) reported that extraction of whole mould infected building blocks with organic solvents is not suitable, due to the large amounts of extracted interfering compounds from extracted materials. Hence, the special care needs to be taken in order to separate fungal biomass from background material, whenever it is possible.

Solvent mixtures used for extraction of different samples collected in the damp indoor environment commonly contain mixture of acetonitrile and water (Gottschalk et al., 2008) or methanol and water (Polizzi et al., 2009). Often formic acid (Nielsen et al., 1999) or acetic acid (Vishwanath et al., 2009) are added to extraction solvent mixtures in order to promote extraction of acidic metabolites. Earlier studies of *Stachybotrys* metabolites mostly based on UV/Vis diode array detection (DAD), clearly suggested spirocyclic drimanes as dominant compounds in the all analysed extracts (Hinkley and Jarvis, 2000; Andersen et al., 2002; Andersen et al., 2003). In order to separate drimanes from MTR and antranones, Hinkley and Jarvis (2000) developed sample clean-up procedure using normal phase solid phase extraction on PEI silica, which is also utilised in some of the more novel analytical methods (Bloom et al., 2007). Only a few existing analytical methods are specifically targeted for *Stachybotrys* metabolites (Bloom et al., 2007; Gottschalk et al., 2008), whilst no method exists that is specifically dedicated to analysis of *Chaetomium* metabolites. Most of the extraction methods are developed as a part of multi-analyte screening method targeting the variety of different secondary metabolites commonly present in the indoor environment (Polizzi et al., 2009; Vishwanath et al., 2009) as such more target approach based on known chemical properties could be beneficial.

1.5.2. *Mass spectrometry based detection of bioactive fungal metabolites*

Most of the existing methods for analysis of fungal secondary metabolites in indoor environment are based on mass spectrometry and are usually combined with gas or liquid chromatography as a separation technique (Nevalainen et al., 2015). Other methods used for measurement of bioactive secondary metabolites from indoor environment include immunochemical methods (Brasel et al., 2005; Charpin-Kadoush et al. 2006; Dietrich et al., 1999) as well as the previously more common HPLC-UV/Vis (Nielsen et al., 1999; Andersen et al., 2002; Andersen et al., 2003; Hinkley and Jarvis, 2000). GC-MS methods for analysis of *Stachybotrys* metabolites (MTR) are reported in the literature (Bloom et al., 2007; Nielsen et al. 2001), however, due to the non-volatile nature of many bioactive secondary metabolites and mycotoxins (Nevalainen et al., 2015), liquid chromatography appears to be the separation technique of choice (Bloom et al.,

2007; Gottschalk et al., 2008; Polizzi et al., 2009; Vishwanath et al, 2009; Polizzi et al., 2014). Different types of mass spectrometers are used in analysis of bioactive secondary metabolites and they generally differ in type of mass analyser. The most commonly used mass analysers in the existing methods dedicated to analysis of bioactive secondary metabolites in the indoor environment are quadrupole and time-of-flight (TOF) mass spectrometers. Most of the existing analytical methods have utilised QqQ instrument, a tandem mass spectrometer based on quadrupole mass analyser (Bloom et al., 2007; Gottschalk et al., 2008; Polizzi et al., 2009; Vishwanath et al, 2009). High resolution TOF combined with quadrupole in tandem mass spectrometer (QTOF) is less commonly used in analysis of bioactive secondary metabolites commonly found in the damp indoor environment (Polizzi et al., 2009; Polizzi et al., 2014).

During this PhD project ultra high performance liquid chromatography (UHPLC) was used as separation technique for all performed analyses. UHPLC was combined with both types of tandem mass spectrometers: QTOF (**Paper 1**, **2**, **3**, **4** and **5**) and QqQ (**Paper 4** and **5**), utilizing both type of mass analysers. In both type of tandem mass spectrometers (QTOF and QqQ) the same type of ionisation technique was used, namely electrospray ionisation (ESI). ESI together with atmospheric pressure chemical ionisation (APCI) is considered as "soft" ionisation technique due to the low amount of energy applied to the compounds, resulting in less fragmentation. This is in contrast to hard ionisation technique such as electron impact ionisation (EI) (de Hoffmann and Stroobant, 2007). ESI is more suitable for polar compounds, whilst APCI works better with medium- and low-polarity compounds (de Hoffmann and Stroobant, 2007). During ESI ionisation, a liquid sample is nebulised, evaporated using the heat and nitrogen gas, and finally ionised under atmospheric pressure using the high electric field (de Hoffmann and Stroobant, 2007). ESI can be performed in positive and negative mode (ESI^+, ESI^-), depending on the chemical properties of the metabolite of interest. All *Chaetomium* and *Stachybotrys* metabolites presented have been analysed in ESI positive mode, as this is considered the most robust and general detection mode . The main challenge with the use of ESI as an ionisation technique is known as matrix effects. Matrix effects are signal suppression or enhancement as a result of the presence of coeluting compounds that may interfere with the signal recorded for the metabolite of interest. Matrix effects will be further discussed in section 1.5.3. The following section will focus on two tandem mass spectrometry techniques used in this PhD study (QTOF and QqQ) and their advantages and disadvantages in analysis of bioactive secondary metabolites and mycotoxins.

1.5.2.1. High resolution tandem MS (MS/HRMS) versus multiple reaction monitoring (MRM)

In the mass analyser, ions are separated based on their mass-to-charge ratio (*m/z*). The determining characteristics of every mass analyser are: sensitivity, mass resolution, accuracy, scan speed or acquisition rate and MS/MS capabilities (de Hoffmann and Stroobant, 2007). TOF mass analyser separates the ions based on the time necessary for each ion to travel through the flight tube in order to reach the detector. TOF instrument provides high mass accuracy and sufficient quality of obtained isotopic pattern to enable correct assignment of recorded masses to elemental composition of the unknown compound. On the other hand, quadrupole ion filters can be used in two ways: i) as a filter that allows only ions with certain *m/z* to pass; ii) to record all passing ions regardless of their *m/z,* thereby obtaining the full mass spectrum (full scan mode). Quadrupole mass spectrometer is cheaper, more robust and easier to use in comparison to TOF mass analyser. They also provide better sensitivity when set to screen only for target analytes, however the obtained mass accuracy and the isotopic pattern quality are insufficient to be used for dereplication.

Both TOF and quadrupole mass spectrometers can be used as a tandem mass spectrometer (MS/MS) instrument, by combining the mass analyser with a collision cell. Q-TOF instrument consists of a quadrupole ion filter and a TOF mass analyser, which can be connected through a collision cell (Fig. 4). This collision cell can be either a quadrupole or hexapole in its design. When a Q-TOF instrument is operated as a tandem mass spectrometer, first, the quadrupole (Q1) isolates the precursor ion, which is further fragmented in the collision cell and finally acquired by a TOF. Thus, Q-TOF provides the combination of a high mass resolution ability of a TOF instrument with the MS/MS capability of a quadrupole, enabling the tentative identification of unknown compounds.

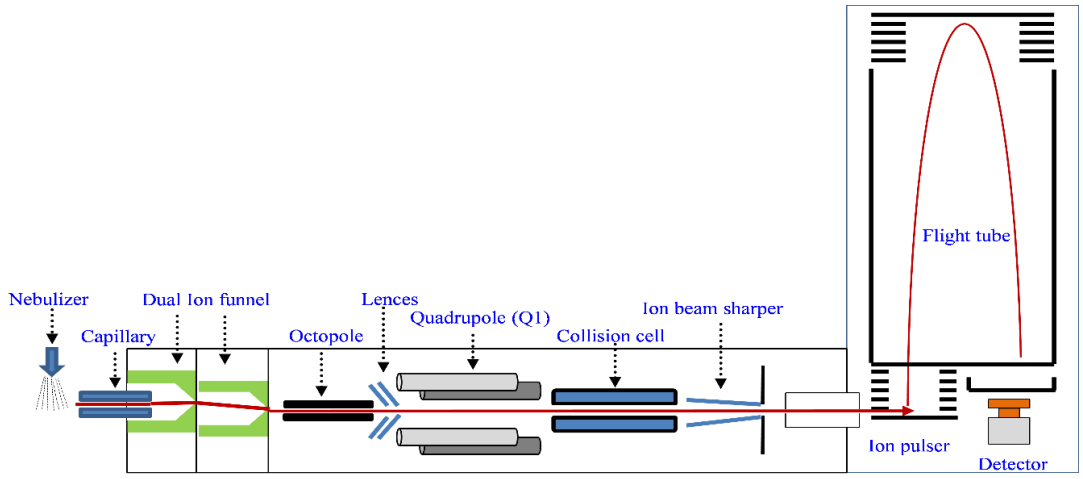


Fig. 4 Schematic overview of a QTOF-MS instrument. Figure modified from 6200Series TOF and 6500 Series Q-TOF LC/MS System, Agilent Concepts Guide (a)

In comparison to Q-TOF, QqQ has TOF mass analyser replaced with another quadrupole mass analyser (Fig. 5). First quadrupole (Q1) has the same function as in the QTOF instrument, selecting the precursor ion, which is passed on to the collision cell where fragmentation occurs. Second quadrupole (Q2) acquires obtained fragments (product ions) from the selected precursor ion.

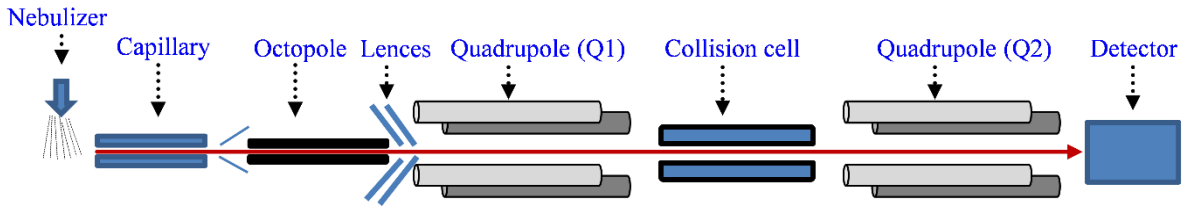


Fig. 5 Schematic overview of a QqQ mass spectrometer. Figure modified from Agilent 6400 Series Triple Quadrupole LC/MS, Agilent Concepts Guide (b)

The different type of experiments that can be acquired with Q-TOF and QqQ instruments are presented in Fig. 6. QqQ spectrometers are traditionally used in targeted quantitative analysis due to increased sensitivity and robustness, where it is usually operated in multiple reaction monitoring (MRM) mode (Fig. 6A). In an MRM method, precursor ion, product ions and collision energies are carefully selected and optimised for each metabolite, in order to provide the best possible signal. The advantage of this method is increased selectivity as the instrument records only ions with selected *m/z*, as well as fast measurement of metabolites from small amounts of sample. The main disadvantage of this method is low resolution of the recorded masses. Another

disadvantage is the limited number of metabolites that can be included in the method. By increasing the number of metabolites in the MRM method, the scan time per MRM decreases, which results in the lower number of data points per peak and may further compromise the quality of the obtained data and quantification.

On the other hand, QTOF instrument can provide both high-resolution mass spectrometry (HRMS) measurement (Fig. 6B) and MS/HRMS measurements (Fig. 6C, D, E). HRMS data provide information about accurate mass and isotopic pattern that can be utilised in dereplication (tentative identification by assigning the recorded mass to the elemental composition). The MS/HRMS data can be obtained by: i) targeted MS/HRMS (Fig. 6C), where precursor ions are manually selected; ii) auto MS/HRMS (Fig. 6D) where HRMS data is recorded together with MS/HRMS data of in cycles, and precursor ions are selected automatically based on their abundance in the complex sample; and iii) all ions MS/HRMS (Fig. 6E), where all ions are fragmented regardless of their intensity.

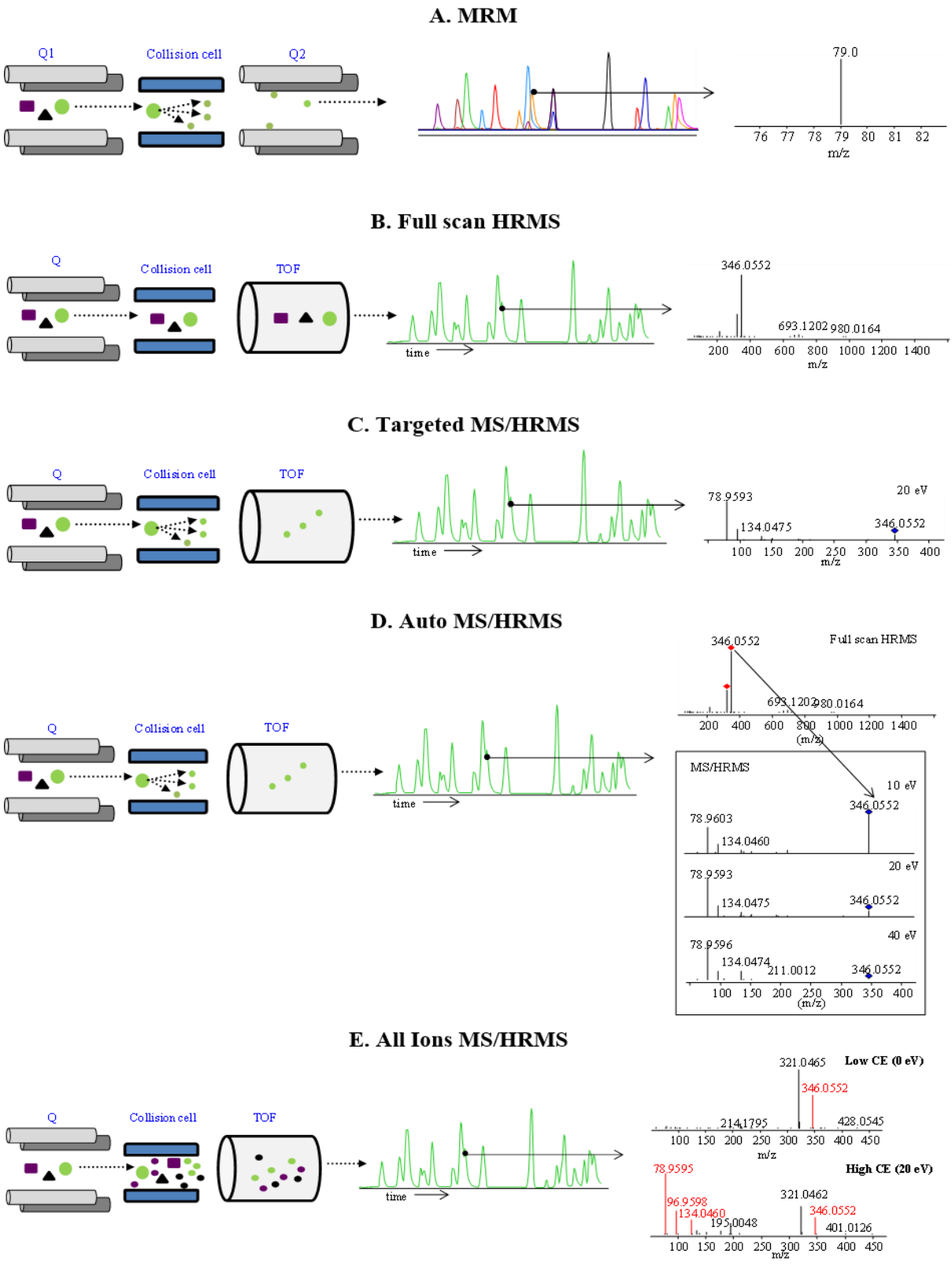


Fig. 6 Schematic overview of the different acquisition experiments possible to obtain by QqQ (A and B) and QTOF instrument (B, C, D and E), showing the different type of data obtained. Figure taken from PhD thesis by Magdenoska, 2015

During this PhD study, the type of experiments acquired on QTOF instrument were HRMS and auto MS/HRMS. HRMS data was acquired in order to obtain accurate mass and isotopic patterns necessary for tentative identification of *Chaetomium* and *Stachybotrys* metabolites present in complex samples, which were not available as standards (aggressive dereplication approach, **Paper 2**, **3**, **4** and **5**). Auto MS/HRMS data was used in identification and detection of *Chaetomium* and *Stachybotrys* metabolites whose MS/HRMS spectra were already available in the MS/HRMS library (MS/HRMS library approach, **Paper 2**, **3**, **4** and **5**). The use of HRMS and MS/HRMS data in data analysis will be further explained in section 2.2.

1.5.3. *Analytical method development, validation and quantification*

The main goal of any analytical method is to provide high reproducibility and reliability without introducing the false positive or negative results. The validation of the analytical method provides the information on method performance parameters, proving its fitness to the intended purpose. When using LC-MS in analysis of mycotoxins and other bioactive fungal secondary metabolites the general validation approach includes determination of : i) linearity, ii) precision and accuracy/recovery, iii) limit of detection and iv) matrix effect (Vishwanath et al., 2009; Varga et al., 2013).

Linearity. The linearity of the analytical method is assessed by creating the calibration curves covering several orders of magnitude (minimum three), depending on the expected amounts of target analytes present in the samples. The approach for creating the calibration curves used throughout this PhD study was external calibration approach. Other approaches include standard addition approach and calibration using internal standard. In external calibration approach, calibration curve is prepared in the surrogate matrix. A surrogate matrix can be either neat solution or extract of the matrix (e.g. extract of dust, wallpaper, building material) which does not contain compounds of interest or contains only the traces of it. Extracted matrix is usually spiked with standards at different levels for matrix-matched calibration. The main challenge in analysis of fungal metabolites present in the indoor environment is unavailability of matrices for matrix-matched calibration. Due to the variety of building materials used in the indoor environment, it is impossible to prepare matrix-matched calibration for every material (matrix). Moreover, the extract of a given matrix (material) can vary significantly depending on the extent to which material was wetted prior to extraction, due to the possible occurrence of material degradation. In the methods dedicated to analysis of fungal secondary metabolites in the indoor environment, the external calibration approach for the assessment of linearity is

often the only possibility (Vishwanath et al., 2009). The use of a standard addition approach would be inefficient, time consuming, and would require large number of samples and would not necessarily provide greater accuracy, whilst calibration using the internal standard is currently not possible due to the unavailability of isotope labelled internal standards.

Precision and accuracy/recovery. The precision and accuracy/recovery are evaluated using non-spiked and spiked samples, which are subsequently subjected to an identical sample preparation procedure. Non-spiked samples are used to check for the presence of target analytes in the spiking matrix. With regard to spiked samples, spiking can be performed at different time points during sample preparation procedure in order to evaluate the extraction efficiency of each step used during that procedure. Precision and accuracy/recovery are usually determined on two or more levels in triplicates and often on three different days to account for inter- and intra-batch variability.

Limit of detection (LOD). LOD is the lowest quantity of the analyte in the sample that can be reliably detected. Several approaches can be used to determine LOD such as LOD determination based on S/N ratio, LOD determination using the standard deviation of the blank or determination from the calibration curve (Boyd et al., 2008).

Matrix effect. As mentioned above, signal suppression or enhancement of the analyte signal (SSE, matrix effect) can occur due to the presence of coeluting compounds in the extract when ESI ionisation is used (Matuszewski et al., 2003). When using the external calibration approach, the presence of coeluting compounds will depend on the type of matrix used. Hence, the MS signal of the same compound due to the differences in matrix can vary between the calibrants and samples, as well as between the samples extracted from different matrices. The matrix effect can cause over or underestimation of the concentration. Vishwanath et al. (2009) investigated matrix effect of three different building materials (mortar, cardboard/gypsum wallboard and soil-wood mixture) as well as dust. Their results showed that matrix effects depend on the material as well as on the target analyte. In their experiments, the use of dust as a matrix clearly showed a greater extent of signal alteration for majority of analytes in comparison to the other three matrices. Although dust showed the greatest matrix effect, signal suppression or enhancement was also noted for certain metabolites in matrices other than dust (e.g. emodin in mortar with 36 % enhanced signal), whilst some metabolites did not show significant SSE in any of the tested matrices including dust (e.g. stachybotrylactam with the signal enhancement from 5-9 % in all tested matrices). Clearly, the use of external calibration does not provide desired quality of obtained data especially in cases of complex matrices such as dust. The use of isotope-labelled internal standards would enable correction for matrix effects. Hence, there availability

would undoubtedly increase the quality of analytical methods for fungal secondary metabolites from the indoor environment.

1.6. Data analysis

When LC-MS is used as an analytical tool, large amounts of data are produced where data handling is often the most time-consuming step. Different software packages such as MassHunter (Agilent Technologies) (Varga et al., 2013) or Analyst® (Applied Biosystems) (Vishwanath et al., 2009) provide data processing tools designed for specific purposes (e.g. qualitative or quantitative analysis). Statistical methods including descriptive statistics (Chi-Square test, Fisher's exact test), univariate (ANOVA) or multivariate statistical approaches (Principle Component Analysis (PCA), Hierachical Clustering Analysis (HCA)) can be used for data evaluation, especially in cases where comparison between data obtained from different indoor environment needs to be performed (Peitzsch et al., 2012; Vishwanath et al., 2011; Kirjavainen et al., 2015). For the purposes of statistical analysis, different statistical softwares such as SAS (Peitzsch et al., 2012), Unscrambler®, R package (Vishwanath et al., 2011) can be utilised.

During this PhD project, all data analyses was performed using MassHunter Qualitative and Quantitative software provided by instrument vendor (Agilent Technologies). MassHunter Qualitative Analysis was mainly used for multi-targeted analyses of both pure agar cultures as well as extracts of different indoor matrices including dust. The program was used for the purposes of tentative identification of compounds, utilising *Find by Formula* and *Find by Auto MS/MS* algorithms. *Find by Formula* feature was used in aggressive dereplication approach for tentative identification of known unknowns where recorded masses from acquired data were matched to empirical formulas in search file database (**Paper 2, 3, 4** and **5**). *Find by Auto MS/MS* was used for MS/HRMS library approach where obtained MS/HRMS spectra at 10, 20 and 40 eV from the sample of interest were matched to existing spectra in MS/HRMS library **(Paper 2, 3, 4** and **5**). The aggressive dereplication and MS/HRMS approach are discussed to details in section 2.2. MassHunter Qualitative Analysis software was also used for targeted analysis for purposes of peak finding, baseline evaluation and evaluation of chromatograms (**Paper 5**). MassHunter Quantitative program was used both for calibration curve creation and quantification (**Paper 3** and **5**) as well as in multi-targeted approach for fast screening of compounds between different samples in large data sets (**Paper 3** and **4**). Statistical analysis was generally not the part of data processing workflow during this PhD project. The only statistical analysis performed included evaluation of created calibration curves using the R package.

2. Results and discussion

2.1. Methodology development

2.1.1. Establishing procedure for sample preparation

Extraction of metabolites was the most important step of the experimental work performed in this PhD study, as good sample preparation protocol was undeniably a prerequisite for successful analysis. Extractions of metabolites were performed from different type of materials for different purposes. An overview of all the sample types from which extraction of metabolites had to be performed, together with extraction solvents used is given in Table 3. The optimization of the extraction for each sample type is discussed individually below.

Table 3 Overview of extraction solvents used for different type of samples throughout PhD study

Sample type	**Sample**	**Extraction solvent***	**Purpose of extraction**
Pure agar cultures	PDA MEA OAT YES V8 DG18 CYA medium 17	EtAc/DCM/MeOH (3/2/1 *v/v/v*) + 1% FA	Metabolite profiling
Building material	Gypsum board/drywall Chipboard Plywood MDF Masonite Cellular concrete OSB plate Newspapers Cardboard Hessian with paint (for insulation) Glass fibre wallpaper Non-woven wallpaper with polystyrene pattern	ACN/MiliQ (75/25 *v/v*) + 1% FA	Qualitative and semi-quantitative analysis
Dust	Dust with Kimwipes® Lite wipes	ACN/MiliQ (75/25 *v/v*) + 1% FA	Qualitative and semi-quantitative analysis

* ACN-acetonitrile; DCM-dichloromethane; EtAC-ethyl acetate; FA-formic acid; MeOH-methanol; MiliQ-deionised water

2.1.1.1. Pure agar cultures

The procedure for the secondary metabolite extraction from pure agar cultures, regardless of what type of agar medium was used, was modified micro-scale extraction (Smedsgård, 1997). The modification was mainly referred to steps following sample extraction and evaporation, (sample re-dissolving and filtering) prior to LC analysis. Thus, the solvent used to re-dissolve samples was matched to the solvents used in LC analysis (ACN/MiliQ 75/25 *v/v* with 1% FA), with the goal of obtaining optimal chromatographic separation. The filtering step was exchanged with centrifuging in order to avoid contamination with filter plasticizers as well as to decrease the time and cost of this step.

In order to confirm the advantage of the use of micro-scale extraction in extraction of *Stachybotrys* metabolites, 3/2/1 mixture (EtAc/DCM/MeOH 3/2/1, *v/v/v* + 1% FA) was tested against EtAc + 1% FA and ACN/MiliQ (75/25 *v/v*) + 1% FA. The results are presented in Fig. 7.

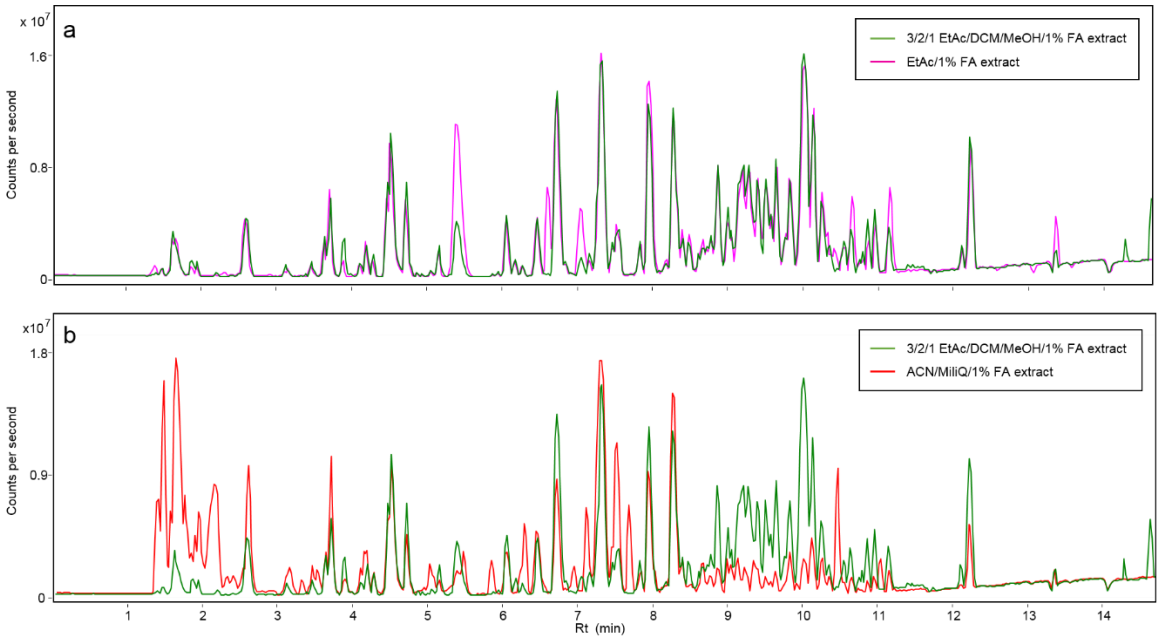


Fig. 7 Comparison of chromatogram from EtAc/DCM/MeOH (3/2/1 *v/v/v*) + 1% FA extract of 14 days old *Stachybotrys* spp. culture grown on DG18 to chromatograms of extracts obtained using different extraction solvents mixtures: a – EtAc + 1% FA; b – ACN/MiliQ (75/25 *v/v*) + 1% FA

Although the mixture of ACN/MiliQ (75/25 *v/v*) seemed to be better in extracting early eluting compounds (Fig 7b), the use of 3/2/1 mixture was clearly advantageous, considering the fact that most of the *Stachybotrys* metabolites eluted between 7 and 10 min (**Paper 4** and **5**). On the

other hand, similar metabolite profile was obtained when comparing 3/2/1 mixture to EtAc extraction (Fig 7a). In fact, pure EtAc seemed to be better for the extraction of compounds with medium polarity (RT 5-7 min). However, the obtained signals of the specific *Stachybotrys* metabolites showed no significant difference between the two solvent mixtures. The 3/2/1 mixture has been used for years at our Institute for pure agar culture extractions of all fungi. Therefore, it was decided to continue with the use of this mixture for pure agar culture extractions of both *Stachybotrys* and *Chaetomium* metabolites throughout this PhD study.

***Stachybotrys* metabolites.** One of the expected challenges in the extraction of *Stachybotrys* metabolites was the successful extraction of all chemical groups of metabolites. Namely, *S. chartarum* exists in two chemotypes S and A, that differ by the type of metabolites they produce (Andersen et al., 2003). At the same time, both chemotypes produce spirocyclic drimanes in significantly greater quantities in comparison to macrocyclic trichothecens (produced only by chemotype S) and atranones and dolabellanes (produced only by chemotype A) (Hinkley and Jarvis, 2000; Andersen et al., 2011; Nielsen, 2003). As a consequence of the higher quantities, drimanes tended to obscure the signal of MTR and atranones, hence increasing the chances of overlooking them in a sample, especially when the samples are analysed with LC combined with less sensitive UV/Vis diode array detection (Andersen et al., 2002; Andersen et al, 2003; Hinkley and Jarvis, 2000). Thus, Hinkley and Jarvis (2000) developed a protocol for separation of spirocyclic drimanes from MTR or atranones using normal phase solid phase extraction on PEI silica.

SPE columns, prepacked with PEI silica, are not commercially available and manual column packing showed to be laborious and time-consuming step. Therefore, separation on PEI silica was tested against separation on NH_2 columns, as a selection of NH_2 columns is commercially available. The SPE protocol used was identical on both type of columns and was adopted from Hinkley and Jarvis method. The results of this experiment are shown in Fig. 8.

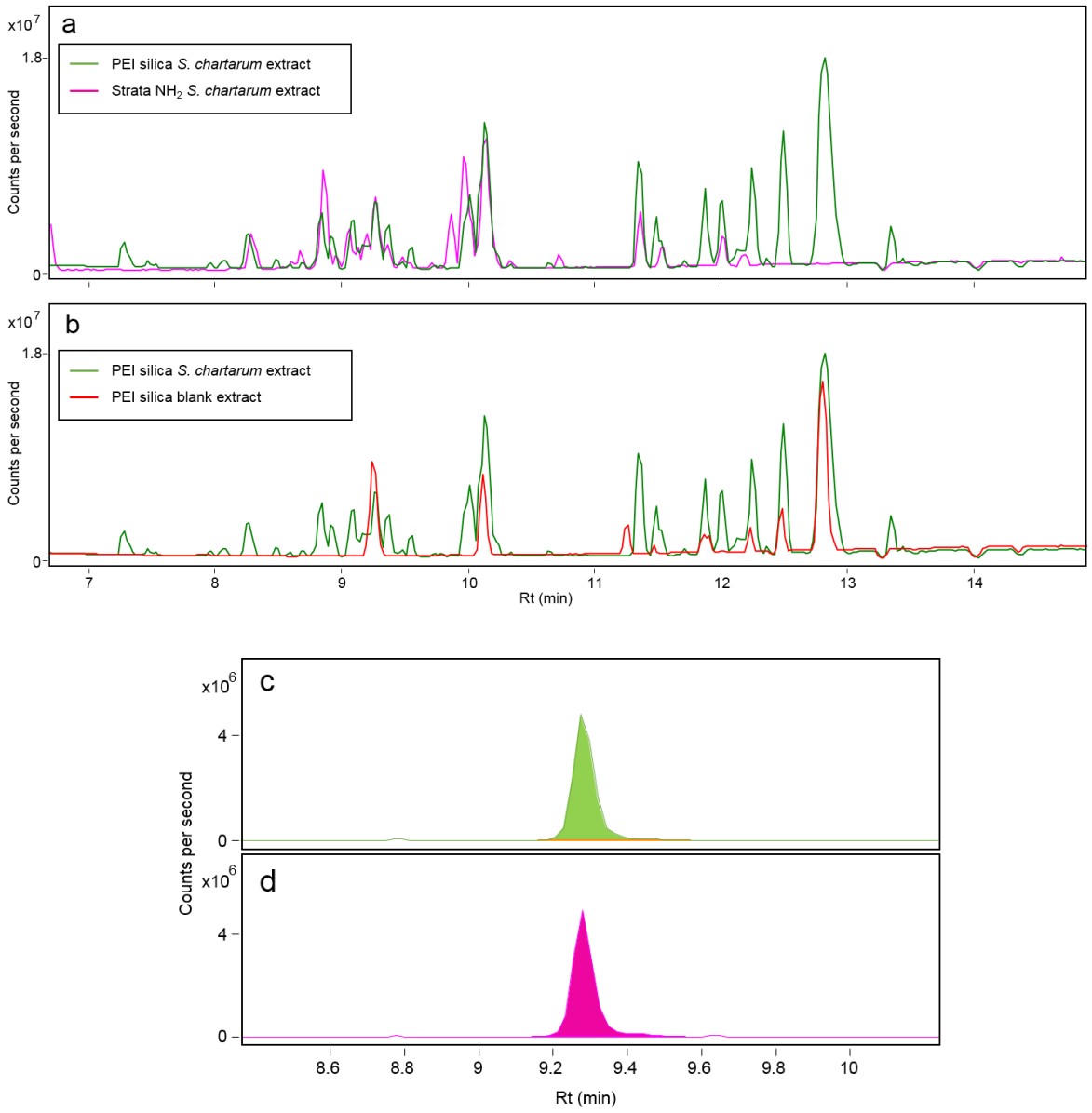


Fig. 8 Comparison of MeOH fractions obtained by normal phase SPE performed on PEI silica and NH_2 column: a – overlaid chromatograms of *S. chartarum* DG18 extract obtained on PEI silica and on Strata NH2; b – overlaid chromatograms of PEI purified *S. chartarum* DG18 extract and blank MeOH on PEI; c – stachybotrydial (**Paper 4** and **5**) in MeOH fraction using PEI silica SPE column; d – stachybotrydial in MeOH fraction using NH_2 SPE column.

The comparison of MeOH fractions from *S. chartarum* DG18 extract obtained using PEI silica and NH_2 column (Fig. 8a) showed slightly different metabolite profile for compounds eluting after 12 min. However, the obtained results were comparable for all the compounds eluting between 8 and 12 minutes, which is the time range when majority of spirocyclic drimanes elutes (**Paper 5**). The comparison to blank MeOH extract from PEI silica (Fig 8b) showed that some of the later eluting peaks (12.25, 12.5 and 12.8 min) were also present in the blank PEI extract. Furthermore, looking at the obtained signals of individual metabolites e.g. stachybotrydial (Fig.

8c and d) there was no significant difference between extracts purified on PEI and NH_2 column. The DCM fraction containing MTR or atranones (Hinkley and Jarvis, 2000) showed similar results, namely, there was no significant difference between extracts obtained on PEI silica and NH_2 column. Therefore, it was concluded that PEI silica SPE column could safely be replaced with NH_2 SPE columns. Thereby, the time necessary for SPE purification of *S. chartarum* extract could be significantly decreased.

However, the use of HRMS instrument for metabolite profiling, especially the possibility of MS/HRMS experiments, enabled easier detection of all metabolite groups as well as greater specificity in identification of all present metabolites, without the need for prior fractionation of the samples. This proved to be true not only for pure agar culture extract but also for much more complex samples such as dust and building material extracts (**Paper 4** and **5**). Thus, with the aim of making *Stachybotrys* sample preparation protocol time efficient, the SPE fractionation was abandoned. The use of HRMS and MS/HRMS in metabolite profiling will be further discussed in section 2.1.2.

2.1.1.2. Building materials and dust

Building materials. Establishing an extraction procedure for *Stachybotrys* and *Chaetomium* metabolites from different types of building materials has also been part of this PhD study. The most important step was certainly finding an efficient technique to remove fungal biomass from background surface, as extraction of whole pieces of building materials with organic solvents proved not to be suitable due to the high amounts of extracted interfering compounds present in them (Nielsen PhD thesis, 2002). Interfering compounds extracted from building materials showed to obscure signal of target metabolites even when only less sensitive UV/Vis detection was used (Nielsen et al., 1998, Nielsen et al., 1999). Thus, this effect could only be increased when QTOF instrument was used for analysis, as it increased sensitivity and visibility of all compounds with sufficient ionisation present in the sample. Scraping off fungal biomass with sterile scalpel blade proved to work the best, especially in case of *Chaetomium* spp. Due to the dense growth and plentiful hairy perithecia visible to the naked eye, *Chaetomium* spp. were easy to detach from building material surface, without sampling large amounts of background material. *Stachybotrys* spp. were more difficult to work with, as only in cases with heavy *Stachybotrys* contamination sufficient amount of biomass could be scraped off without including background material. In case of less dense *Stachybotrys* growth, cutting off small pieces of contaminated surface instead of scraping off was performed. The largest issue observed with

scraping sampling technique was potential loss of scraped biomass especially in cases when the material was dry. The small amounts of interfering compounds from background material did not pose a problem in further analysis as reported previously by Nielsen (PhD thesis, 2002). This can be explained by use of HRMS as detection tool, which enables better distinction between co-eluting compounds in comparison to previously used UV/Vis, despite the fact it increases overall sensitivity of the sample.

Another way of sampling contaminated building material was swabbing across infected surface using sterile swabs (Q-tips). In this case, cotton with adsorbed fungal biomass was carefully removed from the swab using sterile scalper blade and extracted together with the biomass (**Paper 5**). This was done to avoid interaction of the extraction solvent with the plastic from the swab tip and the container and thereby the extraction of possible interfering compounds. Extraction of sterile cotton did not seem to interfere with the metabolite analysis.

The solvent chosen for extraction was acetonitrile-water mixture (ACN/MiliQ 75/25 + 1% FA). Although several extractions with different solvents/solvent mixtures should have been consecutively used in order to fully extract plethora of *Stachybotrys* and *Chaetomium* compounds produced, acetonitrile-water mixture proved to be good extraction solvent for majority of target metabolites, at the same time extracting minimal amounts of interfering compounds (Nielsen PhD thesis, 2002). One could argue that the use of acid in the extraction mixture could cause hydrolysis of certain metabolites and therefore, some of the peaks may be artefacts and not extracted fungal metabolites. The experiment addressing this issue was performed and presented in section 2.1.2.3.

Dust. The extraction procedure for dust samples was the same as for the samples collected from contaminated building materials. However, the dust proved itself to be a very challenging matrix to extract from, with literature reporting heavy influence of dust on extraction of fungal metabolites (Vishwanath et al., 2009). Therefore, the main issue with dust extraction was finding the most suitable way of sampling without introducing extra matrix effect to the extracts. The idea behind dust sampling in this project (**Paper 5**) was not to look for metabolites in settled dust collected from surfaces that are not regularly cleaned, such as corners hidden behind large pieces of furniture or top shelves. The dust sampling was performed from regularly cleaned surfaces, such as tables, cupboards, windowsills etc. (Fig. 9). This approach was chosen in order to estimate how much of the fungal metabolites is being deposited in dust over shorter periods of time. The important part of dust sampling was estimation of sampled area surface prior to every sampling, which was necessary for quantitative analysis. Considering the fact that regularly cleaned surfaces did not contain large amount of dust, clean precision Kimwipes® Lite wipes were used for sampling (**Paper 5**), in order to quantitatively collect as much dust as

possible from available surfaces. For the same reasoning removing dust from wipes proved to be difficult, resulting also in loss of certain amount of collected dust, and thereby in loss of metabolites. Therefore, it was decided not to attempt to remove dust from Kimwipes® Lite wipes but to perform extraction on dust together with the Kimwipes® Lite wipe used for a particular collection. This meant that the possible matrix effects of the Kimwipes® Lite wipes also needed to be assessed (**Paper 5**). Results showed no significant matrix effect of Kimwipes® Lite wipes, thus it was concluded their use represented a safe and effective method for dust sampling. One of the considerations with dust samples extraction was that the amount of solvent for extraction varied from sample to sample, depending on the quantity of dust sampled and size of used Kimwipes® Lite wipe. Care needed to be taken that enough solvent was used for extraction so that wipes together with dust were fully submerged in solvent. This was done in order to ensure the efficiency of the performed extraction.

Fig. 9 Dust sampling using clean precision Kimwipes® Lite wipes from regularly cleaned surfaces

2.1.2. Targeted analysis

During this PhD project, two multitargeted screening methods for metabolite profiling were used: aggressive dereplication and MS/HRMS library approach. The work on MS/HRMS library building was part of this project (**Paper 2**), whilst the aggressive dereplication approach was adopted from Klitgaard et al. (2013). Both methods were used in complementary manner for

metabolite profiling throughout this PhD study (Fig. 10). Each screening method is separately discussed in the following section.

2.1.2.1. Aggressive dereplication

As mentioned previously, aggressive dereplication was developed at our Institute by Klitgård et al. (2013). This method was created with the goal of speeding up the traditionally manually performed dereplication process by quickly highlighting known compounds in fungal extracts and thereby enabling scientists to focus on unknown compounds (Klitgård PhD thesis, 2015). In this PhD study, aggressive dereplication was used as a complementary method to MS/HRMS library screening approach. It was used for the screening of all *Stachybotrys* and *Chaetomium* metabolites that were described in literature but were not included in the MS/HRMS library. For each genus, a separate database was created using different sources, namely in-house fungal database as well as the commercially available Antibase (Laatsch 2012). Furthermore, these databases could be supplemented with tentatively identified compounds. The aggressive dereplication approach was based on UHPLC-DAD-QTOF data acquisition and, as mentioned earlier, MassHunter analysis software (Agilent Technologies) was used for data analysis. Genus specific databases were automatically transformed through the in-house built Excel application (Klitgård et al., 2013) into the search list containing information about compound name and elemental composition. The search lists could easily be changed and made more or less specific (e.g. species or genus specific). These search lists were utilised by MassHunter analysis software using the feature *Find by Formula.* The compound identification was performed by matching accurate mass and isotopic pattern of the compounds present in the analysed sample to the ones present in the search file. In order to increase the confidence of the positive hits, the algorithm was searching not only for molecular ion $[M+H]^+$ but also for other predominant pseudomolecular ions such as $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$ and $[M+H-H_2O]^+$.

The biggest advantage of using this method was the possibility of screening for metabolites that were not included in the MS/HRMS library. This showed to be especially valuable with species that were in terms of metabolite production poorly described, such as *C. elatum* (**Paper 3**) or *S. nephrospora* (**Paper 4**). Matching against all described metabolites within one genus resulted in the detection of good candidates for genus specific biomarkers, both for *Chaetomium* spp. (cochliodone, **Paper 2**) and for *Stachybotrys* spp. (several spirocyclic drimanes, **Paper 4**). Some of these biomarkers (cochliodone in *Chaetomium* spp., **Paper 3**; L-611776 in *Stachybotrys* spp., **Paper 4**), were neither isolated nor found from indoor strains earlier. Consequently, these compounds had not been screened for in earlier chemical analyses of indoor samples. Thus, the

use of aggressive dereplication approach pinpointed the compounds, often produced in high amounts that had been previously continuously overlooked.

One of the disadvantages of this method was certainly the lower confidence of positive matches in comparison to matches obtained by use of MS/HRMS library. The method was unable to differentiate among structural isomers unless their RT were known, which often was not the case (**Paper 3** and **4**). On the other hand, the method was proved to be helpful in providing information about the number of structural isomers produced by a certain species. Although scoring and matching were performed fast and in an automated manner, obtained result lists often required manual inspection, in order to avoid false positives. The result lists often included same compound assigned to several peaks (e.g. chaetoglobosin G, D, U and V were assigned as chaetoglobosin A, **Paper 3**) and vice versa. Metabolites detected by aggressive dereplication, could be included in the MS/HRMS library, provided that the obtained data was of sufficient quality (stachybotrydial, **Paper 5**; cochliodone, **Paper 3**).

2.1.2.2. MS/HRMS library approach

The creation of MS/HRMS library was a part of this PhD project (**Paper 2**), and all data analysis performed throughout this project utilised this library. The MS/HRMS library was created as a response to a need for better dereplication procedure, which could provide higher specificity and confidence of the results in comparison to the aggressive dereplication. The use of spectral library was based on UHPLC-DAD-QTOF analysis, using the data acquired in auto MS/MS mode. All in-house available standards of fungal secondary metabolites (~ 1500) were analysed using three different collision energies (10, 20 and 40 eV), thereby taking into account the difference in energy needed to fragment different compounds. Since the mass of majority of *Chaetomium* and *Stachybotrys* compounds lies within 350 – 650 Da, energy of 10 and 20 eV was usually sufficient to provide specific fragmentation whilst 40 eV appeared to be too much for most of the analysed compounds. All the obtained spectra needed to be inspected and manually added to the library, which was both labour intensive and time-consuming. The use of this method in the compound identification enabled comparison of both accurate mass and obtained fragments of the compounds present in the analysed sample and in the library. The library was managed by PCDL-manager (Agilent Technologies), which enabled easy creation of smaller size sub-libraries such as the indoor sub-library containing only *Stachybotrys* metabolites (**Paper 4 and 5**). The use of sub-libraries slightly increased the speed of data analysis process and eased the data manipulation.

Generally, the use of MS/HRMS library in data analysis provided improved efficiency and confidence of an identified compound. One of the advantages of this method was that it did not require information about RT for correct compound identification. Thus, there is a possibility of using the library with different LC-methods. However, when analysing structural isomers with the similar fragmentation patterns (e.g. chaetoglobosins, cochliodone 1, 2 and 3; **Paper 3**), RT was a necessary parameter for their differentiation. The biggest disadvantage of this method was the size of the library and unavailability of fungal metabolites standards. This appeared to be a challenge especially with data analysis of *Chaetomium* spp., as there were only three *Chaetomium* metabolites (chaetoglobosin A, chaetoglobosin C and chaetoviridin A) available upon library creation. The use of aggressive dereplication approach and subsequent manual inspection of the fragmentation spectra for tentatively identified compounds, aided gradual size increase of the library. As a result of this combined approach there are eleven *Chaetomium* compounds included in the library at present. Hence, this example illustrates how the future increase in the size of the library is expected to proportionally increase the effectiveness of this method. It should also be mentioned that the use of the spectral library allows for different types of matching and scoring (forward, reverse, similarity search), which in the final line may influence the obtained results. The demonstrative examples as well as advantages and disadvantages of both MS/HRMS library approach and aggressive dereplication are further explored and discussed in **Paper 2**.

2.1.2.3. Metabolite profiling

As previously mentioned, metabolite profiling for both *Chaetomium* and *Stachybotrys* metabolites was performed in pure agar culture extracts. Pure agar cultures were chosen with the goal of exploring metabolite production potential of certain strain/species. At the beginning, different agar media were tested in terms of diversity of metabolite production, namely Yeast Extract Sucrose agar (YES), Oatmeal agar (OAT), Malt Extract Agar (MEA), V8 juice agar (V8) and Potato Dextrose Agar (PDA). The results showed that agar media with low nutrient content and high $a_{w,}$ such as MEA and PDA, provided the best coverage of metabolites for both screened genera (*Stachybotrys* spp. and *Chaetomium* spp.). Therefore, after initial testing, only MEA and PDA were used for metabolite profiling. The workflows for combined use of MS/HRMS library and aggressive dereplication in metabolite profiling is presented in Fig. 11. The list of identified metabolites for each analysed species was obtained by combining results of extracts from different media, analysed separately. The results and the challenges faced for *Chaetomium* spp. and *Stachybotrys* spp. are discussed separately in the following section.

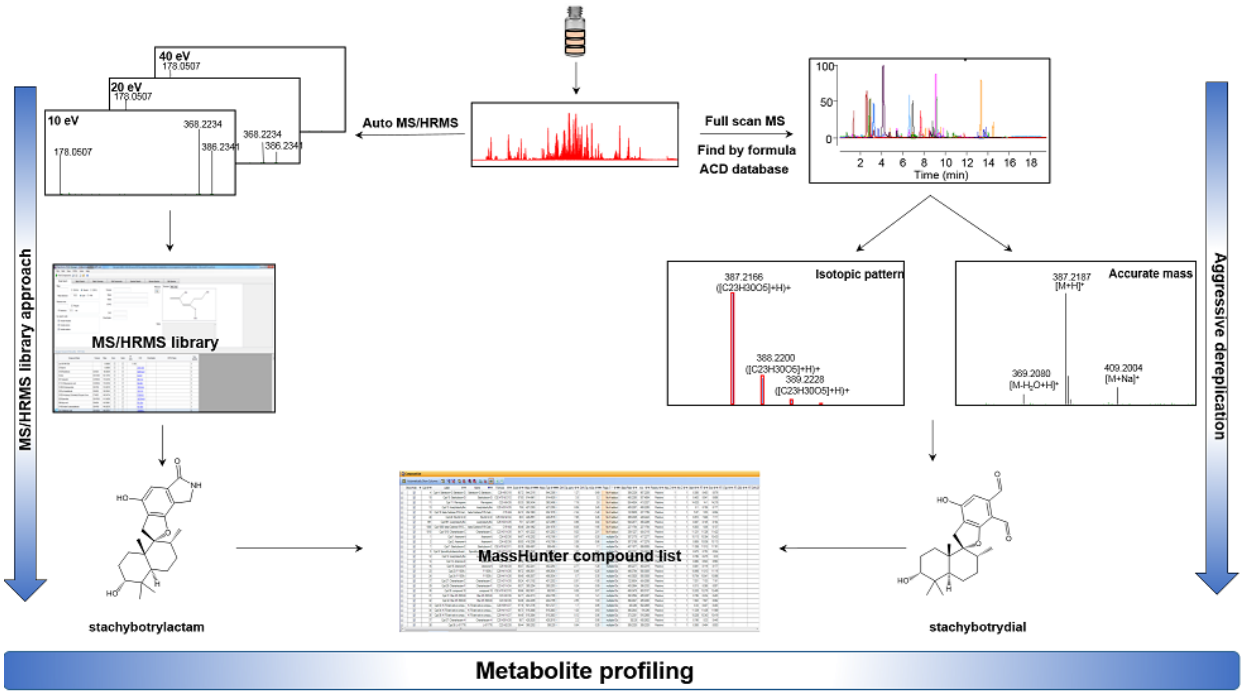


Fig. 10 An example of complementary use of aggressive dereplication and MS/HRMS library workflows in metabolite profiling of fungal extracts. In this example auto MS/HRMS of PDA extract of *S.chartarum* (IBT 9631) was analysed and matched against MS/HRMS library containing 10, 20 and 40 eV spectra of 30 *Stachybotrys* metabolites. The example shows the resulting identification of stachybotrylactam (**Paper 3 and 4**). Additionally, full scan MS was matched against the database containing all *Stachybotrys* secondary metabolites described in the literature (extracted both from in-house database and Antibase) but not included in the library. Matching is performed based on accurate mass and isotopic pattern calculated from elemental composition. The example shows positive hit for stachybotrydial (**Paper 4**). All positive hits from both analyses are compiled in the MassHunter compound list.

***Chaetomium* spp.** The metabolite profiling of three indoor and four reference (non-indoor derived) *Chaetomium* species included 23 strains in total (**Paper 3**). So far, more than 400 *Chaetomium* species have been described (www.indexfungorum.org), producing more than 200 known biological active metabolites (Zhang et al., 2012). Most commonly reported *Chaetomium* species from 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), whilst most commonly reported metabolites from both species are chaetoglobosins (Udagava et al., 1979; Thohinung et al., 2010). The primary goal of study presented in **Paper 3**, when it comes to metabolite profiling, was mapping as many *Chaetomium* metabolites as possible in screened indoor species and strains, by means of the above presented analytical methodology. The obtained metabolite profiles were further compared to metabolites mapped from reference *Chaetomium* strains, in order to establish similarities and/or differences between *Chaetomium* species commonly found indoor and *Chaetomium* species of non-indoor origin. This comparison would possibly provide

good candidate(s) for indoor *Chaetomium* specific biomarkers. The overview of all metabolites produced by different species is given in Table 4.

The main challenge in the detection of *Chaetomium* metabolites was the lack of standards and the large number of structural isomers produced by *Chaetomium* indoor species. As mentioned previously, MS/HRMS library contained only three *Chaetomium* metabolites, whilst the search list with all *Chaetomium* metabolites used in aggressive dereplication contained 220 compounds. This meant that identity confirmation for every metabolite identified by aggressive dereplication necessitated further manual inspection of the fragmentation pattern and/or inspection of UV/Vis data for compounds with UV chromophores. Moreover, many of these compounds were either structural isomers or analogues, making the distinction between them even more challenging. Based on MS/HRMS library matching, at least four compounds with different RT were identified as chaetoglobosin A (**Paper 3**). Chaetoglobosin A and C were correctly assigned by comparison with standards, whilst other two compounds were given several possibilities as one of the structural isomer (chaetoglobosin G/D/U/V). The same situation was observed with chaetoglobosin E/F, chaetoviridin A/chaetomugilin C and chaetoviridin C/chaetomugilin D/S (**Paper 3**, Fig. 1). In all these cases, the compounds were assigned to a group of structural isomers due to the large structural similarities between them and the lack of standards that could provide their unequivocal identification. The results of this study suggested that all these metabolites were produced only by *C. globosum*, despite the literature reporting production of some of them by *C. elatum* too (Thohinung et al., 2010).

Table 4 Metabolite production for different *Chaetomium* species in pure culture (MEA and PDA).

Metabolite	*C. elatum* (2)	*C. erectum* (1)	*C. globosum* (14)	*C. homopilatum* (2)	*C. longicolleum* (2)	*C.malaysiense* (1)	*C. virescens* (1)
Chaetoglobosin A[1, 3]	-	-	+	-	-	-	-
Chaetoglobosin C[1, 3]	-	-	+	-	-	-	-
Chaetoglobosin G/D/U/V[1]	-	-	+	-	-	-	-
Chaetoglobosin E/F[2]	-	-	+	-	-	-	-
Prochaetoglobosin I[2]	-	-	+	-	-	-	-
Prochaetoglobosin II[2]	-	-	+	-	-	-	-
Prochaetoglobosin III[2]	-	-	+	-	-	-	-
Prochaetoglobosin IV[2]	-	-	+/-	-	-	-	-
Chaetoviridin C/ Chaetomugilin D/S[2]	-	-	+	-	-	-	-
Chaetoviridin A/ Chaetomugilin C[1, 3]	-	-	+	-	-	-	-
Chaetoviridin E[2]	-	-	+	-	-	-	-
Chaetoviridin H[2]	-	-	+	-	-	-	-
Cochliodone 1[2, 4]	+	-	+	-	-	-	-
Cochliodone 2[2, 4]	+	-	+	-	-	-	-
Cochliodone 3[2, 4]	+	-	+	-	-	-	-
Chaetoglobin A[2, 4]	+	-	+	-	-	-	-
Cochliodinol[2]	+	-	+/-	-	-	-	-
Longirostrerone A[2]	-	-	-	+	-	-	-
Longirostrerone B[2]	-	-	-	+	-	-	-
Longirostrerone C[2]	-	-	-	+	-	-	-
Versicolorin**[3]	-	-	-	-	+	+	+
Sterigmatocystin[1, 3]	-	-	-	-	+	+	+
3-O-methyl sterigmatocystin[1, 3]	-	-	-	-	-	-	+
Dihydro-sterigmatocystin[1, 3]	-	-	-	-	+	+	-

[1] identified by MS/HRMS library;
[2] identified by aggressive dereplication;
[3] identified by comparison to reference standard and/or by specific UV spectrum;
[4] MS spectrum fitting proposed fragmentation pattern
[5] Several compounds from versicolorin group present

On the other hand, aggressive dereplication revealed other common metabolites for *C. globosum* and *C. elatum*. All analysed strains of both species were able to produce three compounds with the same mass (*m/z* 639.2436) and fragmentation pattern at 10 and 20 eV but with different RT (7.74, 9.53 and 11.39 min; **Paper 3**). All three compounds were identified as cochliodone A/B, two compounds reported in literature as stereoisomers (Phonkerd et al., 2008). In order to confirm their identification, a fragmentation scheme was proposed (Fig. 11) based on the structure of cochliodone A. This fragmentation scheme provided the explanation for the occurrence of all fragments with the intensity of ≥ 5 % in the obtained 20 eV MS/HRMS spectrum. The presence of three peaks was explained by possible existence of more than two stereosiomers, and three compounds were given the provisional names cochliodone 1 (7.74 min), cochliodone 2 (9.53 min) and cochliodone 3 (11.39 min). Furthermore, two additional metabolites were found to be common for *C. globosum* and *C. elatum*: cochliodinol, a derivative of 2,5-dihydroxy-*para*-benzoquinone isolated from both *C. globosum* and *C. cochlioides* (Jerram et al., 1975) and chaetoglobin A, an azaphilone alkaloid dimer previously reported in *C. globosum* (Ge et al., 2008). Their identifications were also confirmed by the proposed fragmentation schemes (cheatoglobin A, **Paper 3**; cochliodinol, Fig. 12) and all identified compounds were added to MS/HRMS library.

In cases of poorly described *Chaetomium* spp. such as *C. erectum* (**Paper 3**), combined use of MS/HRMS library and aggressive dereplication resulted in no hits i.e. none of the compounds in the extract were identified. This suggested that *C. erectum* appeared to have substantially different metabolite profile in comparison to all the *Chaetomium* metabolites so far described in literature. This case illustrates a good example of the main disadvantage of our combined approach to metabolite profiling for poorly described species. Metabolite profiling based on MS/HRMS library and aggressive dereplication can be successful only in cases where produced metabolites have been previously isolated and described, either as known or unknown *Chaetomium* metabolite. In cases like *C. erectum*, where the species produces no known metabolites, other approaches to dereplication need to be considered (NMR).

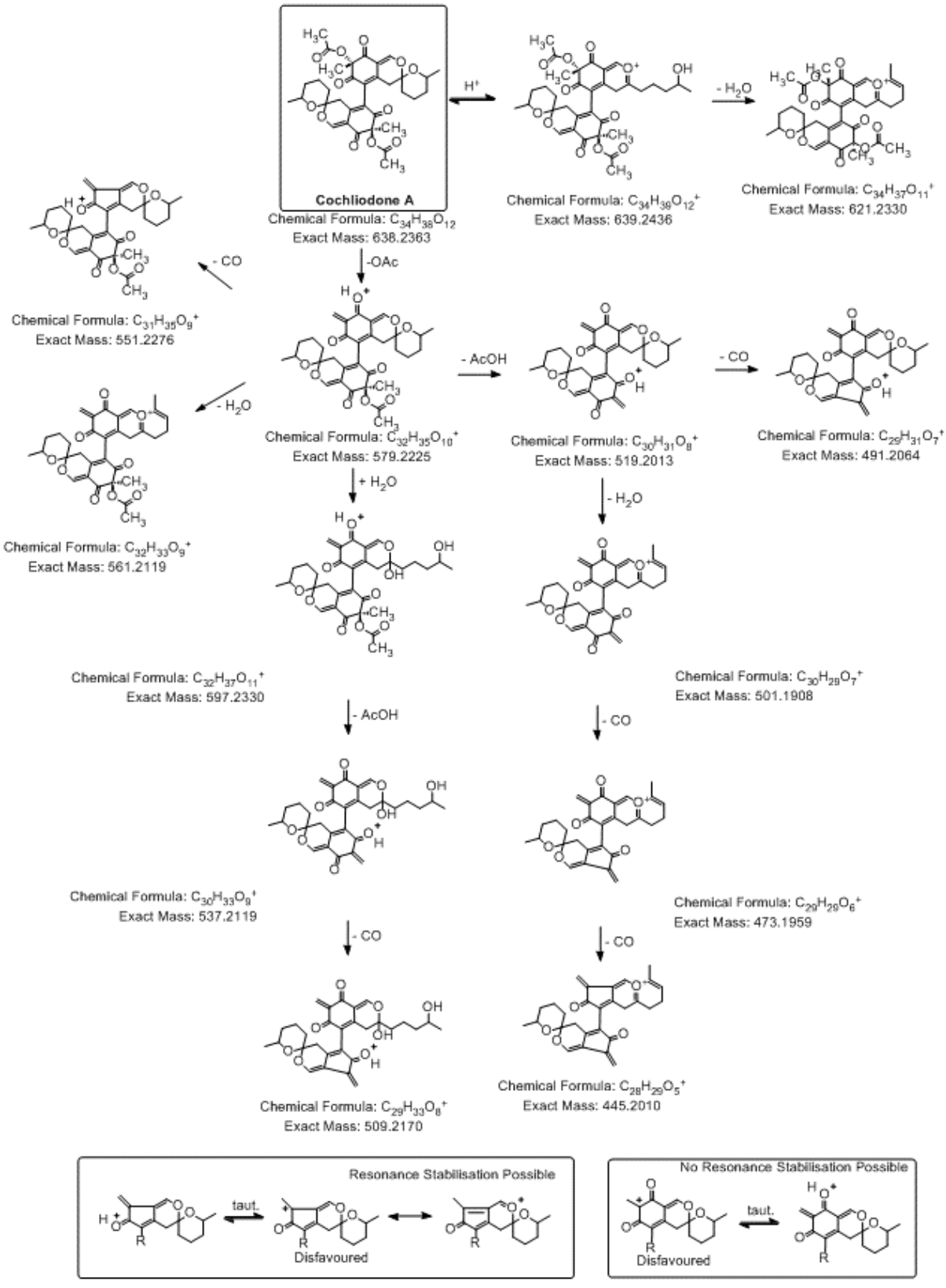


Fig. 11 Proposed fragmentation pattern for cochliodone A

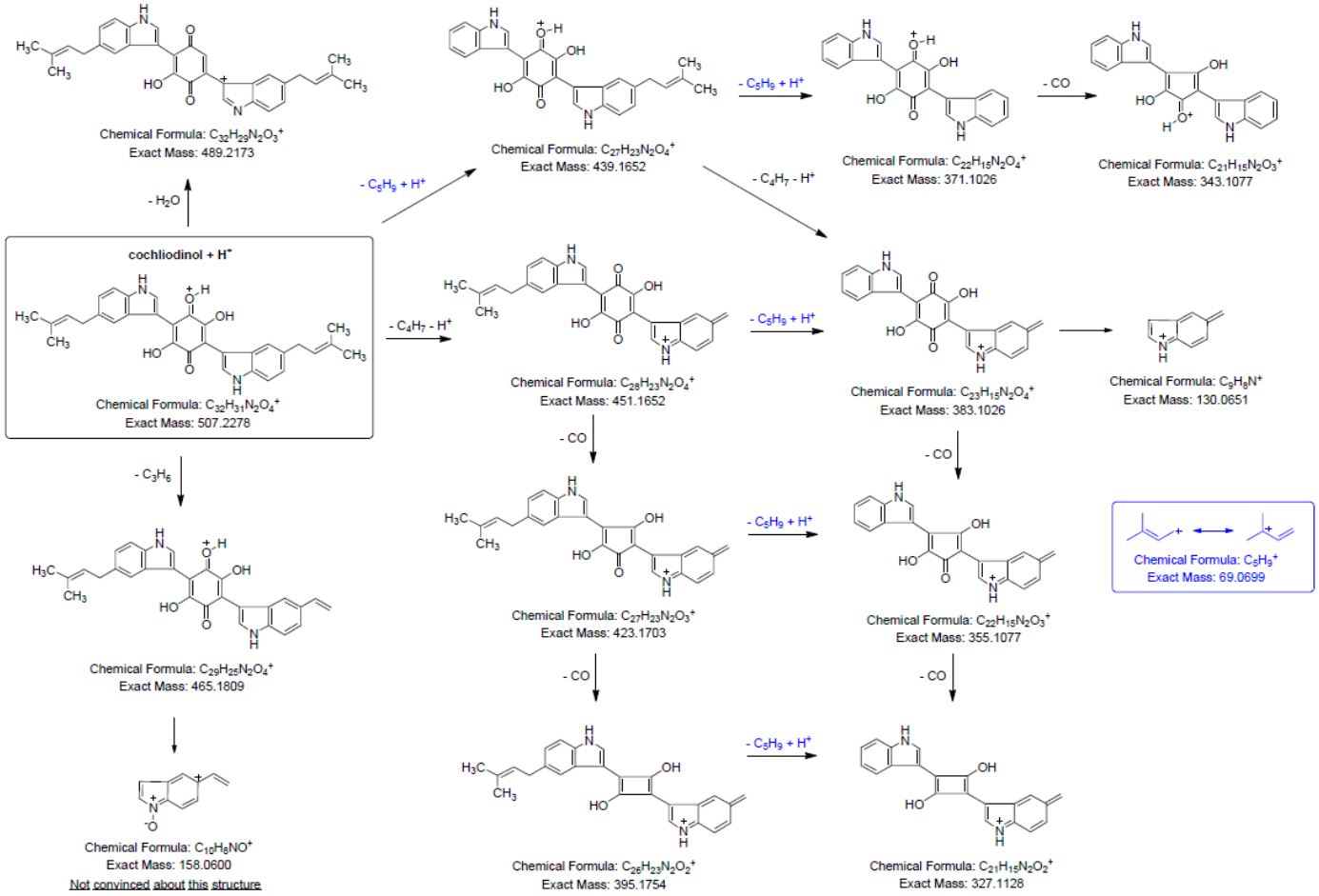
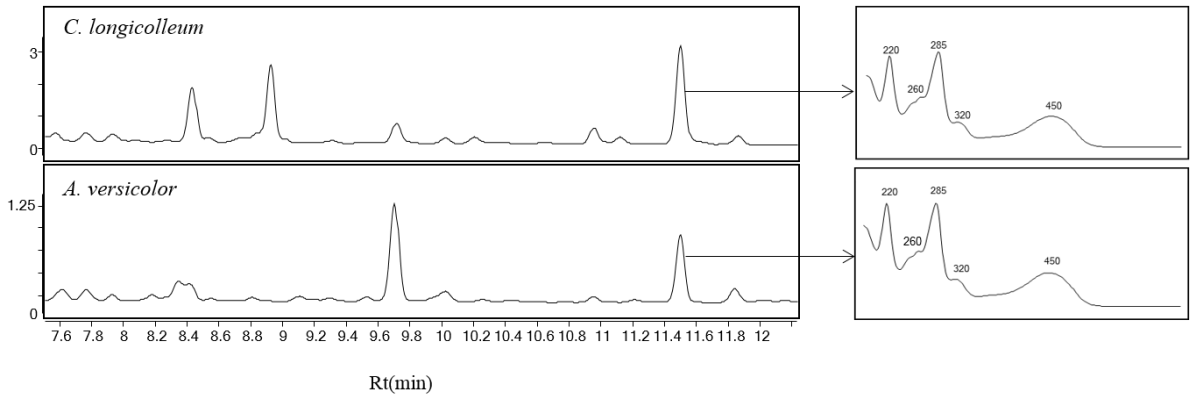
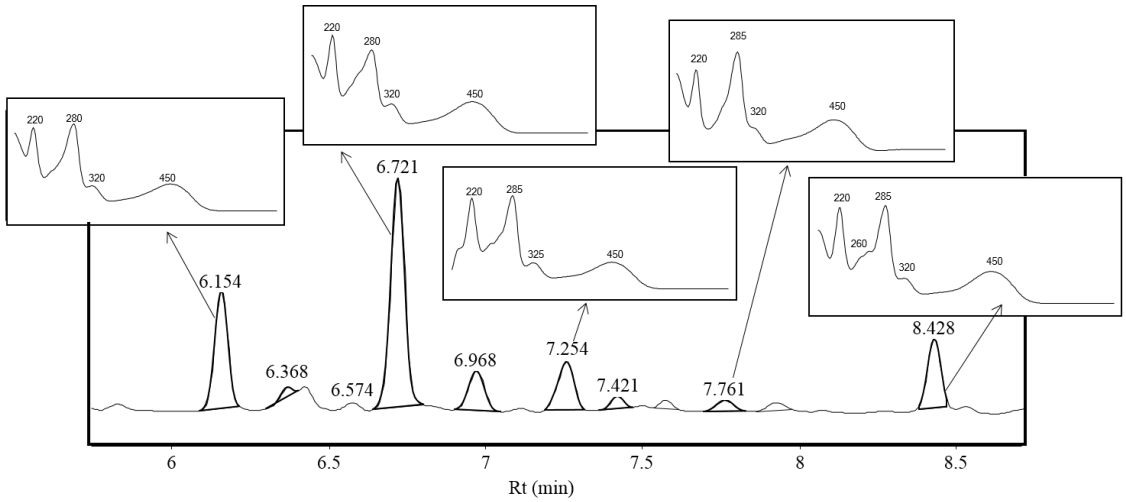


Fig. 12 Proposed fragmentation pattern for cochliodinol

An important part of this study was metabolite profiling of *Chaetomium* reference strains. Six *Chaetomium* strains of four different species (*C. longicolleum, C. homopilatum*, *C. malaysiense* and *C. virescens*) were analysed, mostly for the presence of secondary metabolites common to indoor species. The reference *Chaetomium* strains appeared to produce substantially different secondary metabolites compared to indoor strains, suggesting presence of different genes and thereby different biosynthetic pathways in reference and in indoor *Chaetomium* strains. Sterigmatocystin, an important product of sterigmatocystin/aflatoxin biosynthetic pathway (Rank et al., 2011), was found in extracts of *C. virescens*, *C. malaysiense* and *C. longicolleum,* as well as some other products of this pathway (dihydrosterigmatocystin and 3-O-methyl sterigmatocystin, Table 4). On the other hand, none of the analysed indoor strains was able to produce sterigmatocystin or any other product of this biosynthetic pathway, as shown in Table 4. The importance of this finding lies in the fact that although literature reports production of sterigmatocystin by certain *Chaetomium* spp. (Rank et al., 2011), their metabolite profiles appear to be significantly different from metabolite profiles obtained from most commonly isolated indoor *Chaetomium* spp.. Thus, the presence of sterigmatocystin in indoor environment can only be related to the presence of *A. versicolor* (Nielsen et al., 1999) and not to the presence of *C. globosum* and *C. elatum.* Furthermore, the UHPLC-DAD analysis of *C. virescens, C. longicolleum* and *C. malaysiense* extracts showed several characteristic UV spectra identified as versicolorins. According to the existing literature, there are at least three different versicolorins (A, B and C) as well as several other compounds belonging to the versicolorin group (all biosynthetic precursors of sterigmatocystin and thus also the aflatoxins) (Cole and Cox, 1981). The comparison of our extracts with the extract of *A. versicolor* strain (IBT 29547), known to produce versicolorins (Kingston and Chen, 1976) revealed the presence of the same compound eluting at 11.5 min with identical UV spectrum. Additionally, several compounds with similar UV spectra, eluting between 6 and 8 min, were found in the reference *Chaetomium* extracts, but not in *Aspergillus* extracts (Fig. 13). This suggests that the biosynthetic pathway in *C. virescens, C. longicolleum* and *C. malaysiense* results in the production of more versicolorin compounds than the single compound in *A.versicolo*r strains. Further investigation, however, was outside of the scope of this study, as none of the versicolorin like compounds were found in extracts of *C. globosum* or *C. elatum*.



Verification of versicolorin type of compound in extract of *C. longicolleum* (IBT 41566) by comparison with *A. versicolor* extract (IBT 29547): same compound found at 11.5 min with identical UV spectrum



Compounds belonging to versicolorin group eluting between 6-8.5 min with their characteristic UV spectra

Fig. 13 Versicolorin type of compounds in MEA extract of *C. longicolleum* (IBT 41566)

***Stachybotrys* spp.** Metabolite profiling of *Stachybotrys* spp. was performed on three different species (*S. chartarum, S. chlorohalonata* and *S. nephrospora*) and seven strains in total (**Paper 4**). Four out of seven strains belonged to *S. chartarum* species, with two being chemotype S strains (MTR producer) and two chemotype A strains (atranone producer). As mentioned previously, so far, more than 123 *Stachybotrys* spp. have been described (www.indexfungorum.org), with around 140 *Stachybotrys* compounds currently reported in the literature (Antibase, Laatsch 2012; Hinkley and Jarvis, 2000; Nielsen, 2002; Li et al., 2013; Ma et al., 2013; Wu et al., 2014). *S. chartarum* is most commonly isolated *Stachybotrys* spp. in the indoor environment (Andersen et al., 2000). In the last couple of decades, a lot of attention has been focused on *Stachybotrys*

metabolites, due to their connection to occurrence of negative health effects and possible causal role in occurrence of idiopathic pulmonary haemosiderosis in babies (Jarvis et al., 1998; Etzel, 2007; Miller and McMullin, 2014). About 40 % of indoor isolated *S. chartarum* strains are more toxic MTR producers (chemotype S) while the rest are atranone producers (chemotype A) (Miller et al., 2003). Furthermore, *S. chartarum* chemotype A and *S. chlorohalonata* can only be distinguished morphologically, as they appear to have the same metabolite profiles (Andersen et al., 2003).

The main goal of the study presented in **Paper 4,** similarly to the study performed for *Chaetomium* spp. in **Paper 3,** was to map as many *Stachybotrys* metabolites as possible, based on the available standards and compounds described in literature. The aim was to find candidates for *Stachybotrys* specific biomarkers, as well as to use the obtained metabolite lists to screen for *Stachybotrys* metabolites in the indoor samples, which will be discussed further in Section 2.2.2. All identified *Stachybotrys* metabolites from seven tested strains are presented in Table 5.

Table 5 Metabolite production for different *Stachybotrys* species in pure cultures (MEA and PDA).

Metabolite	*S. chartarum* chemotype S (2)	*S. chartarum* chemotype A (2)	*S. chlorohalonata* (2)	*S. nephrospora* (1)
Atranone A[3]	-	-	+	+
Atranone B[3]	-	-	+	-
Atranone G[2]	-	-	+	-
Dolabellane 1	-	+	+	-
Dolabellane 2	-	+	+	-
Roridine E[3]	+	-	-	-
epi/iso-Roridine E[3]	+	-	-	-
Roridin H[3]	+	-	-	-
Roridin L2	+	-	-	-
Satratoxin H[2]	+	-	-	-
Isosatratoxin H	-	-	-	-
Isosatratoxin F	-	-	-	-
satratoxin G[3]	+	-	-	-
Chartarlactam C[2]	+	+	-	-
Chartarlactam D[2]	+	+	+	-
Chartarlactam K[2]	+	+	+	+
Chartarlactam M/O[2]	+	+	+	+
Stachybotrylactam[1]	+	+	+	+
Stachybotryamide[1]	+	+	+	+
Mer-NF-5003-B[3]	+	+	-	+
Stachybotrydial[2,4]	+	+	+	+
SDHBFL III/Chartarlactam B[2]	+	+	+	+
SDHBFL IV/K 76 compound 1[2,4]	+	+	+	+
K 76 compound 2[2]	+	+	+	+
L-611776[2,4]	+	+	+	+
F-1839-I[2]	+	+	+	+
F-1839-J[2]	+	+	+	+
Compound 2[2]	+	+	+	+

[1] identified by MS/HRMS library;
[2] identified by aggressive dereplication;
[3] identified by comparison to reference standard and/or by specific UV spectrum;
[4] MS spectrum fitting proposed fragmentation pattern

In comparison to *Chaetomium* metabolite profiling, *Stachybotrys* metabolite profiling showed to be less challenging. Although only few *Stachybotrys* compounds were commercially available as standards (e.g. stachybotrylactam), more than 20 years of work on these compounds resulted in a number of qualitative standards, mostly of MTR and atranones (isolated in-house or donated from other research groups). Furthermore, qualitative standards of some other metabolites such as K-76 compounds, were available for purchase. Hence, our *Stachybotrys* MS/HRMS library consisted altogether of 24 *Stachybotrys* standards upon its creation. Therefore, the main challenge in *Stachybotrys* spp. metabolite profiling was not metabolite identification in the pure cultures. As previous studies showed (Hinkley and Jarvis, 2000; Andersen et al., 2002, Andersen et al., 2003) spirocyclic drimanes tend to dominate *Stachybotrys* extracts, often obscuring the signal from MTR and atranones which was also observed in **Paper 4** and **5**. However, the analysis with the QTOF instrument and the use of extracted ion chromatogram (EIC) enabled easier detection of co-eluting compounds. This practically meant that even in cases where the MS/HRMS library failed to identify atranones and/or MTR, due to the masked signal by spirocyclic drimanes, the use of EIC and the confirmation with authentic standards resulted in confident identification. This problem was, however amplified, when indoor samples were screened for presence of MTR and atranones, which will be further discussed in section 2.2.2.

Although the MS/HRMS library contained a significant number of metabolites, very few of those belonged to spirocyclic drimane group of compounds, which, as mentioned earlier, appeared to be the predominant group of metabolites produced by all tested species/strains. The fact that spirocyclic drimanes were found in all tested *Stachybotrys* strains, regardless of species or chemotype, labelled this group as the best candidate for *Stachybotrys* biomarker pool. Some of the major peaks in all screened *Stachybotrys* extracts could not be identified by use of MS/HRMS library and thus, needed to be identified by aggressive dereplication. **Paper 5** gives an example of use of aggressive dereplication in stachybotrydial dereplication (*m/z* 387.2166), whose identification was further confirmed by known UV for this compound (Andersen et al., 2002, Jarvis, 2003). Again, as in case of *Chaetomium* metabolites not included in MS/HRMS library, a fragmentation pattern explaining presence of all fragments with ≥ 5 intensity in 20 eV MS/HRMS spectrum was proposed, serving as final proof of stachybotrydial's identity (Fig. 14) together with the corresponding UV spectrum (**Paper 5**). Dereplication of other *Stachybotrys* compounds was presented in **Paper 4**. Among dereplicated compounds, L-671,776 (*m/z* 389.2323, also known as L-611776), showed to be especially interesting as potential biomarker, as it seemed to be produced in the highest amounts by all tested strains (Fig. 15). L-671,776 was first isolated by Lam et al. (1992) from *S. echinata*, and its structure has been revised by Falck et al. (1997). The compound is the structural analogue of stachybotrydial, with one reduced aldehyde function in comparison to stachybotrydial. It has been isolated from different *Stachybotrys* spp. (Roggo et

al., 1995, Stefanelli et al., 1996) and ever since, it has been focus of attention due to its strong activity as a selective inhibitor of mio-inosytol monophosphatase (Falck et al., 1997) and HIV-protease (Stefanelli et al., 1996). Despite a lot of interest in L-671,776 in different clinical trials, this compound, to the best of our knowledge, has so far never been reported or screened for in samples from indoor environment. Similar UV spectrum and proposed fragmentation pattern (Fig. 16) explaining all fragments with ≥ 5 intensity in 20 eV MS/HRMS spectrum, aided its unequivocal identification. All dereplicated spirocyclic drimanes, whose identities have been with high confidence confirmed by fragmentation pattern and/or UV/DAD data, were added to the MS/HRMS library. Thus, the MS/HRMS library consists of 30 *Stachybotrys* compounds as of present.

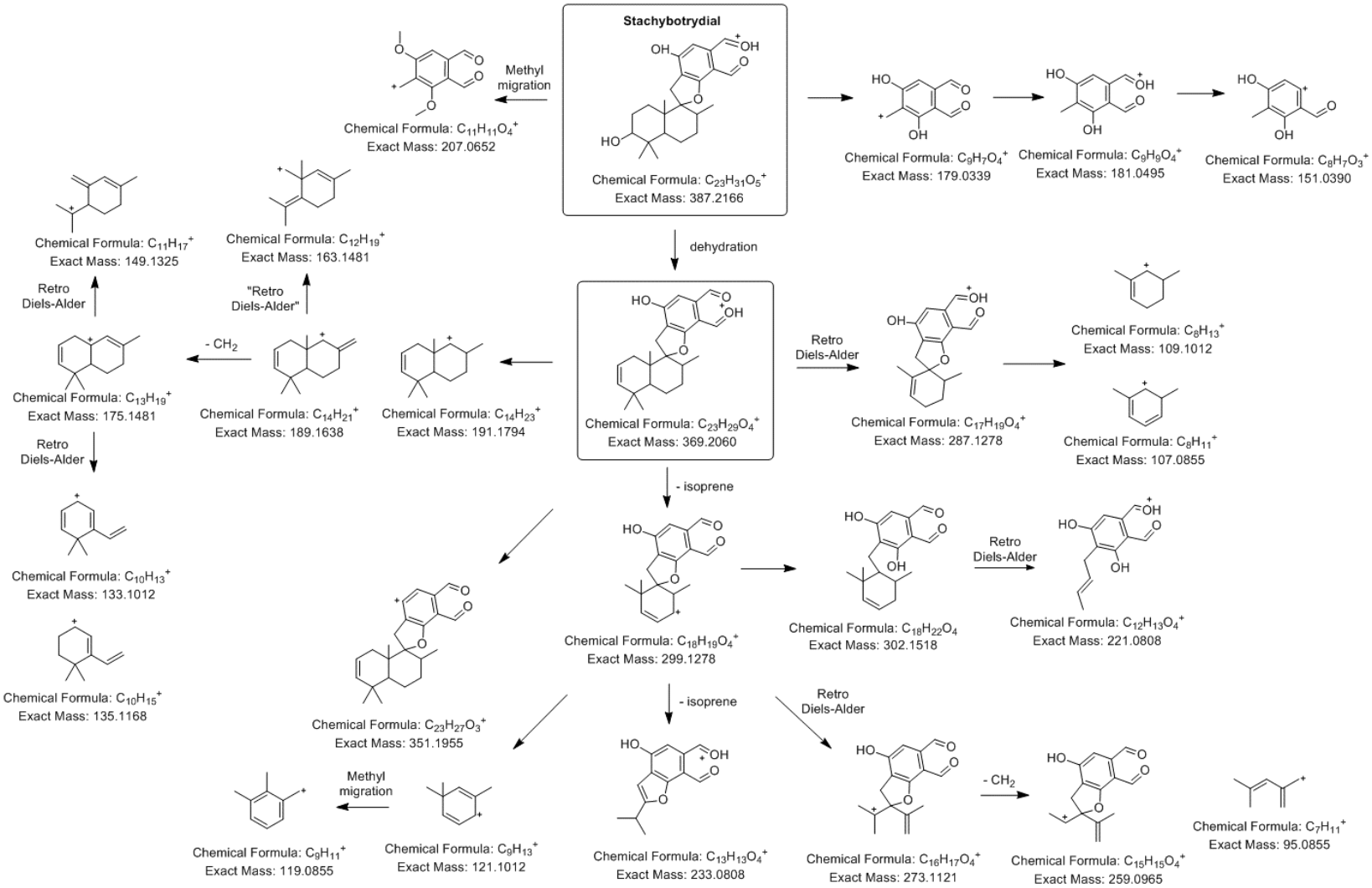


Fig. 14 Proposed fragmentation pattern for stachybotrydial

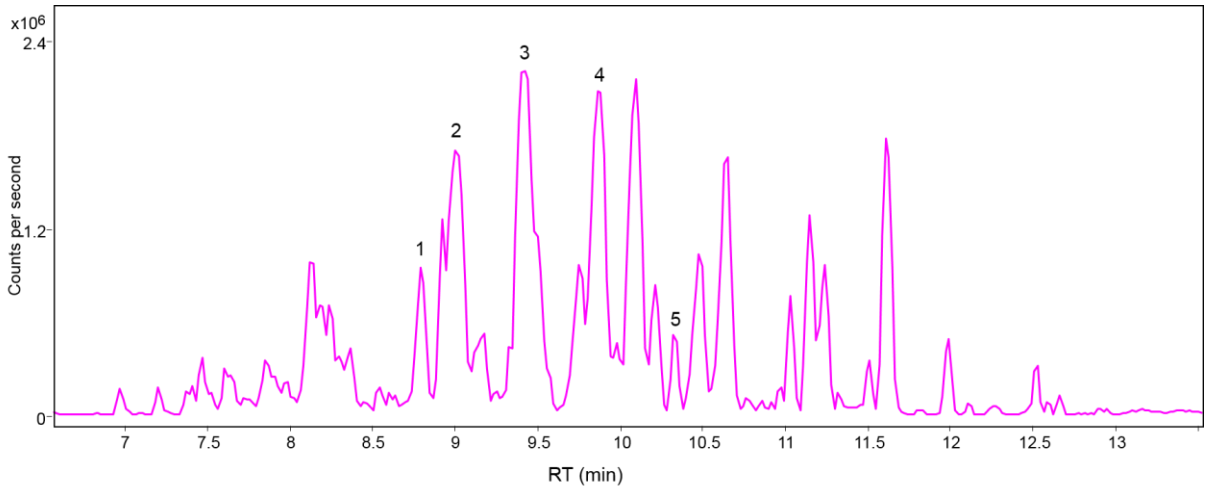
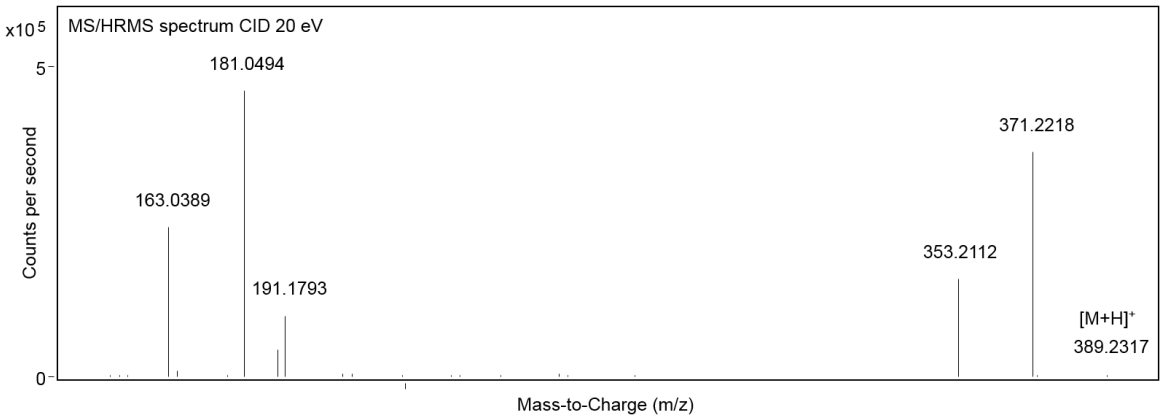


Fig. 15 Compounds identified by aggressive dereplication and confirmed by fragmentation pattern and/or UV spectra in PDA extract of S. chartarum (IBT 9631): 1 – K-76 compound 1, 2 – compound 2 (Jarvis et al., 2003), 3 – L-611775, 4 – unknown *m/z* 471.2393, 5 – F-1839 I.



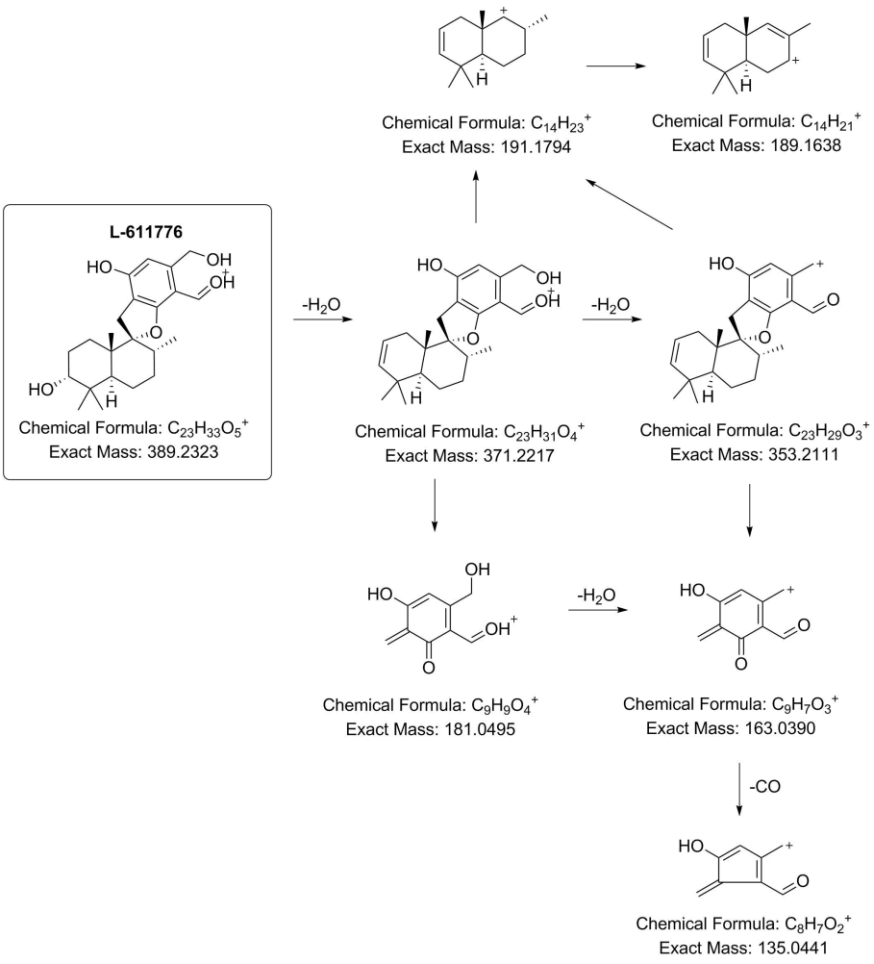


Fig. 16 MS/HRMS 20 eV spectrum of L-611776 and proposed fragmentation pattern explaining the occurrence of all fragments with ≥ 5 % intensity

As previously seen with *Chaetomium* spp. extracts, structural isomers and/or stereoisomers were also observed in extracts of *Stachybotrys* spp.. *Stachybotrys* compounds that shared the same empirical formula were stachybotrylactam/chartarlactam O/M, stachybotryamide/chartarlactam N/H, spirodihydrobenzofuranlactam III/chartarlactam B, spirodihydrobenzofuranlactam IV/K 76 compound 1 and spirodihydrobenzofuranlactam V/chartarlactam A. In some cases, more than one peak having the same mass was observed, e.g. two observed peaks with *m/z* 386.2326 corresponding to stachybotrylactam/chartarlactam O/M (**Paper 4**). In other cases, only one peak whose mass could correspond to several structural isomers was observed (e.g. *m/z* 430.2588 that can correspond to stachybotryamide, chartarlactam N or H). Common for all these cases is the fact that it was impossible to correctly assign the peak(s) without the presence of authentic standards. In order to further investigate whether some of the peaks with the same mass truly represent multiple structural isomers present in the sample or just the artefacts due to the extraction procedure, a short time extraction was performed. Namely, *S. chartarum* (IBT 9466) was grown for 14 days on PDA, after which plugs were taken for three variations of extraction: i) micro-scale extraction (Smedsgaard, 1997) adopted for *Stachybotrys* metabolites with a duration of 60 min (**Paper 4** and **5**); ii) extraction with ACN with a duration of 5 min; and iii) extraction with ACN:MiliQ (75/25 *v/v*) + 1% FA with a duration of 5 min. Fig. 17 shows the results of the comparison of these extractions. It is clear from the results that short-term extraction resulted only with the extraction of spirocyclic drimanes normally present in high amounts in extracts. The extraction with acetonitrile-water mixture (ACN:MiliQ (75/25 *v/v*) + 1% FA) yielded only L-611776 (Fig 17c), whilst the extraction with pure ACN resulted with several other spirocyclic drimanes (Fig 17b). Better extraction with pure ACN for later eluting compounds was expected due to the increase in their lower polarity. The experiment was inconclusive when it comes to multiple peaks for earlier eluting spirocyclic drimanes, normally present in smaller amounts such as stachybotrylactam/chartarlactam O/M. However, the presence of structural analogues (L-611776 and stachybotrydial) as well as some stereoisomers/structural isomers (*m/z* of 429.2272 at 9.1 and 11.4 min) suggests that the plethora of *Stachybotrys* compounds in pure culture extract is indeed the result of the fungal biosynthesis and not just the sample preparation induced artefacts.

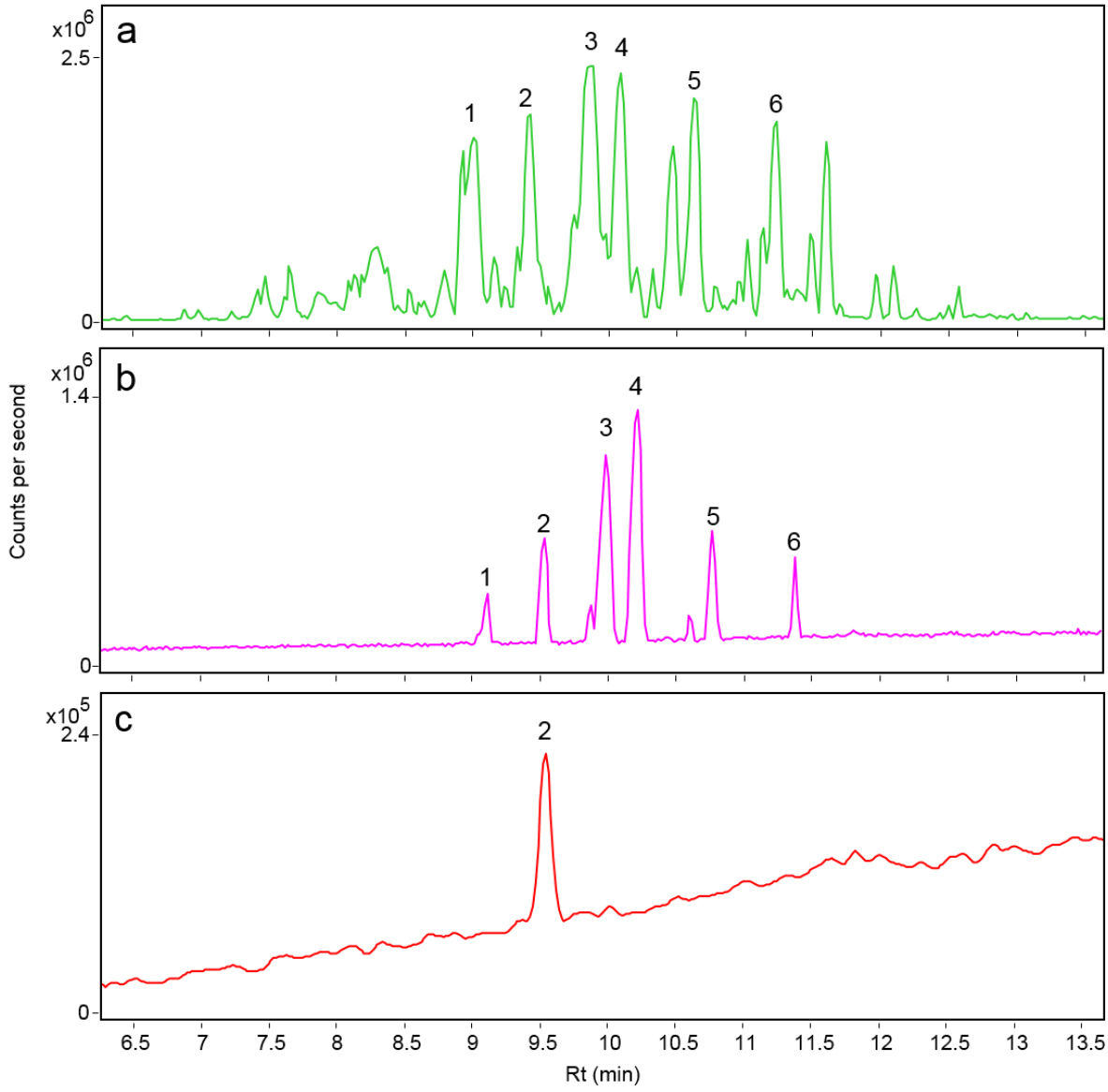


Fig. 17 *S. chartarum* (IBT 9466) extracted with a) microscale extraction (Smedsgaard, 1997) for 60 min; b) with pure ACN for 5 min; c) with ACN:MiliQ 75/25 *v/v* + 1% FA for 5 min. Metabolites identified in at least two extracts: 1 – *m/z* 429.2272, tentative compound 2 (Jarvis et al., 2003); 2 – L-611776; 3 – unknown 471.2393; 4 – stachybotrydial; 5 – *m/z* 487.2326, tentative compound 4 (Jarvis et al., 2003); 6 – *m/z* 429.2272, tentative compound 2 (Jarvis et al., 2003).

2.1.3. Quantification of secondary metabolites

This section discusses used approaches for preparation of calibration curves for *Chaetomium* and *Stachybotrys* metabolites as well as the challenges faced during their validation and quantification. All the calibration and validation experiments were based on an external calibration approach (either prepared in neat solvent or matrix-matched), without using an internal standard. Quantification of *Chaetomium* metabolites was performed using the QTOF instrument while quantification of *Stachybotrys* metabolites was performed on the QqQ instrument.

2.1.3.1. QTOF method for Chaetomium *secondary metabolites*

The semi-quantification of *Chaetomium* metabolites from artificially inoculated building materials is presented in **Paper 3**. As mentioned above, semi-validation and quantitative measurements were performed using the external calibration approach. It should be noted that all calibration curves were prepared without the use of isotope-labelled internal standard due to the unavailability of the internal standard compounds. This meant that any possible loss of target compounds during the sample preparation or due to possible differences in matrix effect between calibrants and analysed samples could not be accounted for. External calibration curves were prepared only for two quantitatively available metabolites: chaetoglobosin A and chaetoviridin A. All other *Chaetomium* metabolites were quantified based on structure similarity with one of these two compounds, which represented the main challenge of this approach.

Using calibration curve of chaetoglobosin A to calculate concentration of all identified chaetoglobosins was based on the same elemental composition for chaetoglobosin A, C, G, U and V therefore providing the same molecular ion which was used as quantifier, or in case of hydrogenated analogues, molecular ion with two *m/z* units more (chaetoglobosin E and F). Furthermore, all chaetoglobosins appeared to have very similar fragmentation patterns and UV spectra, due to the minor differences in their structures. Based on all these facts, the assumption of similar ionisation properties for all chaetoglobosins was made. Since the area under the peak in case of MS analysis is not directly proportional to the concentration and is highly dependent on the ionisation properties of the compound of interest, relative comparison of the peaks for these metabolites needed to be confirmed by UV/Vis data (**Paper 3,** Supplementary information).

Concentrations of metabolites other than chaetoglobosins were calculated based on the calibration curve for chaetoviridin A, although these metabolites showed less similarity in elemental composition and chemical structure to chaetoviridin A in comparison to structural similarities among chaetoglobosins. Assuming similar ionisation properties of chaetoviridin A and other identified chaetoviridins and chaetomugilins seemed plausible, considering their structural similarities. On the other hand, the only similarity between chaetoviridin A on one side and cochliodones and chaetoglobin A on the other, was azaphilone moiety, making the prediction of ionisation relative to each other almost impossible. Clearly, calculated concentrations, especially in cases where structural differences between metabolites were more prominent, are not exact. However, the purpose of this study was not to determine exact concentrations of each metabolite but to make rough estimation of their amounts on building materials. Therefore, this approach showed to fit its intended purpose, despite its obvious pitfalls (**Paper 3,** Supplementary information).

When it comes to method performance and validation (**Paper 3,** Supplementary information), our method performance testing was limited and far from full analytical method validation. Besides the lack of internal standard, recovery and accuracy could not be determined due to the nature of the analysed samples. Testing the recovery of the method would require blank matrix spiked before and after extraction. Since it was impossible to obtain blank matrix (fungal mycelium free from metabolites), experiments determining recovery and accuracy were not included in method performance testing. Signal suppression/enhancement (SSE) was evaluated for five representative materials included in the artificial inoculation experiment: non-treated plywood, non-treated chipboard, gypsum, nonwoven woodchip wallpaper (wallpaper type 1) and nonwoven wall-covering with pattern (wallpaper type 2).

The peak area to concentration response for chaetoglobosin A was linear across all levels (0.01 – 10 μg/mL, R^2 = 0.9908), with the limit of detection being 0.09 μg/cm^2 and a limit of quantification of 0.3 μg/cm^2. Chaetoviridin A did not exhibit linearity across the entire calibration range due to the detector saturation, but good linearity was observed across first four levels (0.01 – 1 μg/mL, R^2 = 0.9932), which were used for calibration curve with the limit of detection being 0.01 μg/cm^2 and the limit of quantification 0.02 μg/cm^2. When it comes to the signal suppression/enhancement all results lied within allowed limits (± 20 %), except in the case of low level spiked chipboard where signal enhancement outside of the allowed limits were noted for both metabolites (52 % for chaetoglobosin A and 35 % for chaetoviridin A, **Paper 3**, Supplementary information) . The least signal alteration caused by matrix was observed in gypsum. Generally, no material showed significant signal enhancement/suppression abilities. Vishwanath et al. (2009) also observed similar results for some of the materials (gypsum, wood).

It should also be noted that the effect of matrix on signal suppression/enhancement in this experiment presented in **Paper 3** was on purpose largely overestimated. Although the presence of small amounts of background due to the wetness of the sampled material in the samples could not be avoided, they contained mostly scraped *Chaetomium* biomass as *Chaetomium* is known to provide substantial amounts of biomass on very wet materials (Nielsen et al., 1999). Therefore, it can be said with certainty that the spiked matrix samples contained more matrix than any of the samples of *Chaetomium* grown on building materials (**Paper 3**). On the other hand, since the method was dedicated for the analysis of real life fungal infected building material extracts, other factors were also expected to contribute to the matrix effect. These include co-extracted constituents of possibly present bacteria and other microorganisms, as well as dust, which was previously reported to heavily alter the signal (Vishwanath et al., 2009).

Overall, the semi-quantitative QTOF method developed and presented in **Paper 3** could clearly benefit from further optimisation. Use of isotope-labelled internal standard, may provide better linearity over the wider concentration range and account for the losses during different stages of the sample prep, as well as for differences between different matrices. Availability of other *Chaetomium* metabolite standards would result in a method calibrated for each individual compound included in it. This would eliminate uncertainty in calculated concentrations of metabolites, whose quantification is currently based on calibration curve of structurally similar compounds. Despite all limitations, this method has proved fit to the intended purpose, namely to provide a rough estimation of amounts of *Chaetomium* metabolites on building materials. Until all requirements for better method optimisation are provided, the method can be used as a valuable tool for estimating the amounts of *Chaetomium* metabolites in the indoor environment.

2.1.3.2. QqQ method for Stachybotrys *secondary metabolites*

The development of the method for quantification of *Stachybotrys* metabolites was presented in **Paper 5.** The method was used to quantify *Stachybotrys* metabolites in dust samples (**Paper 5**) as well as in samples collected from building materials (scrape-offs, swabs; **Paper 4** and **5**). As in the case of the method for *Chaetomium* metabolites, all validation and quantification measurements were based on the external calibration approach. Calibration curves and measurements were performed without the use of isotope-labelled internal standards, again due to their unavailability. External calibration curves were made for four quantitatively available standards: roridin E, satratoxin H, stachybotrylactam and trichodermin. All other metabolites were quantified based on the calibration curve of structurally similar metabolite, if any (Table

6). This resulted in the method that was quantitative for all observed MTR and spirocyclic drimanes, whilst atranones and dollabellanes were only screened qualitatively, due to the large structural difference with four compounds quantitatively available as standards (roridin E, satratoxin H, stachybotrylactam and trichodermin). Challenges of quantifying other MTR based on structural similarities to satratoxin H or roridin E and other spirocyclic drimanes based on structural similarity to stachybotrylactam are discussed in greater detail in **Paper 5**.

As mentioned in previously, spirocyclic drimanes are produced in larger amounts in comparison to both atranones and MTR. Therefore, they tend to obscure the signal of less abundant atranones and MTR, especially when analysed on the QTOF instrument. To address this issue and increase sensitivity of less abundant compounds in extracts, the method was transferred from QTOF to the more sensitive QqQ instrument. This was done by choosing the mass transitions based on information retrieved from QTOF MS/HRMS 10, 20 and 40 eV spectra. Information about precursor ions, best fragments as well as collision energies at which certain fragments were obtained, were taken directly from MS/HRMS library and further optimised. The required optimisation of collision energy was minimal and in all cases included changes of less than 5 eV (**Paper 5**). This example demonstrates the value of the information that can be retrieved from MS/HRMS analyses obtained on the QTOF and proves the concept of direct MS/HRMS library to QqQ transfer.

When it comes to method performance and validation, the situation is the same as with *Chaetomium* quantitative method. The performed experiments were, due to the nature of the analysed samples, limited and far from true method validation. Recovery of the method could not be obtained, as it was impossible to obtain blank matrix for spiking (mycelium free from metabolites). Signal suppression/enhancement was tested only for clean precision Kimwipes® Lite wipes used for dust collection (**Paper 5**), which appeared to have no impact on signal suppression/enhancement. It was approximated that signal suppression/enhancement of different building materials was not significant, based on results reported by Vishvanath et al. (2009) as well as our on results obtained for *Chaetomium* metabolites and presented in **Paper 3**. **Paper 5** provides a thorough discussion on *Stachybotrys* method performance parameters. Major method performance results are presented in Table 6.

Table 6 Matrix effect, signal suppression/enhancement (SSE), standard error of regression line (Sy), average observed accuracy (Acc) and maximum observed accuracy (Acc_{max}), coefficients of variation (CV), limit of detection (LOD) and lower limit of quantification (LLOQ) values for *Stachybotrys* metabolites.

Metabolite	SSE[d] (%)	Sy[e]	Acc (Acc_{max})[f](%)	CV[g] (%)	LOD[h] (ng/cm^2)	LLOQ[i](ng/cm^2)
Satratoxin H[a]	134	1.8	28 (79)	2.2	15	50
Satratoxin G[a]	134	1.8	28 (79)	2.2	15	50
Roridin E[b]	104	4.9	29 (114)	2.2	0.1	0.2
Roridin L2[b]	104	4.9	29 (114)	2.2	0.1	0.2
Stachybotrylactam[c]	89	36	21 (77)	2.5	2	6
Stachybotrylactam isomer[c]	89	36	21 (77)	2.5	2	6
Stachybotryamide[c]	89	36	21 (77)	2.5	2	6
Stachybotrydial[c]	89	36	21 (77)	2.5	2	6
Mer-NF-5003-B[c]	89	36	21 (77)	2.5	2	6
L-611776[c]	89	36	21 (77	2.5	2	6
K-76 compound 1[c]	89	36	21 (77	2.5	2	6
K-76 compound 2[c]	89	36	21 (77	2.5	2	6
Compound 2[c]	89	36	21 (77	2.5	2	6
F-1839 I[c]	89	36	21 (77	2.5	2	6
F-1839 J[c]	89	36	21 (77	2.5	2	6
Trichodermin	79	40	13.4 (28)	4.4	5	17

[a]Compounds calibrated against satratoxin H standard

[b]Compounds calibrated against roridin E standard

[c]Compounds calibrated against stachybotrylactam standard

[d]SSE%: signal suppression/enhancement calculated as the slope ratio of the linear functions for matrix-matched standards and liquid standards multiplied by 100

[e]Standard error of regression line

[f]Average accuracy on all levels. Accuracy on each level was calculated as ratio of calculated concentration and expected concentration multiplied by 100. Acc_{max} represents maximum observed value regardless of level including the values outside allowed limits (20 %)

[g]CV%: calculated as the average of the ratios of standard deviation and average concentration multiplied by 100 for each level

[h]LOD: Limit of detection calculated at the lowest concentration levels as concentrations corresponding to a signal-to-noise ratio (S/N) 3/1

[i]LLOQ: Lower limit of quantification calculated at the lowest concentration levels as concentrations corresponding to a signal-to-noise ratio (S/N) 10/1

The main goal of the QTOF to QqQ method transfer was the possibility of increased sensitivity, which would enable screening for *Stachybotrys* metabolites in indoor samples containing very low concentrations of them, such as dust samples. This was demonstrated in **Paper 5**, where atranone A and dolabellanes were detected, whilst MTR and spirocyclic drimanes were both detected and quantified in the dust samples collected from a water-damaged kindergarten. Similarly to the *Chaetomium* quantitative method, quantitative measurements obtained by the *Stachybotrys* quantitative method are not exact. The method provides rough estimates of amounts of *Stachybotrys* metabolites found in different indoor samples. Undoubtedly, the use of

isotope-labelled internal standards, as well as the availability of authentic quantitative standards for other *Stachybotrys* metabolites would increase the quality of the data obtained with this method. However, despite its disadvantages, the method proved to be a valuable tool in providing information about *Stachybotrys* mycotoxins and other bioactive metabolites present in contaminated buildings.

2.2. *Chaetomium* and *Stachybotrys* metabolites in the indoor environment

2.2.1. *Chaetomium* spp. in indoor environment

This section discusses the results presented in **Paper 3**, obtained on *Chaetomium* spp. present on building materials and/or in indoor environment. The results are divided in three separate sections, discussing the growth of *Chaetomium* spp. on different types of building materials, *Chaetomium* metabolites produced on artificially inoculated building materials and on naturally contaminated indoor materials. Table 8 provides an overview of all the results obtained for *Chaetomium* metabolites from different damp indoor materials gathered during this PhD study.

2.2.1.1. Growth on artificially inoculated materials

The growth of three different *Chaetomium* spp. was evaluated on five different building materials (**Paper 3**). Two strains of *C. globosum* (IBT 7029 and 41801), one *C. elatum* (IBT 41944) and one reference strain of *C. virescens* (IBT 26237) were each separately inoculated on sterilised (1 × 40 kGy γ-irradiation) building blocks of chipboard, plywood, gypsum wallboard, MDF and masonite. The artificial inoculation was performed with the purpose of determining growth patterns on the above-mentioned materials as well as what type of metabolites different species were able to produce under controlled conditions *in vitro*.

Prior to sterilization, building blocks were tested for pre-contamination with fungal spores by addition of double-distilled water, and thereby creating favourable conditions for fungal growth. In this experiment, untreated building blocks were added 100 mL of double-distilled water and left to absorb overnight. After 24 h, the excess of water was decanted and building blocks were incubated in closed plastic boxes at room temperature for 14 days. The results revealed presence of several fungal species, as presented in Fig. 18. As confirmed in another study on gypsum wallboard, presented in **Paper 1**, the building materials seemed to come pre-inoculated

with fungal spores. The fungi appeared to be in hibernation state on the materials, waiting for the favourable condition for their growth to occur (increased a_w). Fig. 18 shows chipboard, plywood, MDF and masonite after 14 days period of incubation. As visible from the Fig 18, chipboard and plywood were predominantly contaminated with *Chaetomium* spp. (Fig. 18a and b, red circles), whilst MDF was predominantly contaminated with *Eurotium* spp. (Fig. 18c, pink circle). Two different strains of *Chaetomium* spp. could clearly be distinguished visually, due to the difference in their colour and size of perithecia. Gypsum wallboard showed sporadic *Chaetomium* growth, which was overtaken by *Stachybotrys* spp. after two weeks (Fig. 21, Section 2.2.2.1). Masonite (Fig. 18d) did not yield abundant fungal growth, but only sporadic growth of *A. niger* (circled in blue), which was also seen on other three materials together with few isolated *Penicillium* spp. colonies (green circles), also observed on plywood and MDF.

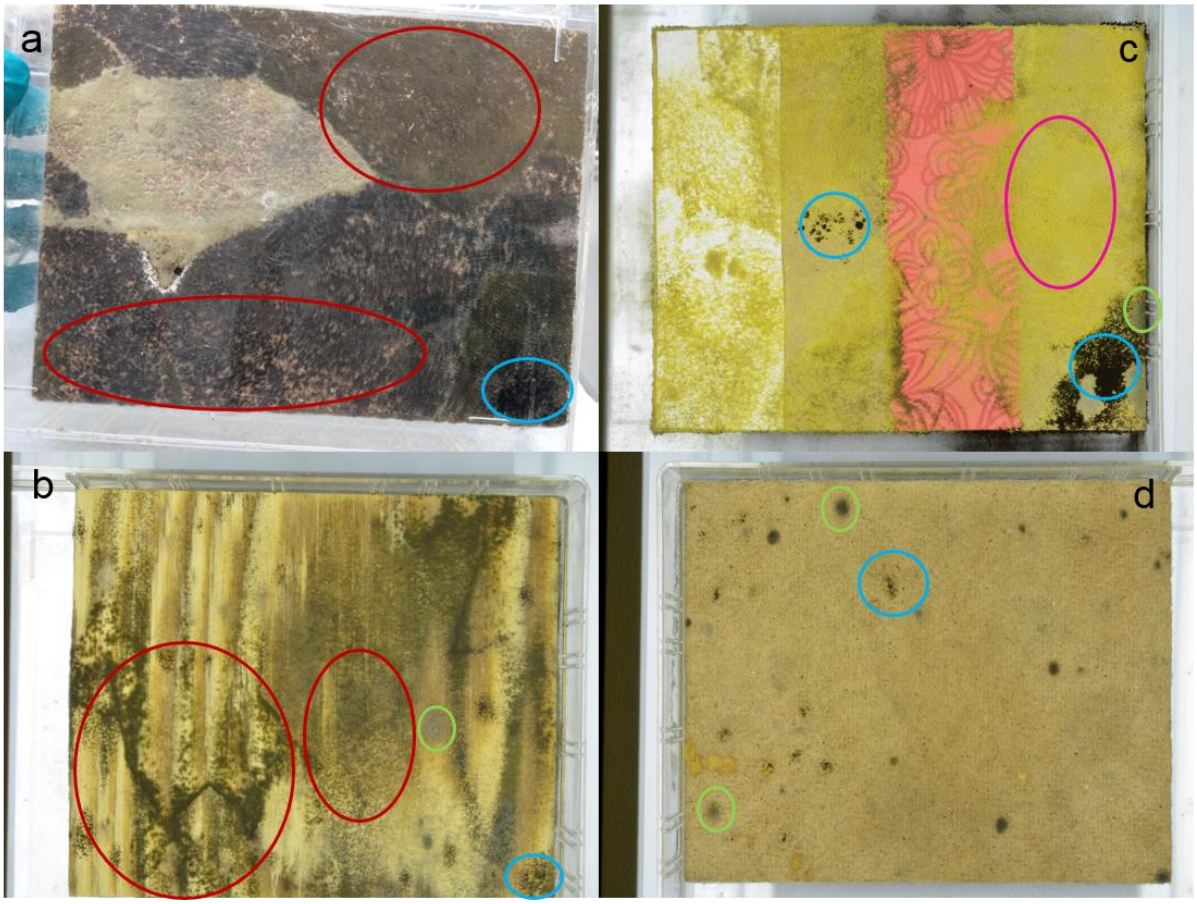


Fig. 18 Different fungal species found on artificially wetted building materials after growth period of 14 days: a) chipboard; b) plywood; c) MDF; d) masonite. Marked areas represent different species: red – *Chaetomium* spp.; blue – *A. niger*; pink – *Eurotium* spp.; green – *Penicillium* spp..

The artificial inoculation study on sterilized materials (**Paper 3**) corroborated the findings of the pre-sterilization experiment. In this study, both *C. globosum* and *C. elatum* showed preference for

wood-based materials, namely chipboard and plywood, where both species showed best growth and yielded the highest amounts of biomass (Table 7). Gypsum wallboard also proved to be good substrate for *C. globosum* and *C. elatum,* however, the growth was visually less dense in comparison to chipboard and plywood. Our results were in agreement with existing literature, which reports that *C. globosum* present in damp indoor environment is most commonly found on solid wood, manufactured wood, textiles, and wallboard as well as on insulation (Miller et al., 2008; Flannigan and Miller, 2011). MDF and masonite did not support any growth of either *C. globosum* or *C. elatum,* which was confirmed both macro- and microscopically. Inability of MDF to support *Chaetomium* growth could be explained by low water absorption ability of the material (5 %, Table 7), whilst masonite showed to be a poor substrate despite the water absorption ability equal to gypsum (17 %). *C. virescens* did not yield any growth on any of the five materials, suggesting that this fungus thrives the best in conditions different from the ones found in indoor environment.

Furthermore, this study aimed to test how the presence of parameters such as wallpapers and/or adhesive on building material influenced growth/metabolite production. The obtained results and findings are thoroughly discussed in **Paper 3**.

Table 7 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 (**Paper 3**)

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 8 Overview of all *Chaetomium* metabolites found on different building materials throughout this PhD study

	Building material							
Metabolite	pure agar culture	cardboard[b]	ceiling tile[c]	cellular concrete[a]	chipboard[a,b]	gypsum [a,c]	OSB[a]	plywood[a,b]
Chaetoglobin A	+	+	-	+	+	+	+	+
Chaetoglobosin A	+	-	-	-	+	+	+	+
Chaetoglobosin C	+	-	-	-	+	+	+	+
Chaetoglobosin E/F	+	-	-	-	+	+	+	+
Chaetoglobosin G/D/ U/V	+	-	-	-	+	+	+	+
ChaetoviridinA/ Chaetomugilin C	+	-	-	-	+	+	+	+
ChaetoviridinC/ Chaetomugilin D/S	+	-	-	-	+	+	+	+
Chaetoviridin E	+	-	-	-	-	+	-	-
Chaetoviridin H	+	-	-	-	-	+	-	-
Cochliodone 1	+	+	-	+	+	+	+	+
Cochliodone 2	+	+	-	+	+	+	+	+
Cochliodone 3	+	+	+	+	+	+	+	+
Cochliodinol	+	-	-	-	-	-	-	-
Prochaetoglobosin I	+	-	-	-	+	-	-	+
Prochaetoglobosin II	+	-	-	-	+	-	-	-
Prochaetoglobosin III/III$_{ed}$	+	-	-	-	+	-	-	+
Prochaetoglobosin IV	+	-	-	-	-	-	-	-

[a]produced by *C. globosum*

[b]produced by *C. elatum*

[c]*Chaetomium* spp. (not identified to the species level)

2.2.1.2. Chaetomium *metabolites on artificially inoculated materials*

Secondary metabolites production on artificially inoculated materials was evaluated for chipboard, plywood and gypsum wallboard, as no growth of either *C. globosum* or *C. elatum* was observed on MDF or masonite. Generally, both species were able to produce the majority of metabolites detected in pure agar cultures, on at least one of the materials (**Paper 3**). The only exception was cochliodinol that was not detected on any of the three inoculated materials (Table 8). Obtained results corroborated findings of growth evaluation, showing the highest number of metabolites for both *C. globosum* and *C. elatum* on chipboard and the lowest on gypsum (**Paper 3**, Table 3). Chaetoglobosins and chaetoviridins/chaetomugilins were produced only by *C. globosum* strains. It should be noted that chaetoviridins/chaetomugilins were found only on materials inoculated by IBT 41801, which was in accordance with results obtained from pure culture extracts. This could possibly be explained by better adaptation of *C. globosum* IBT 41801 to the conditions of damp indoor environment in comparison to *C. globosum* IBT 7029. These results are also in accordance with findings of Nielsen et al. (1999). Both *C. globosum* and *C. elatum* produced cochliodones 1, 2 and 3 on all three materials (chipboard, plywood and gypsum wallboard). Chaetoglobin A, another common metabolite for *C. globosum* and *C. elatum*, based on results from pure agar cultures, was found on all three materials inoculated with *C. globosum*, whilst *C. elatum* was able to produce this metabolite only on chipboard.

During growth of *C. globosum* and *C. elatum* on artificially inoculated building materials, especially on chipboard, production of condensate four weeks after inoculation was observed. This condensate probably occurred as the result of fungal degradation of the material, which resulted in the production of water that occurred in the form of condensate on the lid of the plastic box where the building block was placed (Fig. 19). The same was observed during growth of *Chaetomium* spp. on agar media where the presence of the condensate was noted on the lid of Petri dish. In order to investigate whether this condensate contained any of the *Chaetomium* metabolites found on the materials or in pure agar cultures, an experiment was made where *C. globosum* (IBT 42176) was grown on V8 for 30 days in the hermetically sealed container, after which produced condensate was directly analysed for presence of *Chaetomium* metabolites. The results showed no presence of any *Chaetomium* metabolites in the produced condensate.

Fig. 19 Occurrence of condensate as the result of fungal degradation of chipboard: a – *C. elatum* (IBT 41944); b – *C. globosum* (IBT 41801)

Quantification of *Chaetomium* metabolites on artificially inoculated building materials showed that three metabolites, namely chaetoglobosin A, chaetoviridin A and cochliodone 3 were produced in significantly higher amounts in comparison to other metabolites (**Paper 3**). The highest estimated concentrations for chaetoglobosin A, chaetoviridin A and cochliodone 3 were 95 µg/cm^2, 19 µg/cm^2 and 21 µg/cm^2 respectively. Table 9 represents estimation of concentration range (µg/cm$^{2)}$ for four major metabolite groups found on artificially inoculated building materials. Concentration estimates for individual metabolites can be found in **Paper 3** (Supplementary information, Table S2).

Table 9 Estimation of concentration range (μg/cm2) for four major metabolite groups found on artificially inoculated building materials

Materials		Concentration range (μg/cm2)							
		Chaetoglobosins		Chaetoviridins		Cochliodones		Chaetoglobin A	
		C. globosum	*C. elatum*	*C. globosum*	*C. elatum*	*C. globosum*	*C. elatum*	*C. globosum*	*C. elatum*
Chipboard	wp type 1	0.3-2.9	NA	0.6-2.7	NA	0.1-4.4	0.3-4.8	1	1.3
	adhesive	1 -95.4	NA	1.4-1.8	NA	0.1-4.5	0.1-3.7	2.3-2.3	3
	wp type 2	1.1-6.5	NA	0.5-8.5	NA	0-3.7	0.9-2.7	2-7	2.2
	blank	0.7-81.7	NA	1.6-19.2	NA	1.9-21	0 -2.6	3-3.19	2.54
Plywood	wp type 1	0.2-10.6	NA	0.2-9.3	NA	1.4-7	0.1-3.5	NA	NA
	adhesive	0.7-39.8	NA	0.7-13.4	NA	0.2-11.7	0.1-3	0.1-0.3	NA
	wp type 2	0.8-36.3	NA	0.4-14.1	NA	0 -14.6	0.6-1.9	NA	NA
	blank	1.1-62.4	NA	1.3-15.3	NA	0.2-12.9	0.6-2	0.02	NA
Gypsum	wp type 1	2.3-2.6	NA	0.1-6	NA	0.1-4.8	0.1-1.7	NA	NA
	adhesive	6-9.9	NA	0.2-8.8	NA	0.1-6.8	NA	NA	NA
	wp type 2	2.2-2.6	NA	0.3-3.9	NA	0.3-3.5	NA	NA	NA
	blank	2.2-2.7	NA	0.5-0.5	NA	0.7-4	0.2-2.8	NA	NA

NA-not analyzed due to the absence or insufficient growth

Results of *Chaetomium* spp. study on artificially inoculated building materials provided two important conclusions. Firstly, the results strongly suggested the production of chaetoglobosins and chaetoviridins/chatomugilins only by *C. globosum*. Furthermore, chaetoglobosin A and chaetoviridin A were produced in significantly higher amounts in comparison to the other chaetoglobosins and chaetoviridins/chaetomugilins. Therefore, these compounds appear to be good candidates for *C. globosum* specific biomarkers. On the other hand, cochliodones and chaetoglobin A were shared metabolites for both *C. globosum* and *C. elatum*. Since cochliodone 3 was found in significantly higher amounts in comparison to cochliodone 1, 2 and chaetoglobin A, this compound is so far the best candidate for *Chaetomium* genus-specific biomarkers in indoor environment.

2.2.1.3. Chaetomium *spp. on real life water-damaged building materials*

Analysis of damp building materials and other samples naturally contaminated with *Chaetomium* spp. confirmed all previously obtained results on *Chaetomium* spp. from artificially inoculated materials. In all cases where the contaminant was *C. globosum* the samples were positive on several chaetoglobosins, chaetoviridins/chaetomugilins as well as on cochliodones and in most cases on chaetoglobin A too (Table 8). On the other hand, in cases where the

contaminant was identified as *C. elatum* or was not identified to species level, samples were positive on presence of cochliodones and occasionally on chaetoglobin A. The type of material found in water-damaged indoor environment and contaminated with *Chaetomium* spp., was either wood based materials (chipboard, OSB, cardboard) or gypsum board (Table 8). These results are both in agreement with the above presented results as well as with literature findings (Miller et al., 2008; Flannigan and Miller, 2011).

The main challenge during the study of *Chaetomium* metabolites found on real life water-damaged building materials was the low number of reported cases (Table 4 and 5, **Paper 3**). During this PhD study the samples from only four real-life cases where *Chaetomium* spp. was identified as main contaminant were collected (Table 5, **Paper 3**). Therefore, materials artificially wetted but naturally contaminated with *Chaetomium* spp. were also included in the study (Table 4, **Paper 3**). When it comes to real life water-damaged indoor environment the obtained results undoubtedly confirmed cochliodones as *Chaetomium* spp. biomarkers as well as chaetoglobosins and chaetoviridins/chaetomugilins as *C. globosum* specific biomarkes. As already mentioned in the introduction, the insufficient data on toxicology is currently available only for chaetoglobosins (Udagava et al., 1979; Thohinung et al., 2010), whilst none is available for chaetoviridins/chaetomugilins, cochliodones and chaetoglobin A. Hence, the role, if any, of these *Chaetomium* metabolites in building dampness-related illness and/or discomfort remains to be determined.

2.2.2. *Stachybotrys* spp. in indoor environment

This section discusses the results obtained on *Stachybotrys* spp. from water-damaged indoor environment and/or from artificially inoculated building materials. The results are presented in **Paper 4** and **5**. As it was previously done for the findings on *Chaetomium* spp., the results are divided in three sections discussing *Stachybotrys* spp. growth on different building materials, metabolites found on artificially inoculated building materials and metabolites found in extracts of samples collected from real life water-damaged indoor environment. Table 10 gives an overview of all *Stachybotrys* metabolites found on different indoor materials collected during this PhD study.

2.2.2.1. Growth on artificially inoculated materials

Growth of four *Stachybotrys* spp. on five artificially inoculated materials: chipboard, plywood, gypsum wallboard, MDF and masonite was evaluated in **Paper 4**. *Stachybotrys* spp. used in this study were *S. chartarum* (IBT 9631, chemotype S), *S. chartarum* (IBT 9466, chemotype A), *S. chlorohalonata* (IBT 40825) and *S. nephrospora* (IBT 9458). The concept of the experiment was the same as previously explained for *Chaetomium* spp., except that in this case two strains were inoculated on each building block. The combination of two strains inoculated on the same sample block represented different species within same genus (*S. chartarum* IBT 9631*/S. chlorohalonata* IBT 40285*, S. chartarum* IBT 9466*/S. nephrospora* IBT 9458) with different metabolite profiles when applicable (satratoxin producer/atranone producer, Fig. 20; **Paper 3**).

The pre-sterilisation experiment on chipboard, plywood, gypsum wallboard, MDF and masonite showed that only gypsum board was naturally pre-contaminated with *Stachybotrys* spores (Fig. 21), whilst wood-based materials appeared not to be pre-contaminated with *Stachybotrys* spp. (Fig. 18). After 14 days, the gypsum wallboard showed presence of both *Stachybotrys* spp. and *Chaetomium* spp., however the material was fully overgrown with *Stachybotrys* spp after 28 days. Pre-contamination of the gypsum wallboard with several different fungal species, among which there are also *Stachybotrys* spp., is presented in the greater detail in **Paper 1**.

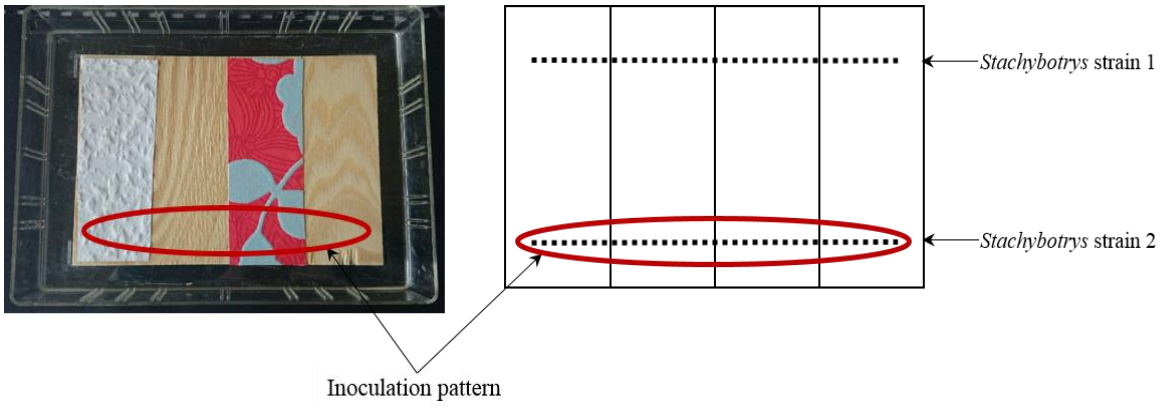


Fig. 20 Artificially inoculated building blocks with inoculation pattern and distribution of strains across sections. Sections from left to right: wallpaper adhesive + nonwoven woodchip wallpaper (wp1), wallpaper adhesive, wallpaper adhesive + nonwoven wall-covering with pattern (wp2), non-treated material

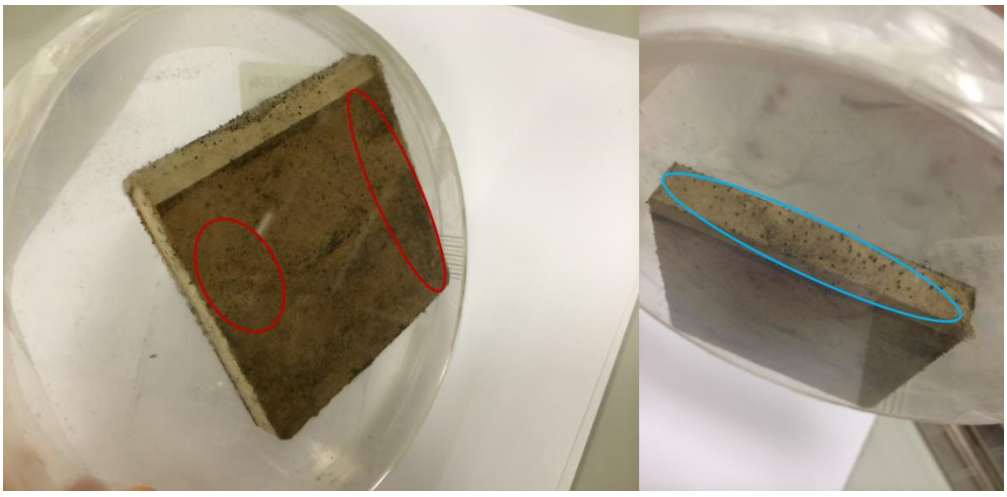


Fig. 21 *Stachybotrys* spp. and *Chaetomium* spp. found on artificially wetted naturally contaminated gypsum board after growth period of 14 days: red – *Stachybotrys* spp.; blue – *Chaetomium* spp

The growth of *Stachybotrys* spp. on artificially inoculated materials after sterilization confirmed *Stachybotrys* spp. preference for gypsum wallboard, which was also in accordance with the existing literature (Andersen et al., 2011). The fungal growth was both slower and less dense on wood-based materials (plywood and chipboard, **Paper 3**) in comparison to gypsum wallboard. In general, there was no visual difference in growth between two strains inoculated on the same building block. When it comes to the other two materials, masonite supported very scarce growth for all used *Stachybotrys* strains, whilst no *Stachybotrys* growth was observed on MDF. Lack of any *Stachybotrys* growth on MDF could be explained, as in case of *Chaetomium* spp. by material's poor water absorption ability (5 % *w/w*) (**Paper 3**, **Paper 4**). As it was also observed earlier with *Chaetomium* spp. (**Paper 3**), comparing the growth on the two types of wallpapers used (nonwoven woodchip wallpaper - wp1 and nonwoven wall-covering with pattern - wp2) revealed more prominent growth on wp1. Growth on wp2 was inhomogeneous, appearing only on the flat surfaces in between polystyrene pattern and never on the pattern itself, due to the lack of nutrients in inorganic parts of wallpaper (**Paper 4**). Evaluation of the types of materials contaminated with *Stachybotrys* spp. and collected from real life water-damaged environment corroborated general preference of *Stachybotrys* spp. for gypsum board and insulation materials (Table 4, **Paper 4**).

Table 10 Overview of all *Stachybotrys* metabolites found on different building materials throughout this PhD study

	Indoor material							
Metabolite	pure agar cultures	cardboard /insulation	photoalbum cardboard	chipboard	dust	gypsum	masonite	plywood
Atranone A	+	+	-	+	+	+	+	+
Atranone B	+	+	-	+	+	+	+	+
Atranone G	+	-	-	-	-	-	+	+
Dolabellane 1	+	+	-	+	+	+	+	+
Dolabellane 2	+	+	-	+	+	+	+	+
Roridine E	+	-	-	+	+	+	+	+
epi/iso-Roridine E	+	-	-	+	+	+	+	+
Roridin H	+	-	-	+	-	+	+	+
Roridin L2	+	-	-	+	-	+	+	+
Satratoxin H	+	-	-	-	-	+	-	-
Isosatratoxin H	+	-	-	-	-	-	-	-
Isosatratoxin F	+	-	-	-	-	-	-	-
satratoxin G	+	-	-	-	-	+	+	+
Chartarlactam C	+	-	-	-	-	-	-	-
Chartarlactam D	+	-	-	-	-	-	-	-
Chartarlactam K	+	+	-	+	-	+	+	+
Chartarlactam M/O	+	+	-	+	+	+	-	-
Stachybotrylactam	+	+	-	+	+	+	+	+
Stachybotryamide	+	+	-	+	+	+	+	+
Mer-NF-5003-B	+	+	-	+	-	+	-	-
Stachybotrydial	+	+	-	+	+	+	+	+
SDHBFL III/Chartarlactam B	+	+	-	+	-	+	+	+
SDHBFL IV/K 76 compound 1	+	+	-	+	-	+	+	+
K 76 compound 2	+	+	-	+	-	+	+	+
L-611776	+	+	-	+	-	+	+	+
F-1839-I	+	+	-	+	-	+	-	+
F-1839-J	+	+	-	+	-	+	+	+
Compound 2*	+	+	-	+	-	+	+	+

*Jarvis, 2003

2.2.2.2. Stachybotrys *metabolites on artificially inoculated materials*

The results of secondary metabolite production on artificially inoculated building materials corroborated the findings on *Stachybotrys* growth on different materials (**Paper 4**). The metabolite production was evaluated on chipboard, plywood, gypsum wallboard and masonite, whilst MDF could not be sampled due to the absence of any *Stachybotrys* growth on it. The highest number of metabolites per building block was found on the gypsum board, confirming the *Stachybotrys* spp. preference for this material. This was again in accordance with results observed for Stachybotrys spp. in **Paper 1**. Among wood-based materials, the best metabolite production was observed on plywood while the lowest number of metabolites was observed on chipboard (Table 2, **Paper 4**). Generally, all three species were able to produce all metabolites detected in pure agar cultures, on at least one of the four materials (chipboard, plywood, gypsum wallboard and masonite), suggesting that used *Stachybotrys* spp. are well adapted for growth on wet building materials (**Paper 4**).

The biggest challenge of this study was cross-contamination of two inoculated strains on the same building block. Namely, the presence of atranones and dolabellanes was detected in gypsum extracts of chemotype S *S. chartarum* strain (IBT 9631), whilst several MRTs were detected in gypsum and plywood extracts of *S. chlorohalonata* (IBT 40285). According to Andersen et al. study (2002), performed on more than 200 isolates of *Stachybotrys* spp., no *Stachybotrys* isolate is able to produce both MTR and atranones. Thus, the only possible explanation for presence of both groups of metabolites in the same extract was cross-contamination. The evaluation was even more complicated when two atranone producers were combined on the same building blocks (*S. chartarum* IBT 9466 and *S. nephrospora* IBT 9458) (**Paper 4**). The results, however, showed no predomination or suppression of one strain towards the other, suggesting plausible coexistence of several *Stachybotrys* species/strains on the same building materials. This was also confirmed in real-life cases of water-damaged building materials (**Paper 4** and **5**).

Spirocyclic drimanes were produced by all strains and species on both gypsum board and wood-based materials. Among them, the most abundant compounds in all analysed extracts were L-611776 and stachybotrydial, suggesting them as the best candidates for *Stachybotrys* indoor biomarkers so far.

2.2.2.3. Stachybotrys *spp. in real life water-damaged indoor environments*

During this PhD study, samples from seven separate cases of fungal contamination in indoor environment with *Stachybotrys* spp. as main contaminant were collected (**Paper 4**, Table 3). The samples included following materials: gypsum, cardboard, insulation and chipboard as well as dust. Spirocyclic drimanes were detected in all analysed samples except in the case of photoalbum cardboard (Table 10, **Paper 4**, Table 3). They showed once more to be the dominant group in all analysed samples, which corroborated findings previously reported in literature (Hinkley and Jarvis, 2000; Andersen et al., 2002). The total estimated amount of all spirocyclic drimanes in wall-swabs from study-case presented in **Paper 5** was 2.5 μg/cm^2. The results obtained for spirocyclic drimanes from real-life water damaged building materials supported the findings from artificially inoculated materials. Namely, the most abundant compounds found in six out of seven cases were stachybotrydial, L-611776 (**Paper 4**) along with stachybotrylactam and chartarlactam M/O (stachybotrylactam isomer, **Paper 5**). These findings suggested once again stachybotrydial and L-611776 as the best candidates for *Stachybotrys* indoor biomarkers.

Atranones and dolabellanes were detected in all samples in six out of seven reported cases, whilst MTR were detected in samples of four out of seven cases. This is in accordance with the literature reporting atranone producing strains (chemotype A) as more frequently found in the indoor environment in comparison to MTR producing strains (chemotype S) (Andersen et al., 2002; Miller et al., 2003). The highest estimated concentration found on building materials was found for satratoxin H (180 ng/cm^2, **Paper 5**), whilst atranones and dolabellanes could not be quantified due to the unavailability of chemically related reference standards (**Paper 4** and **5**). All results suggested the coexistence of chemotype A and S, without obvious predominance of any of the chemotypes when it comes to metabolite production.

The major breakthrough in this PhD project was the detection and quantification (estimated amounts) of MTR in dust samples (**Paper 5**). Paper 5 presented the case-study of water-damaged kindergarten contaminated with both atranone and MTR producing *Stachybotrys* spp (Fig. 22). Settled dust collected from the water-damaged room, as well as from neighbouring rooms, contained all three groups of metabolites: atranones, MTR and spirocyclic drimanes (Table 4, **Paper 5**). The amounts of spirocyclic drimanes found in dust were for one order of magnitude higher in comparison to the amounts of MTR represented by roridine E**,** whilst estimated amounts of both spirocyclic drimanes and MTR were three to four magnitudes lower in dust in comparison to the amounts found on the contaminated gypsum wallboard (Table 4, **Paper 5**). Although hydrolysis products of MTR (verrucarol and trichodermol) have been

previously reported in dust (Bloom et al., 2007; Bloom et al., 2009; Peitzsch et al., 2012), this is the first study where MTR were directly quantified in dust. Furthermore, this is the first time that spirocyclic drimanes other than stachybotrylactam are reported in dust as well as the first time that a comparison between the amounts of mycotoxins produced on contaminated wall surfaces and the amounts of mycotoxins that actually become airborne has been made (**Paper 5**). This study-case demonstrates the importance of sampling and analysis of dust as it clearly depicts wide distribution of mycotoxins in detectable amounts (roridin E) in adjacent rooms to the water-damaged room (**Paper 5**). The dust, as the indoor matrix, can provide a good estimate of the bioactive fungal metabolites and other fungal components that become airborne in the indoor environment. Hence, the importance of collection of sufficient amounts of dust samples from damp indoor environments and their analysis for presence of different mycotoxins and other bioactive secondary metabolites could not be stressed enough. The presence of spirocyclic drimanes in dust samples confirms them as good candidates for *Stachybotrys* indoor biomarkers.

Fig. 22 The case of water-damaged kindergarten presented in Paper 5: to the left gypsum wallboard contaminated with Stachybotrys spp., to the right one of the adjacent rooms where sampling of dust took place

3. Conclusion and future perspectives

Based on the obtained results, especially results from dust, the used analytical methodology has proven to be a valuable tool in analysis of *Chaetomium* and *Stachybotrys* secondary metabolites produced in the conditions of the damp indoor environment. The main advantage of the presented methodology is its applicability to the compounds produced by other fungal species, as well as other microorganisms commonly found in the indoor environment. The obtained results identified good candidates for genus and species specific biomarkers for future exposure/health effects studies. Some of these compounds (cochliodone, L-611776) are reported for the first time in the indoor environment. Finally, used analytical technology enabled the detection and the estimation of amounts of several *Stachybotrys* metabolites in dust samples, which have never been reported in dust before. Clearly, the presented research provides a minor contribution to an attempt to describe the complexity of fungal compounds found in the damp indoor environment. Whether one or more of the identified metabolites found in the indoor environments during this PhD project have toxicological significance and can act as causal factors for some of the reported health effects is yet to be determined.

Despite the extensive research within this field, we still seem to be far from understanding what are the key factors associated to the damp building related illness and/or discomfort and how big is the role of indoor fungi and their metabolites in the negative health effects occurrence. Existing analytical methods (Polizzi et al., 2009; Vishwanath et al., 2009), targeting a variety of fungal and bacterial metabolites are with no doubt extremely helpful in explaining microbiota of damp indoor environment. However, during the course of my PhD study, one question seemed to be constantly emerging when it comes to damp indoor environment: are we measuring all relevant metabolites present and is there something that we are missing?

All existing, as well as the analytical methods presented here, serve the purpose of better characterisation and explanation of what can be found in wet building environment. This knowledge can further be used in exposure assessment studies as well as for determination of species and genus relevant indoor biomarkers. Certainly, the effects of bioactive secondary metabolites cannot and should not be studied individually, considering the complexity of indoor microbiota. However, the combined effects of different fungal components as well the combined effects of fungal and other microbial components (bacteria, mites) cannot be assessed before the effect of each individual component is known. Therefore, however helpful multi-targeted multi-organism analytical approaches are, currently there is a lack of knowledge on extensive metabolite profiles of frequent indoor fungal contaminants. Future research should therefore focus on thorough studies of metabolites produced by commonly found indoor species such as

Penicillium spp., *Aspergillus* spp., *Cladosporium* spp. etc. Utilising the same approach presented in this thesis, these studies will result in addition of the new compound data to the existing MS/HRMS library. This will further result in increased confidence and effectiveness of the method as well as less time necessary for data processing.

The important step in future research is the investigation of all identified metabolites in samples collected in field studies. In order to reach statistically relevant conclusions about indoor present metabolites, large sets of field samples need to be included in comprehensive studies as done by Peitzsch et al. (2012). Especially important for exposure studies is the presence of different metabolites in dust. Settled dust easily becomes airborne by mechanical disruption, which is relevant for the overall exposure assessment. Dust also seems to provide the accurate estimate of the indoor mycobiota in terms of presence of spores, micro-particles and metabolites. Therefore, the thorough investigation of presence of bioactive fungal metabolites in dust is crucial.

From the analytical chemist point of view, better quantification and validation procedures are absolutely necessary in the future, in order to increase quality, accuracy and validity of the acquired quantitative data. The availability of isotope-labelled internal standards are required in order to resolve issues with matrix effects as well as with the accuracy of the obtained data. The availability of reference material and standards is also crucial for further analytical method development.

Finally, interdisciplinary approach to the complexity of presence of bioactive metabolites in the damp building environment should result in finding species-specific biomarkers, which will certainly provide a necessary breakthrough within this field of research.

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5. Papers

5.1 Paper 1 - Pre-contamination of new gypsum wallboard with potentially harmful fungal species

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Pre-contamination of new gypsum wallboard with potentially harmful fungal species

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Practical implications

This study shows that wet gypsum wallboard, regardless of type or brand, is prone to fungal growth because fungi are already incorporated into the material during production. This study demonstrates the importance of securing buildings constructed with high amounts of gypsum wallboard against water-damage and high humidity. It also shows that industry has a major task ahead in ensuring that fungal spores are not built into their products and that a hazard analysis and critical control points approach, as used in the food industry, would seem to be a good strategy to ensure minimal fungal contamination of new building materials.

ABSTRACT

Gypsum wallboard is a popular building material, but is also very frequently overgrown by *Stachybotrys chartarum* after severe and/or undetected water damage. The purpose of this study was to determine if *Stachybotrys* and other fungi frequently isolated from wet gypsum wallboard are already present in the panels directly from the factory. Surface disinfected gypsum discs were wetted with sterile water, sealed and incubated for 70 days. The results showed that *Neosartorya hiratsukae* (≡ *Aspergillus hiratsukae*) was the most dominant fungus on the gypsum wallboard followed by *Chaetomium globosum* and *Stachybotrys chartarum*. Our results suggest that these three fungal species are already embedded in the materials, presumably in the paper/carton layer surrounding the gypsum core, before the panels reach the retailers/building site.

1. Introduction

Gypsum wallboard (drywall or plasterboard) is used extensively in both new builds and renovation projects throughout the world because it is cheap, easy to work with and fire resistant. It is, however, also prone to fungal growth if exposed to high humidity or water ingress. Growth of the filamentous fungus *Stachybotrys chartarum* is particularly associated with wet gypsum wallboard worldwide (Flannigan and Miller, 2011) and research has shown that in Danish water damaged buildings *S. chartarum* occurs on 25 % of the gypsum wallboard samples, compared to only 4 % in other samples examined (Andersen et al., 2011). Other filamentous fungal genera, such as *Penicillium*, *Chaetomium* and *Ulocladium*, have also been associated with wet gypsum wallboard (Gravesen et al., 1999; Flannigan and Miller, 2011), but these associations are less significant (Andersen et al., 2011).

Many filamentous indoor fungi can produce species specific bioactive metabolites during growth. *Chaetomium globosum* produces chaetoglobosins and *Penicillium chrysogenum* produces PR-toxin, roquefortines and penicillins, whereas *S. chartarum* is known for its production of macrocyclic trichothecenes and atranones (Nielsen et al., 1998; Samson et al., 2010). Some of these metabolites (roquefortine A, chaetoglobosin A and roridin E (a macrocyclic trichothecene) (Polizzi et al., 2009)) as well as fungal cell wall components (β-glucans (Rand and Miller, 2011)) have been detected in indoor air and fungal growth in buildings is therefore problematic, as some of these compounds may have a negative impact on the health and well-being of the occupants (Nikulin et al., 1997; Mussalo-Rauhamaa et al., 2001; Carey et al., 2012; Rosenblum Lichtenstein et al., 2015). Especially *S. chartarum* is of concern, because it is also able to produce hemolytical proteins (Nayak et al., 2013) and macrocyclic trichothecenes that have previously been associated with of a number of animal and human health problems, e.g. leukopenia in horses, sheep and rabbits (Drobotko, 1944; Forgacs et al., 1958; Harrach, 1983; Jarvis et al., 1986) and pulmonary hemorrhage and hemosiderosis in infants in Cleveland (Jarvis et al., 1998; Etzel, 2007).

S. chartarum can be found on mouldy cellulose rich materials, like hay, plant debris, enriched soil and paper (Ellis, 1971; Domsch et al., 2007), but is rarely detected in air (Baxter et al., 2005; Viegas et al., 2014), because the spores are borne in sticky slime heads (Samson et al., 2010). Detection of viable spores in air samples is usually only possible when large areas (>m^2) covered with *Stachybotrys* growth are disturbed and sampled close-up (Dill et al., 1997; Tiffany and Bader, 2000). *C. globosum* can be found in similar cellulose rich habitats as *S. chartarum* (Domsch et al. 2007) and is equally difficult to get airborne since its spores are borne in ascomata formed in cracks and cavities (von Arx et al., 1986). In nature, both species are thought to have beetles, ants and mites as dispersal vectors (von Arx et al., 1986; McGinnis, 2007).

Common belief has it that fungal growth in damp/wet buildings happens because fungal spores from outside drift indoors and randomly start growing on any available material. This may be true for genera, such as *Aspergillus, Cladosporium* and *Penicillium*, which are readily airborne and much more common in the environment (Samson et al., 2010). In the case of *Chaetomium* and *Stachybotrys* on gypsum wallboard, however, the lack of viable airborne spores in the outer environment and the overrepresentation of *Stachybotrys* on wet gypsum wallboard point to another source than outside air/environment as the origin of contamination. Price and Ahearn (1999) found *Chaetomium* and *Stachybotrys* in their untreated gypsum samples and noticed in a passing remark: "*These species were presumably part of the inherent bioburden on the gypsum wallboard following manufacturing and storage.*" The purpose of this study is therefore to determine if *Stachybotrys* and other indoor fungi are already present in unused and undamaged gypsum wallboard.

2. Materials and Methods

2.1. Building materials, treatment and incubation

Thirteen different panels of gypsum wallboard (900/1200 × 2400 × 13 mm) were bought in four different *do it yourself* (DIY) shops around Copenhagen over a period of six months. Two different brands and three different types of gypsum wallboard were used in the studies (see Table 1). Each panel was divided into four sections (900/1200 × 600 mm) and three circular discs (70 mm in diam.) were cut from each section (twelve discs in total per panel). Each set of 12 discs was surface disinfected for 30-45 sec. by submerging one disc at a time in 1000 ml of 96 % ethanol and gently rubbing both surfaces. Three additional sets of twelve discs from panel # 9 were also surface disinfected in Rodalon (according to the directions for use), household bleach (according to the directions for use) or sterile water following the same procedure as for ethanol.

After surface disinfection, the discs were placed in a rack and allowed to air dry to constant weight, usually overnight, in a sterile laminar air flow (LAF) bench (23-25 °C and 30-35 % RH). Each disc was placed aseptically into an empty, sterile Petri dish (plastic, 90 mm in diam.) and weighed. Sterile water was added to the Petri dish to reach approximate 23 % water content (w/w of the constant weight) and weighed again. Each Petri dish was then sealed with parafilm and incubated at room temperature (22-23 °C) for 70 days. Discs of the first 9 planes were evaluated qualitatively for fungal growth every other week (presence or absence of each genus on a disc), through the lid without disturbing the disc, using a stereo microscope. Fungal colonies were identified to genus level based on their morphology. Discs of the last four planes were also evaluated on day 3 and 7. After 70 days the Petri dishes were weighed again, opened and representative colonies isolated for species identification using morphology and metabolite profiling according to Andersen and Nissen (2000), Andersen et al. (2002; 2003), Samson et al. (2007), Samson et al. (2010) and Wang et al. (2016).

3. Results

Analysis of the thirteen different planes of gypsum wallboard showed that there was fungal growth of one or more fungal species, on either the face, the reverse or on both, on all tested planes (Table 2). Plane 9 (moisture resistant, brand B) had the highest total fungal count (64 fungal counts on 12 discs), whilst plane 3 (fire resistant, brand B) had the lowest (21 fungal counts on 12 discs). The analyses also showed that there were no major differences between brands or between same types of plane purchased from different DIY outlets. During the 70 days each experiment lasted fungal growth and dissemination were followed and recorded using a stereo microscope. Only six genera, *Neosartorya*, *Aspergillus*, *Chaetomium*, *Penicillium*, *Cladosporium* and *Stachybotrys*, reoccurred on three or more of the thirteen panels (Table 2). *Alternaria*, *Botrytis*, *Phoma* and *Ulocladium* were found on only one panel and one or two discs, while *Harzia, Paecilomyces* and *Pochonia*, were found only once.

3.1. Fungal identification

At day 70 the seals of the Petri dishes were broken and representative colonies were isolated and identified to species. The most abundant fungal species found on gypsum wallboard was *Neosartorya hiratsukae.* This fungus was found on all thirteen panels (100 %) and on most discs; both face and reverse. It appeared first on the reverse of the discs, but within days it was also visible on the face. The fungus had covered the whole disc with small (100-230 µm in diam.) white ascomata (cleistothecia) (Fig. 1A/D) in 3-7 days after first appearance. Only on panel 5 was *N. hiratsukae* more abundant on the face than on the reverse (Table 2). The most abundant *Aspergillus* turned out to be *A. hiratsukae*, which is the asexual state of *N. hiratsukae* (i.e. the same organism as *N. hiratsukae*), and was found around the edge of the discs and constituted most of the recorded "*Aspergillus"* in Table 2. *C. globosum* was the second most abundant species, found on eleven panels

(85 %) and was equally common on both face and reverse. It took ca. 14 days after onset for the fungus to cover the whole surface of the disc with large (300-500 µm in diam.) dark ascomata (perithecia) with green curly hair (Fig. 1B/E). Both *P. chrysogenum* and *Cladosporium cladosporioides* showed the same pattern by occurring mostly on the face of nine (69 %) and eight (62 %) panels, respectively, and only as discrete, slow growing colonies that stopped expanding after 14-21 days (Fig. 1B). *S. chartarum* (both chemo types) was found on seven (54 %) of the thirteen panels and would cover the whole surface of the disc within 7 days after onset with black clusters of slime heads (Fig. 1C/F). *Alternaria infectoria*, *Aspergillus versicolor* and *Cladosporium sphaerospermum* were found on only one panel and on one or two discs, while *Aspergillus fumigatus*, *Chaetomium elatum, Penicillium polonicum* were found only once.

3.2. Surface disinfection

After the emergence of *N. hiratsukae* ascomata on the first 5 panels, it was speculated whether the ethanol was triggering the growth of this fungus or if it could be present in the ethanol. Therefore two other surface disinfectants, household bleach and Rodalon, and autoclaved water were also used on subsamples of panel 9. Analysis of the three subsamples showed similar results compared to ethanol disinfection (Table 3). With water and bleach the ascomata of *N. hiratsukae* appeared just as rapidly (14 days) as they did with ethanol, whereas the Rodalon treatment delayed the ascomata production by ca. 7 days. The subsample washed in autoclaved water did not show any additional fungal growth and neither bleach nor Rodalon hampered the growth of *S. chartarum*. Table 3 also shows that the water content of the discs decreased slightly from 22.5 % to 20.8 % on average during the 70 day long incubation period despite several layers of parafilm around the edge of the Petri dishes.

3.3. Onset of fungal growth

The last four panels (panels 10-13) were also examined on day 3 and day 7 to see how quickly the fungi could germinate, grow and sporulate. Figure 2 shows the onset of sporulation for each genus and the end time where no new fungal colonies appear. The first undifferentiated hyphal growth was evident on day 3 (graph not shown) and conidial heads of *A. hiratsukae* were visible after only 7 days of incubation, whereas *N. hiratsukae* ascomata started to appear after 14 days. Also *C. cladosporioides* and *P. chrysogenum* were visible after 14 days with conidiophores and the first chains of conidia. The first slime heads of *S. chartarum* were visible after 21 days, while *C. globosum* ascomata were visible after 28 days (panels 6-9, result not shown). No new colonies appeared after 42-45 days.

4. Discussion

The results of this study show that gypsum wallboard is already contaminated with fungal spores before the panels reach their end-users, since the same fungal species, *Neosartorya hiratsukae* (≡ *Aspergillus hiratsukae*)*, Chaetomium globosum* and *Stachybotrys chartarum*, were found repeatedly in the paper/cardboard of all surface disinfected samples irrespectively of type, brand or outlet.

N. hiratsukae, which is an uncommon fungus in the environment, has to our knowledge never been reported on gypsum wallboard before. It has previously been isolated from soil, fruit juice and indoor air (Samson et al., 2007). *N. hiratsukae* is also reported to be pathogenic to humans (Guarro et al., 2002) and to produce avenaciolide (Samson et al., 2007). One reason for occupants and surveyors not to realize growth of this fungus could be that the small white ascomata are evenly distributed across the white paper surface of the gypsum wallboard and therefore not readily visible to the naked eye (Fig. 1A) and difficult to see without a slanted light source on the stereo microscope. The pale green anamorphic state of the fungus, *A. hiratsukae*, is produced only

sparingly on gypsum wallboard. During the 70 day incubation period it was noted how the ascomata slowly disintegrated and released vast numbers of ascospores. This suggests that *N. hiratsukae* spores (both live and dead) and micro-particles from the ascomata can easily become airborne and constitute a health risk in buildings with water damaged gypsum wallboard without the knowledge of the occupants.

A limited number of research studies have been published on the occurrence of *C. globosum* in water damaged buildings and even fewer have reported *C. globosum* on paper and gypsum wallboard (Price and Ahearn, 1999; Jerusik, 2010; Flannigan and Miller, 2011). One reason for not detecting *C. globosum*, even though it is very conspicuous on gypsum wallboard when mature (Fig. 1B), may be its long lag phase on both gypsum and laboratory media. Furthermore, samples from water damaged buildings often contain other fungi (e.g. *Penicillium* spp.) that grow much faster on laboratory media and thereby obscure any growth of *C. globosum*.

The association between *S. chartarum* and gypsum wallboard, on the other hand, is well documented and it was therefore expected that *S. chartarum* would be the dominant fungus. Still, more than 50 % of our panels were contaminated with *S. chartarum*, which is more than some studies report (Gravesen et al., 1999; Andersen et al., 2011), but less than the 60-77 % Flannigan and Miller (2011) found in their study. **If** our results are representative for a panel (1 out of 12 discs is contaminated with one viable *S. chartarum* spore) it would suggest that a whole panel would be contaminated with 40-60 viable spores depending on the size of the panel (ca. 20 spores/m^2). This corresponds well to our field observations in buildings with severe water-damage. There we have seen simultaneous outgrowth of numerous discrete *Stachybotrys* colonies on the same gypsum panel indicating that *Stachybotrys* spores are distributed throughout the material. On our most contaminated sample, panel 5, where 10 discs were contaminated, the same estimation would give approximately 200 viable *S. chartarum* spores per m^2. This combined with the rapid growth rate on

gypsum wallboard (covering a disc in less than a week) might explain why *S. chartarum* can dominate a water damaged building so quickly.

Most gypsum wallboard manufacturers advertise that they use recycled materials in their production. Recycled paper/cardboard is often collected in big bails at the recycling centre and stored under less than optimal conditions (Jerusik, 2010) where bails are exposed to rain, soil and insects, which results in high microbial loads (Betz and Cerny, 1999). *N. hiratsukae*, as well as *C. globosum* and *S. chartarum,* can survive at temperatures around 40 °C (Samson et al., 2007; Domsch et al., 2007) and even if a small percentage of the fungal spores survive pulping and drying, there could be invisible fungal growth on the gypsum wallboard after as little as 7 days after major water-damage. However, since it is not known where in the process the different fungi enter or what their contamination and survival rates are, further research into the specific production methods of the raw materials is needed. One approach to safer gypsum wallboard is for the manufacturers to use hazard analysis and critical control points (HACCP), which is a system used by the food industry for decades to ensure food safety, but other types of industry are increasingly using HACCP as a competitive parameter.

Ongoing chemical analyses of the discs at our department will show which fungal metabolites (e.g. avenaciolide, chaetoglobosins, atranones and satratoxins) these inbuilt fungal contaminants are able to produce during their growth on the gypsum wallboards and if it is consistent with previous findings on *Stachybotrys* spp. (Nielsen et al., 1998). Also a rapid detection method using DNA sequencing directly on the paper/cardboard surface is being developed at our department.

5. Conclusions

The results of this work show that gypsum wallboard is contaminated with *Neosartorya, Chaetomium* and *Stachybotrys* and suggest that the spores of these fungi are embedded in the paper/cardboard surrounding the gypsum core. Even though the manufacturers do not market their gypsum wallboard as "sterile" or "fungal free", most consumers trust that there are no potentially harmful fungi in their building materials. Since growth of these fungi can result in large quantities of micro-particles and bioactive compounds, which may be released into the indoor air after water damage, there is a need for manufacturers to undertake a stricter quality control of their raw materials and finished products. However, even the best efforts of the manufacturers would be rendered futile if proper/correct shipping, handling and storage are not equally strictly controlled. During storage, construction and occupancy, gypsum wallboard must be kept dry, clean and undamaged in order to provide safe and healthy buildings.

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Figure legends

Fig. 1. Gypsum discs (face up) with fungal growth after 70 days. A/D: *Neosartorya hiratsukae* (panel 3); B/E: *Chaetomium globosum* (panel 1) with *Penicillium chrysogenum* encircled; C/F: *Stachybotrys chartarum* (panel 3).

Fig. 2. Time (days) until first appearance of fungal growth/sporulation on discs (both face and reverse) from panels 10-13 (n=96).

Table 1. Type and origin (anonymised) of the gypsum wallboard panels used in this study.

Panel #	Type of wallboard	Brand	Outlet	Purchase date
1	Fire resistant	A	DTU*	14-01-2015
2	Fire resistant	A	DIY-1	20-01-2015
3	Fire resistant	B	DIY-2	17-03-2015
4	Moisture resistant	B	DIY-2	17-03-2015
5	Fire resistant-spacer	A	DIY-1	23-03-2015
6	Fire resistant	A	DIY-3	30-04-2015
7	Moisture resistant	A	DIY-3	30-04-2015
8	Fire resistant	B	DIY-4	30-04-2015
9	Moisture resistant	B	DIY-4	30-04-2015
10	Fire resistant	A	DIY-1	13-07-2015
11	Regular	A	DIY-1	13-07-2015
12	Fire resistant-spacer	A	DIY-1	13-07-2015
13	Fire resistant	B	DIY-2	13-07-2015

* Clean, unused surplus panel from a building site at the Technical University of Denmark.

Table 2. Number of discs with fungal growth on face (F) and reverse (R) from 13 different gypsum wallboard panels. Twelve discs from each panel were surface disinfected with ethanol, wetted with sterile water and incubated for 70 days.

	% Water	*Neosartorya*		*Aspergillus*		*Chaetomium*		*Cladosporium*		*Penicillium*		*Stachybotrys*	
Panel #	**day 1**	**F**	**R**	**F**	**R**	**F**	**R**	**F**	**R**	**F**	**R**	**F**	**R**
1	21.6	4	10	-	1	7	8	3	-	5	1	7	7
2	21.3	8	12	-	-	5	3	2	-	3	1	1	1
3	27.0	2	9	-	-	-	-	-	-	1	1	4	4
4	21.8	8	8	7	5	4	4	3	1	6	4	1	2
5	24.2	11	1	3	1	2	3	2	-	2	-	10	11
6	22.8	12	12	2	-	2	1	1	-	-	-	-	-
7	23.2	12	12	-	1	2	1	-	-	-	1	-	-
8	23.6	3	7	1	-	1	1	9	-	1	-	-	-
9	23.0	12	12	12	12	3	4	-	-	8	-	1	-
10	23.2	12	11	12	-	2	1	-	-	-	-	1	1
11	22.5	12	12	4	-	-	-	4	-	-	-	-	-
12	22.5	12	12	4	2	1	-	-	-	-	-	-	-
13	23.0	6	12	1	2	1	2	8	-	3	1	-	-

Table 3. Number of discs with fungal growth on face (F) and reverse (R) from panel 9 after treatment with 4 different surface disinfectants. Twelve discs for each treatment were surface disinfected, wetted with sterile water and incubated for 70 days.

Surface disinfectant	% Water (w/w)		*Neosartorya*		*Aspergillus*		*Chaetomium*		*Cladosporium*		*Penicillium*		*Stachybotrys*	
	day 1	day 70	F	R	F	R	F	R	F	R	F	R	F	R
Ethanol	23.0	21.6	12	12	12	12	3	4	-	-	8	-	1	-
Water	21.6	19.9	12	12	12	12	3	5	2	-	10	9	-	-
Bleach	22.3	21.0	12	12	12	12	3	2	-	-	6	-	2	2
Rodalon	23.1	20.7	12	12	12	-	2	-	-	-	7	-	-	1

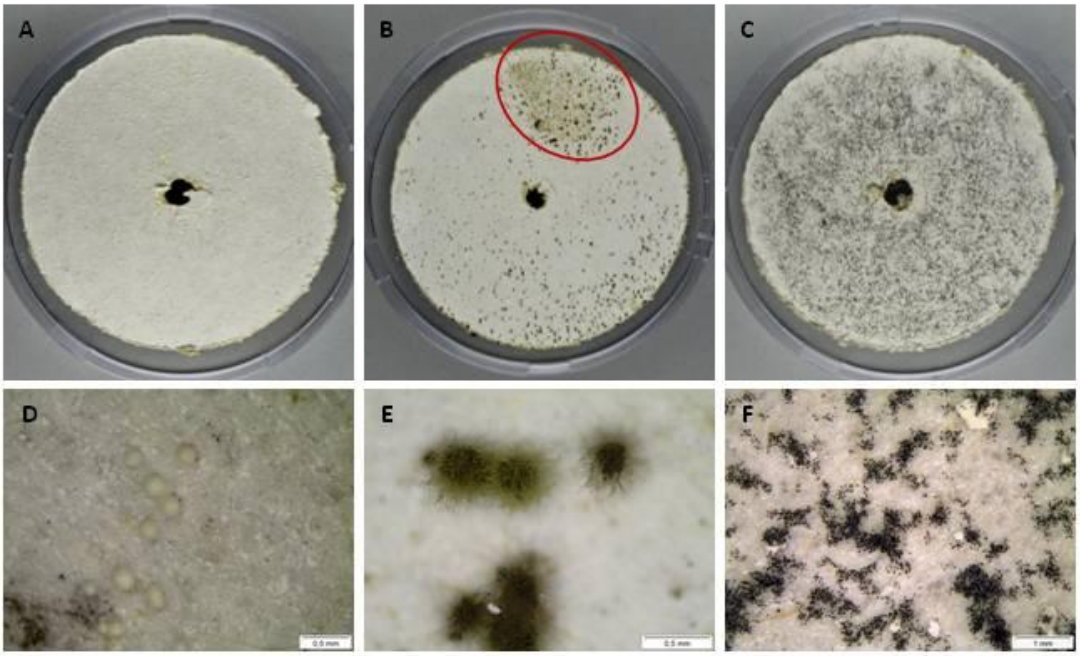

Fig. 1. Gypsum discs (face up) with fungal growth after 70 days. A/D: *Neosartorya hiratsukae* (panel 3); B/E: *Chaetomium globosum* (panel 1) with *Penicillium chrysogenum* encircled; C/F: *Stachybotrys chartarum* (panel 3).

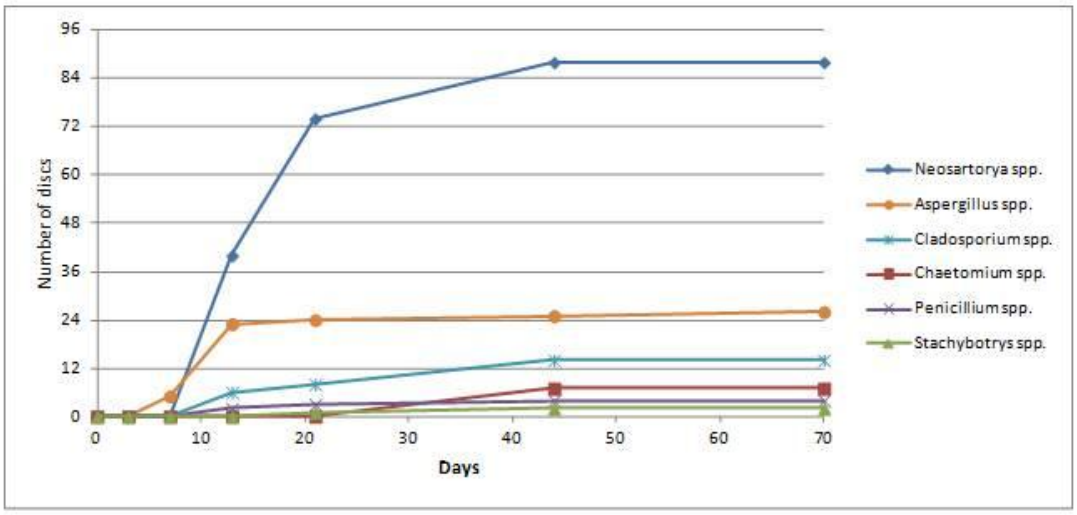

Fig. 2 . Time (days) until first appearance of fungal growth/sporulation on discs (both face and reverse) from panels 10-13 (n=96).

5.2 Paper 2 - Accurate dereplication of bioactive secondary metabolites from marine-derived fungi by UHPLC-DAD-QTOFMS and a MS/HRMS Library

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Article

Accurate Dereplication of Bioactive Secondary Metabolites from Marine-Derived Fungi by UHPLC-DAD-QTOFMS and a MS/HRMS Library

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Abstract: In drug discovery, reliable and fast dereplication of known compounds is essential for identification of novel bioactive compounds. Here, we show an integrated approach using ultra-high performance liquid chromatography-diode array detection-quadrupole time of flight mass spectrometry (UHPLC-DAD-QTOFMS) providing both accurate mass full-scan mass spectrometry (MS) and tandem high resolution MS (MS/HRMS) data. The methodology was demonstrated on compounds from bioactive marine-derived strains of *Aspergillus*, *Penicillium*, and *Emericellopsis*, including small polyketides, non-ribosomal peptides, terpenes, and meroterpenoids. The MS/HRMS data were then searched against an in-house MS/HRMS library of ~1300 compounds for unambiguous identification. The full scan MS data was used for dereplication of compounds not in the MS/HRMS library, combined with ultraviolet/visual (UV/Vis) and MS/HRMS data for faster exclusion of database search results. This led to the identification of four novel isomers of the known anticancer compound, asperphenamate. Except for very low intensity peaks, no false negatives were found using the MS/HRMS approach, which proved to be robust against poor data quality caused by system overload or loss of lock-mass. Only for small polyketides, like patulin, were both retention time and UV/Vis spectra necessary for unambiguous identification. For the ophiobolin family with many structurally similar analogues partly co-eluting, the peaks could be assigned correctly by combining MS/HRMS data and *m*/*z* of the $[M + Na]^+$ ions.

Keywords: marine-derived; MS/MS; dereplication; library; peptaibiotics; metabolomics

1. Introduction

Due to the cosmopolitan occurrence of many bioactive compounds, most natural product extracts contain compounds that have previously been characterized, despite intelligent selection of new organisms. This is of particular importance in primary screens where the target is usually non-selective, which inevitably leads to a high rediscovery rate of generally toxic compounds [1,2].

Microorganisms from the marine environment are a promising source of new bioactive compounds based on new chemical scaffolds [3–5], with the majority of known compounds originating from bacterial species such as *Salinospora* [6], *Pseudoalteromonas* [7,8], and *Vibrio* [9]. However, the subject of marine fungi is of much debate as most marine isolates have been found in mangrove and intertidal zones [4,10,11], rather than in true marine habitats; thus, no strict definition of "true marine fungi" currently exists [12]. Nonetheless, marine-derived fungal strains have yielded a plethora of biologically active compounds [5,13], with isolates of *Penicillium* and *Aspergillus* as the most common sources. These have mainly been isolated from substrates such as driftwood [14] and macroalgae [15], but also in deep-sediments [3,16,17]. *Aspergillus sydowii* is probably the most well-known example, identified as the cause of sea fan disease [18], but also the source of bioactive compounds [19]. It remains obscure whether these represent true marine isolates or just opportunistic strains that have adapted to the marine conditions [12]. From a drug discovery perspective, this might be of less importance, if the opportunistic strains produce different bioactive compounds than their terrestrial counterparts.

Several approaches to the dereplication process exist; for fast screening of extracts the aggressive dereplication approach can be very efficient [20]. This approach is based on accurate mass, isotopic patterns, and preferably selective adducts used for large batch searches of possible metabolites (up to 3000 compounds), e.g., based on all compounds described by a single genus. Yet, it returns false positives that need to be sorted away. The approach is currently not suited for organisms with limited taxonomic information. False positives can be circumvented by adding tandem MS with accurate mass determination of fragment ions (MS/HRMS) which can be automatically co-acquired using auto-MS/HRMS experiments (data-dependent acquisition of MS/HRMS spectra) [21]. This can now be achieved on both time-of-fight (TOFMS) and fourier transform (FTMS) mass spectrometers as well as Orbitrap and Q-Exactive instruments [22–25]. To achieve high quality MS/MS spectra, Agilent Technologies have chosen to acquire spectra at three different fragmentation energies, 10, 20 and 40 eV, as this often provides significant higher quality than e.g., a ramped spectrum from 10 to 40 eV [26]. The acquired MS/HRMS data can then be matches with the possible candidates using *in silico* fragmentation tools that can sort out poor matches [27,28].

For fast tentative identification of natural products, an automatic MS/HRMS spectral library search would be very efficient, if suitable natural products libraries existed. However, Massbank [29] and Metlin metabolomics library [30] (~10,000 compounds with spectra) only contain few microbial natural products. The current status will persist until it is required to publish MS/MS data with novel structures, for which there are now public depositories such as MetLin, Massbank and/or Global

Natural Products Social Molecular Networking (GnPS) [31] in the making at time of writing). Nevertheless, a major barrier is that MS/MS spectra of small molecules are inconsistent between instruments, in particular between ion-trap and collision cell-based instruments [32]. Also, compared to fragmentation of linear peptides [33] and lipids [34], fragmentation of natural products are much less predictable, since they often contain more condensed and highly complex ring systems: In consequence *in silico* predictors cannot predict a fragmentation spectrum, but to some extent, verify some fragments from a structure in a spectrum [27,28].

For smaller natural products libraries, different algorithms have been used to search MS/MS spectra for the tentative identification (absolute identification always requires a nuclear magnetic resonance (NMR) validated reference standard). Fredenhagen *et al.* [35] searched low resolution MS/MS data with the National Institute of Standards and Technology (NIST) algorithm developed for full scan EI^+ spectra and the Mass Frontier software for MS^n spectra and found the latter to be superior. El-Elimat *et al.* [2] used ACD-IntelliXtract that also includes accurate mass of the fragments, but does not use the parent ion data as search entry. A comprehensive review on algorithms can be found in Hufsky *et al.* [28]. Recently, a networking MS/MS strategy has been published from the Dorrestein/ Bandeira labs [36,37], where MS/MS spectra are compared pairwise to yield clusters of structurally related compounds. However, back integration/deconvolution of raw data to find corresponding full scan data and linking MS/MS spectra of adducts belonging to the same molecular feature as well as retention time still needs to be done manually and is thus very time consuming.

In this current study, we demonstrate the use of our MS/HRMS library search to dereplicate known compounds in bioactive extracts from marine-derived *Aspergillus*, *Penicillium*, and *Emericellopsis* strains. Extracts were selected from a screening conducted as a part of the PharmaSea project [38].

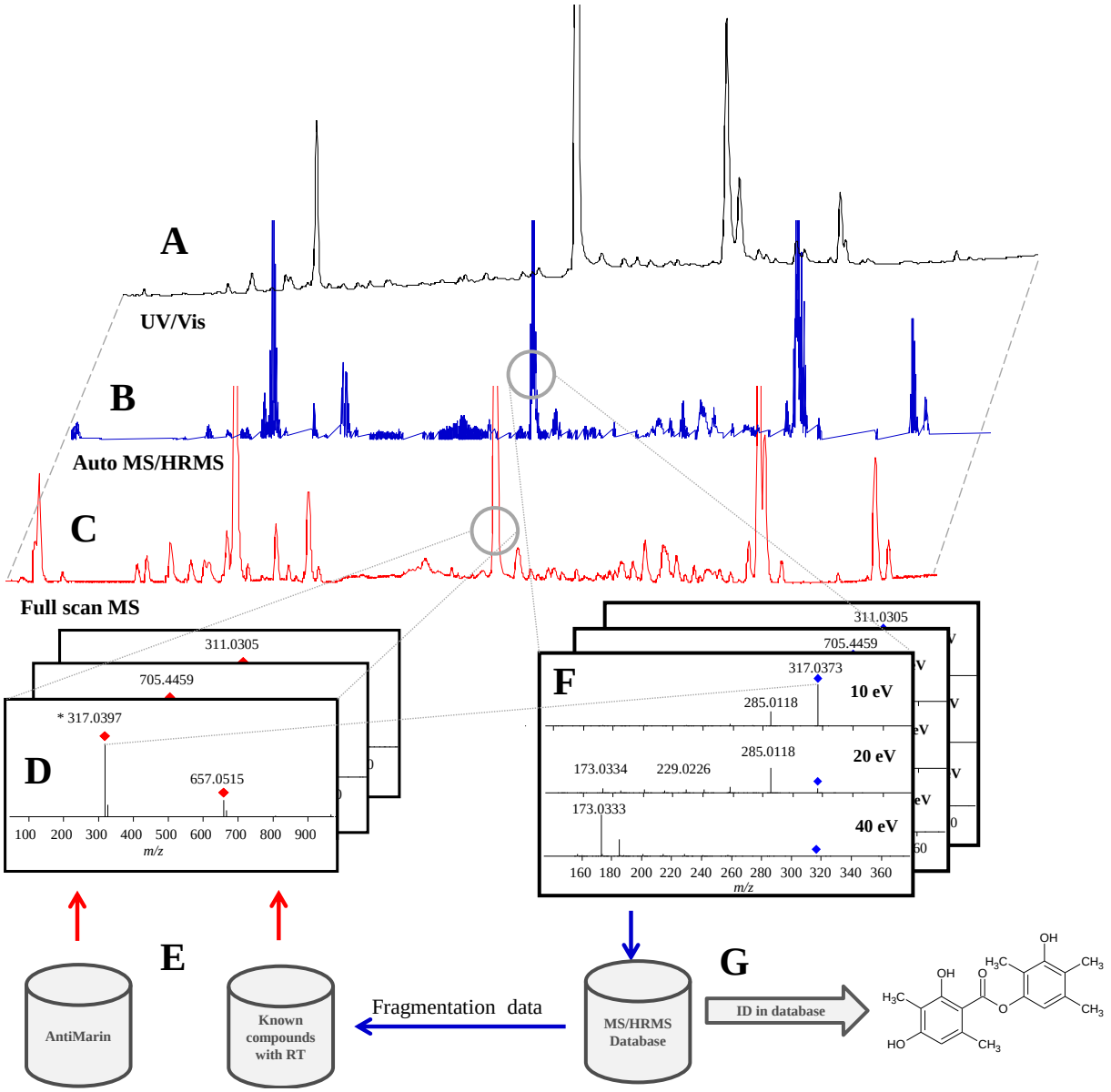
Ultra-high performance liquid chromatography-diode array detection-quadrupole time of flight mass spectrometry (UHPLC-DAD-HRMS) with auto- tandem high resolution mass spectrometry (MS/HRMS) analysis was used to screen the extracts and subsequently, MS/HRMS data was matched against a newly constructed library of 1300 compounds (10, 20, and 40 eV spectra) using the Agilent search algorithm. This algorithm is an integral part of the Agilent MassHunter software, which can subtract background and merge spectra over a chromatographic peak into a single spectrum prior to automatic search against the library. To assess the limitations and inherent bias of the library, we compare the results with the aggressive dereplication approach [20] based on accurate mass, isotope pattern, and lists of taxonomically relevant compounds. Specificity is tested on a number of small polar analytes, showing the importance of including retention time and appropriate search parameters for compounds with less characteristic spectra. Finally, comparison with UV/Vis detection was done for a number of poorly ionizing compounds showing the value of this additional cheap detector.

2. Results and Discussion

Figure 1 illustrates the overall screening concept used in this study, where UHPLC-DAD-QTOF data are analyzed in three different ways: (i) MS/HRMS data searched directly in MS/HRMS library; (ii) aggressive dereplication of the full scan HRMS data using search lists of known compounds; (iii) UV/Vis detection for poorly ionizing compounds. Finally, an unbiased peak-picking algorithm was used to highlight completely novel compounds. For dereplication of previously described

compounds and novel isomers, all four approaches were combined as illustrated in the examples of *Penicillium bialowiezense* (Section 2.2.1) and *Aspergillus insuetus* (Section 2.2.2). Specificity problems with MS/HRMS searching are illustrated for patulin and compounds with the same elemental composition (Section 2.1.5).

Figure 1. Overview of the screening setup where ultra-high performance liquid chromatography (UHPLC) with three detection methods is used. (**A**) ultraviolet/visual (UV/Vis) for poorly ionizing compounds; (**C**,**D**) full scan high resolution mass spectrometry (HRMS) screening; (**B**,**F**) MS/HRMS identification using the MS/HRMS library (**G**). Elemental compositions from compounds known from literature and previous studies were searched for in the full scan data (**E**,**D**).



2.1. Data Acquisition and Library Creation

2.1.1. Chromatographic Separation

The gradient was developed to provide the highest peak capacity in extracts from *Aspergillus niger* and *A. nidulans* with emphasis on not losing polar alkaloids (e.g., pyranonigrins and nigragillins) and small organic acids. This led to the use of the more polar phenyl-hexyl Poroshell column (compared to C_{18}) as well as a low start of the gradient (10% acetonitrile). This retains highly polar compounds such as patulin and type B trichothecenes slightly better than C_{18}. However, the long column required a longer gradient and equilibration time leading to half the productivity, but better opportunities for more MS/MS experiments. The high temperature of 60 °C was needed in order to keep the back pressure below the limit of the 2.7 μm Poroshell column. The method yielded an excellent peak distribution and narrow peak width compared to other methods [2], which allowed for higher quality spectra of most compounds in an extract. Injection volume had to be kept low (1 μL) to avoid peak broadening of polar peaks as samples were dissolved in methanol. However, in some projects less had to be injected (as little as 0.1 μL) as strongly ionizing compounds in high concentration resulted in broad peaks due to peak broadening in the ion-source which was further enhanced by the limited linearity of the time of flight (TOF) detector.

2.1.2. Mass Accuracy and Isotopic Ratio

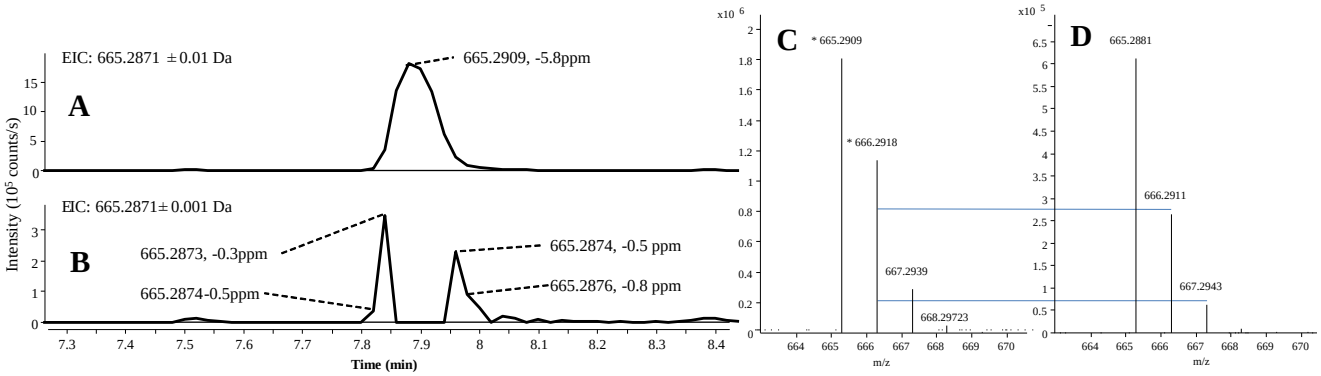
Currently, time of flight mass spectrometry (TOFMS) and fourier transform mass spectrometry (FTMS) instruments provide similar mass accuracy when using a lock mass, but the TOFMS instruments still have problems with detector overload [39,40] as illustrated in Figure 2, where the mass accuracy and isotope ratio is compared between overloaded and non-overloaded parts of a chromatographic peak. As high intensity peaks are unavoidable, MassHunter was set to handle this by using only non-overloaded MS scans from the front and end of the chromatographic peaks during the peak picking and integration, similar to other TOFMS manufacturers like Waters. Currently, this cannot be handled by any third-party software like ACD-IntelliXtract or open source software like XCMS and MZmine.

On the up-side, quadruple time of flight mass spectrometry (QTOFMS) instruments have a much higher scan frequency of both full scan and MS/HRMS scans without losing resolution as is the case on the FTMS instruments (resolution proportional to scan time). When not using overloaded ion clusters (Figure 2) our data provided isotopic ratios $< \pm 2\%$ relative to the theoretical distribution as also observed elsewhere [20] while for Orbitrap data it might be as much as ±35% [24]. Since an accurate isotope ratio is the most efficient way to differentiate candidate elemental compositions within the instrument accuracy [41], the QTOFMS instruments are superior to the FTMS instruments in this point.

In some samples, high intensity peaks suppressed the lock mass ions in certain scans, resulting in up to 100 ppm mass error in cases where the instrument had not been tuned and calibrated for several days. Since MassHunter cannot automatically find scans with intact lock mass in other places in the data file, one needs to be aware of this problem to manually correct it if needed. Here, the MS/HRMS

library still identified the correct compounds, underlining how this approach is very robust against mass errors from over-loaded peaks.

Figure 2. Ultra high performance liquid chromatography-electrospray ionization extracted ion chromatograms (UHPLC-ESI$^+$ EIC) of asperazine $[M + H]^+$ in an extract from *Aspergillus tubingensis*, showing the excellent mass accuracy until saturation in the peak apex. (**A**) EIC at ±0.01 Da; (**B**) EIC at ±0.001 Da; (**C**) spectrum at peak apex; and (**D**) spectrum at a non-saturated part of the peak. The vertical lines between **C** and **D** indicate the theoretical isotopic abundance of the A + 1 and A + 2 isotopomers.



2.1.3. Precursor Selection

A major challenge when using liquid chromatography-mass spectrometry (LC-MS)/MS libraries is the reproducibility of fragmentation patterns between different instruments and different instrument manufactures [42]. A major goal for the establishment of this library was to minimize variability due to changes in mobile phase composition and ion-source settings. This was done by including not only MS/HRMS from $[M + H]^+$, but also from the other predominant pseudo molecular ions such as $[M + Na]^+$ and $[M + NH_4]^+$ (for intensities >50% of $[M + H]^+$). Likewise, were MS/HRMS spectra of $[M + H - (H_2O)_n]^+$, $[M + H - HCOOH]^+$, and $[M + H - CH_3COOH]^+$ ions included when the full scan signal(s) were more intense than $[M + H]^+$, similar to Fredenshagen *et al.* [35]. When fragmentation of $[M + Na]^+$ only resulted in the loss of Na^+ to give the neutral molecule, the search algorithm gave false positives from any ion at the right *m*/*z*. MS/HRMS data from the stable $[M + Na]^+$ was therefore only included when resulting in specific fragments (~50% of the cases). Still, *m*/*z* of $[M + Na]^+$ is important for correct mass assignment of fragile molecules, where $[M + H]^+$ is not present due to spontaneous losses. Furthermore, in cases where in-source fragmentation of Compound A coincidentally results in production of Compound B also present in the library, the *m*/*z* of $[M + Na]^+$ can assist in correct assignment, as demonstrated.

In the negative ionization mode, $[M - H]^-$ is most often the dominant ion detected [43] while the formation of $[M - HCOO]^-$ (if formate is used as a buffer) seems to be very interface dependent [20], but very important for molecules not containing any acidic protons, and it was included when more than 50% of $[M - H]^-$ occurred. This resulted in the detection of highly active compounds like Type A and C trichothecenes, patulin, and aphidicolins not detected in other studies [2,35].

Part of the library (277 compounds) is available in PCDL format for download from the homepage of the Technical University of Denmark [44].

2.1.4. Fragmentation

In order to compensate for the high variation in energy needed to fragment natural products, the library was based on three distinct fragmentation energies (10, 20, and 40 eV) unlike existing microbial MS/MS libraries that are based on a single, fixed energy [2,35]. The high energy of 40 eV is needed to fragment larger, more stable molecules, while 10 and 20 eV are more gentle settings for smaller, more fragile molecules. This combination of energies also meant that the forensic science [26] and the Metlin libraries [35] which are also available for the MassHunter could be directly used. The latter, in particular, contains many lipids, prostaglandins, intracellular primary metabolites, small aromatics, amino acids, vitamins, *etc.*, which are also produced by fungi. The only cases where insufficient fragmentation was observed for all three energies were fusigen, SMTP-7 and 8, where only low intensity losses of formate and one other ion were observed in ESI^+. Thus, projects analyzing compounds with masses above 1000 Da should include additional fragmentation energies of e.g., 60–80 eV, as large single charged molecules are less disposed to fragment on the collisions with N_2. This is mainly due to simple energy kinetics ($E_{kin} = ½ \times m/z \times v^2$) where the ion-velocity in the collision cell is proportional to the square root of the mass.

Small (<200 Da) aromatic acids, pyrones, and lactones will statistically have less specific fragmentation reactions, which is observed in practice as loss of H_2O, HCOOH, and CO_2 [43]. Combined with an increase in the number of natural products with the same mass with decreasing mass (down to 220 Da) there is a double bias towards poor specificity of MS/MS of low mass compounds [43].

2.1.5. Library Scoring

Searching MS/HRMS spectra against the MS/HRMS library in MassHunter allows for three types of scorings: (i) using the parent mass and forward scoring that matches peaks in the unknown spectrum against the library spectrum; (ii) using parent mass and reverse scoring that matches peaks in the library spectrum against the unknown spectrum [28]; (iii) using reverse scoring but not the parent mass, called similarity, for finding compounds sharing fragment ions but having different molecular masses.

The pitfalls of scoring can be illustrated with patulin, a bioactive "nuisance" compound that is widely distributed in fungi that cause interference in many types of bioassays [45–47]. Patulin was identified in ESI^- in marine-derived strains of *Penicillium antarcticum* (Figure 3). Patulin shares the same elemental composition ($C_7H_6O_4$) with six other compounds included in the library (Table 1), which all to a certain extent exhibited similar fragmentation patterns under the same CID condition. Using reverse and forward scoring, all library spectra belonging to compounds with the same elemental composition are in the matching pool. For reverse scoring there is an increased risk of wrong compound identification compared to forward scoring as the search algorithm in this case only looks for peaks present in the library spectra, disregarding peaks present in the unknown spectrum that are not present in the library spectra.

As seen in Figure 3, patulin and 2,3-dihydroxybenzoic acid had a similar ratio of the *m*/*z* 109.0287 fragment ion corresponding to the loss of CO_2 (CID 10 eV). 2,3-dihydroxybenzoic acid does not show any additional peaks in the 10 eV spectrum while patulin produces several. Reverse scoring only matched the two shared peaks in the unknown spectrum, resulting in 2,3-dihydroxybenzoic acid as the

best match, while forward scoring, where all peaks in the spectrum are matched with the library spectrum, yielded patulin as the best match (Figure 3). The identification was verified by an authentic standard of patulin matching full scan MS, MS/HRMS, retention time and the UV spectrum where the slow slope from 200 to 240 nm prior to the main absorption at 276 nm. Deconvolution of all ions in the patulin full scan spectrum showed that it was not a false positive detection due to two or more co-eluting compounds.

Figure 3. UV/Vis spectrum (**A**) and MS/HRMS spectrum at 10 eV (ESI^-); (**D**) of unknown peak identified as patulin, compared to the patulin reference standard (**B**,**E**); and 2,3-dihydroxybenzoic acid (**C**,**F**). Identity was confirmed by correct retention time.

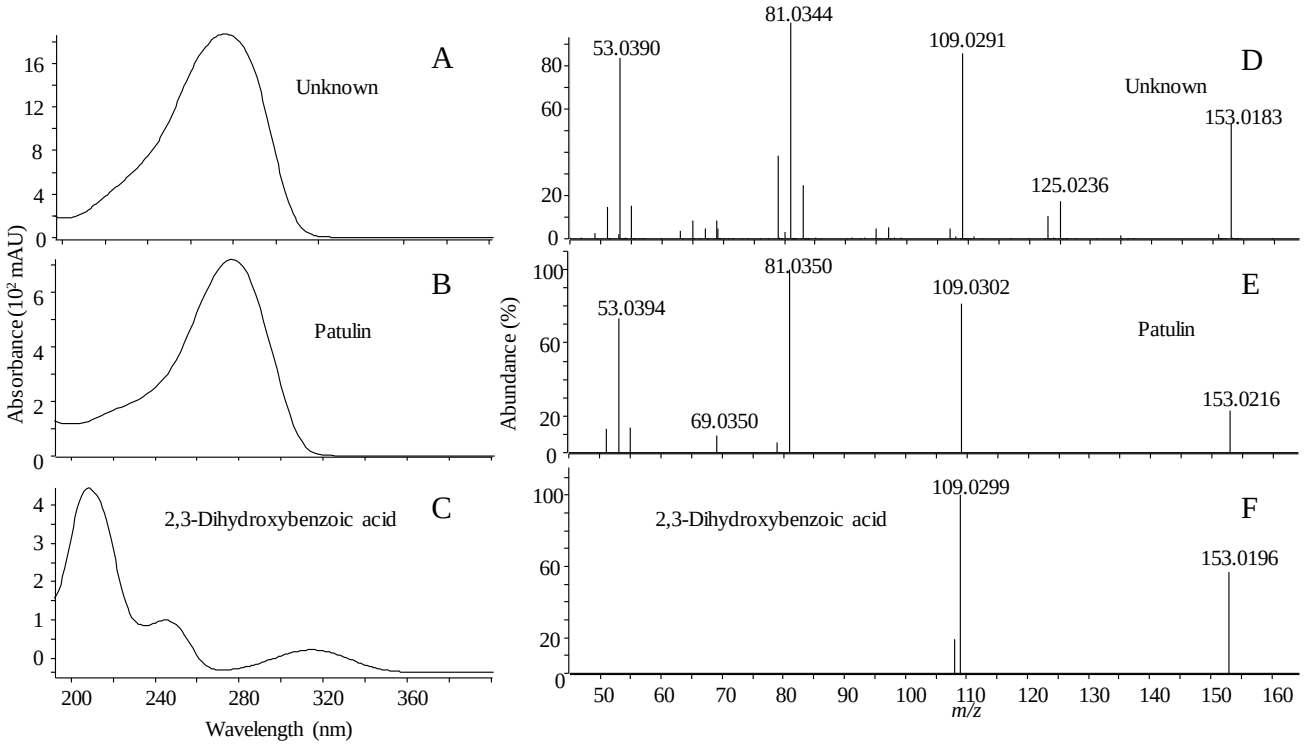


Table 1. Comparison of MS/HRMS spectra of all $C_7H_6O_4$ compounds in the MS/HRMS database against each other using forward and reverse scoring.

Name	RT (min)	Compound	Forward/Reverse Scoring (%)						
			1	**2**	**3**	**4**	**5**	**6**	**7**
Patulin	3.15	1	100	28/50	20/62	27/65	29/90	28/87	25/52
2,3-dihydroxybenzoic acid	3.85	2	28/32	100	60/68	63/71	97/90	76/86	0/0
2,4-dihydroxybenzoic acid	3.74	3	20/29	60/86	100	86/86	55/78	88/88	6/14
2,6-dihydroxybenzoic acid	3.87	4	21/29	63/86	86/98	100	58/79	80/92	6/14
3,4-dihydroxybenzoic acid	2.80	5	29/33	97/97	55/61	58/64	100	78/87	0/0
3,5-dihydroxybenzoic acid	2.63	6	29/33	97/97	55/61	58/64	78/87	100	0/0
Terreic acid	3.99	7	25/44	0/0	6/39	6/38	0/0	0/0	100

Inevitably, an unknown spectrum will contain more noise from co-eluting compounds compared to the library spectrum, another reason why reverse scoring is also valuable. This underlines the importance of using both forward and reverse scoring when evaluating matches from library searches

in order to get the correct identification, thus multiple search types are recommended (e.g., using a minimal score of 50% for forward and 70% for reverse). It is further demonstrated that there is a need for orthogonal data like UV/Vis for dereplication of certain compound classes.

2.2. Dereplication of Marine-Derived Fungi

Fifteen marine-derived strains from different species belonging to *Penicillium*, *Aspergillus*, and *Emericellopsis* were fractionated and screened for their anti-microbial [48], anti-inflammatory [49], central nervous system (CNS) [50], and anticancer activity (unpublished assay based on glioblastoma stem cells), that resulted in 35 active fractions to be evaluated for their chemistry. Here, we present three of those as cases to illustrate the advantages and challenges using a MS/HRMS library for screening and dereplicating active fractions during a screening campaign. Analyzing the data file for MS/HRMS data, including peak picking, integration, and the final matching against 1300 compounds took 30–60 s on a standard laptop, thus providing a fast and easy first examination of active fractions.

2.2.1. Active Components from a Marine-Derived *Penicillium bialowiezense* Strain

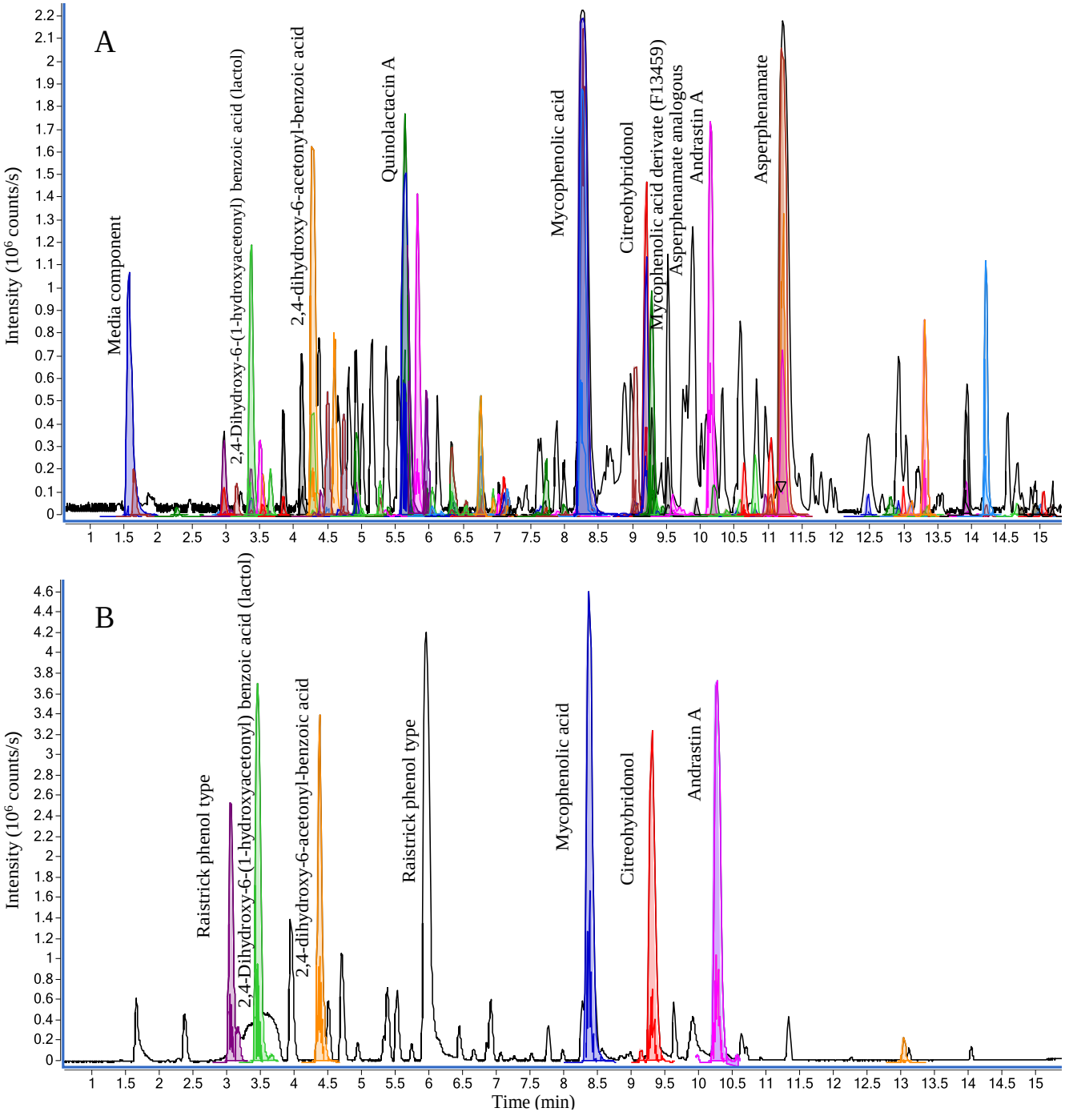
The extract of a *Penicillium bialowiezense* strain (IBT 28294) from a North Sea water sample displayed activity in a CNS assay [51] and anticancer assay (unpublished assay). *P. bialowiezense* is closely related to *P. brevicompactum*, and they are morphologically, genetically, and chemically very difficult to differentiate [52]. They are cosmopolitan species found across an amazing number of habitats such as seaweed, humid indoor environments, soil, and various vegetables and fruits [52,53]. Thus the marine-derived isolate used in this study is likely an opportunist in the marine environment, making the exclusion of known compounds even more important.

The crude extract analyzed in both positive and negative ionization mode can be seen in Figure 4 with the tentative identification of all major peaks: mycophenolic acid, mycophenolic acid derivate (F13459), asperphenamates, andrastin A, quinolactacin A, citreohybridonol, and raistrick phenols [54], all of which have previously been reported from terrestrial fungi [55].

The active fractions were found to contain mycophenolic acid (Figures 4 and 5), which is the active compound in the prodrug CellCept® (Mycophenalate mofetil) used as immunosuppressant in transplant medicine [56]. Several other activities have been reported including antiviral, antitumor [57], and CNS [56,58], in line with the activity observed in this study. The extracted MS/HRMS spectra (Figure 5A) in ESI^+ were compared to the mycophenolic acid standard in the MS/HRMS library (Figure 5B) with high scores (>90%) using both reverse and forward searching based on the accuracy of the parent ion (−0.31 ppm for $[M + H]^+$ 321.1328) and specific and abundant fragment ions at *m*/*z* 207.0649 $[C_{11}H_{11}O_4]^+$ and 159.0436 $[C_{10}H_7O_2]^+$.

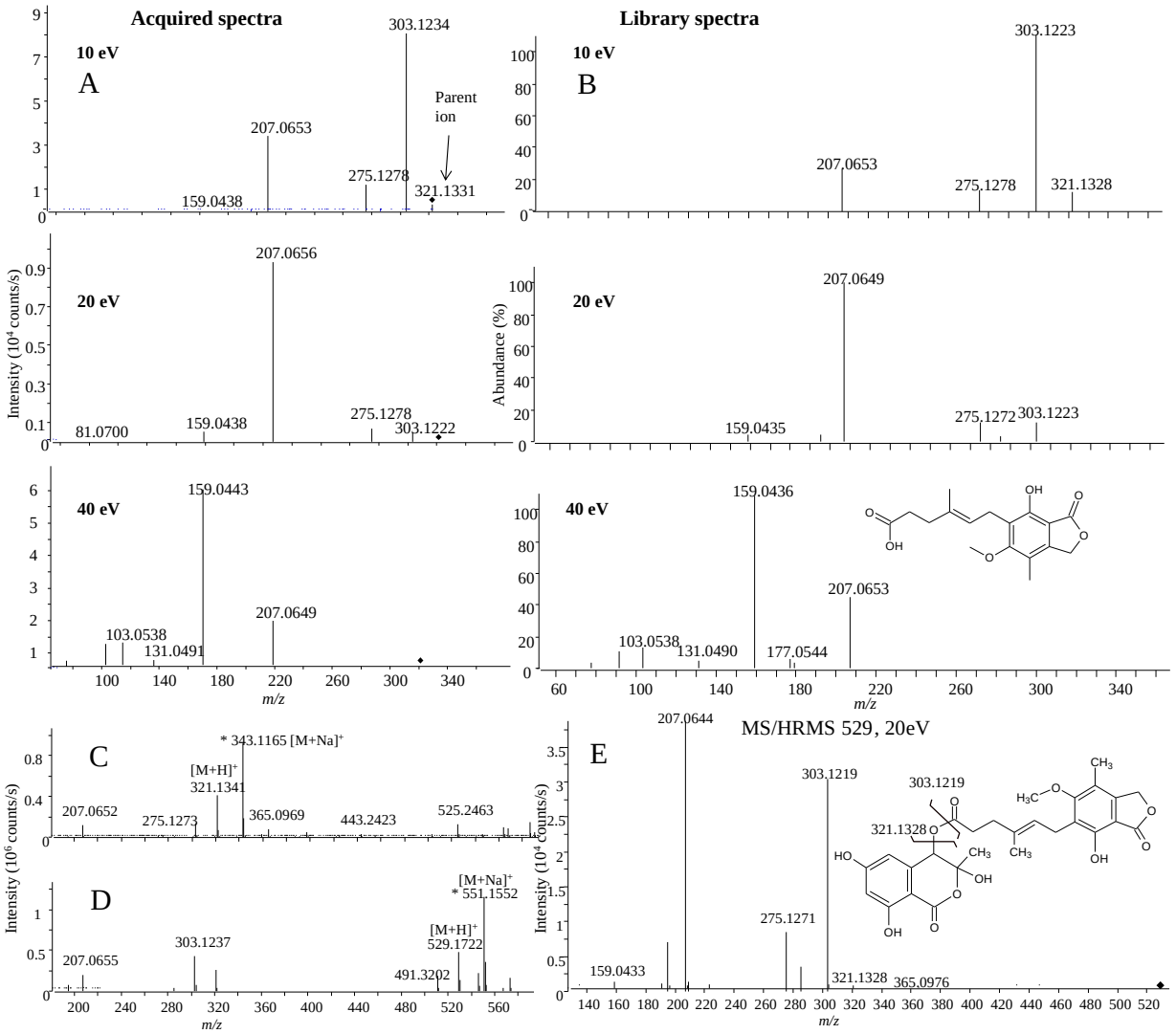
In addition, with ESI^+ reverse and forward scoring, a second compound was detected as mycophenolic acid itself but at a wrong retention time and not producing a $[M + Na]^+$ ion (Figure 5D), indicating that it was a fragment from a larger molecule. HRMS of the $[M + H]^+$ at *m*/*z* 529.1722 (Figure 5D) was used to tentatively identify the compound as a mycophenolic acid derivate F13459 previously isolated as a racemate from *Penicillium* sp. [59,60].

Figure 4. Base peak chromatograms (BPC) of the crude extract of *P. bialowiezense* in both positive (**A**) and negative (**B**) ESI modes. Peaks of compounds identified by MS/HRMS using forward scoring are colored.



The identity was verified by MS/HRMS fragmentation of *m/z* 529.1722 into the same ions observed from MS/HRMS of $[M + H]^+$ for mycophenolic acid (Figure 5E). F13459 might act as a natural prodrug that by hydrolysis loses the isocoumarin portion, leaving the active compound, mycophenolic acid (Figure 5E). The lost portion corresponds to the lactol form of the raistrick phenol, 2,4-dihydroxy-6-(1-hydroxyacetonyl) benzoic acid(Figure 5E) that was also detected in the extract (Figure 4).

Figure 5. MS/HRMS spectra (*m/z* 321) for mycophenolic acid in the active fraction (**A**) compared to library spectra (**B**) at 10, 20 and 40 eV; (**C**) full scan spectrum of mycophenolic acid showing a $[M + Na]^+$ ion at *m/z* 343; (**D**) full scan spectrum of F13459 showing a $[M + Na]^+$ ion at *m/z* 551; (**E**) MS/HRMS at 20 eV for $[M + H]^+$ of F13459 including structure of the compound.

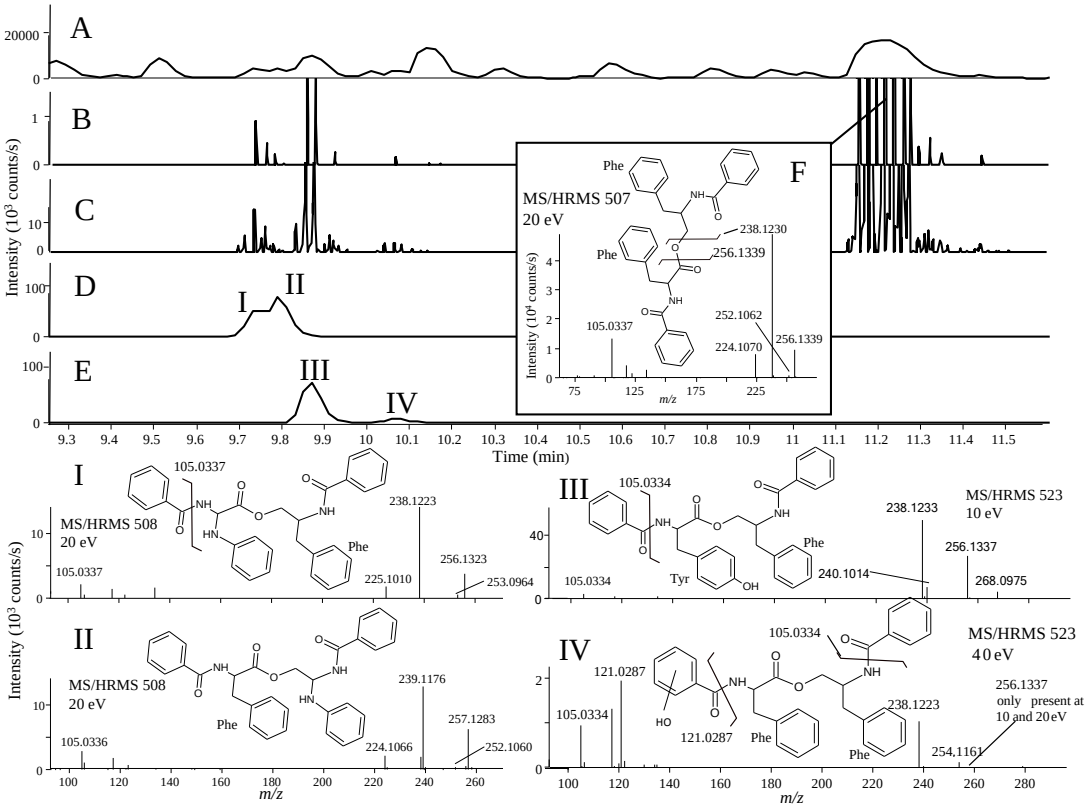


In the fraction displaying anticancer activity (unpublished assay), the library analysis led to the tentative identification of the fungal anticancer metabolite, asperphenamate (Figure 6F) [61]. A group of peaks eluting close to asperphenamate shared their major fragment ions as found using similarity searching (parent ion not used), which showed the presence of four novel asperphenamate analogues with the tentative structures I to IV (Figure 6). Unambiguous structure verification of course requires isolation and elucidation using nuclear magnetic resonance (NMR) spectroscopy.

Asperhenamate and three of the analogues (I, III, and IV) shared dominant fragment ions at *m/z* 238.1230 and 256.1339 (Figure 6B,C), corresponding to $[C_{16}H_{18}NO_2]^+$ and $[C_{16}H_{16}NO]^+$ formed from the right side of the molecule by cleavage of the ester-bond followed by water loss. The most abundant asperphenamate analogue (III) had a $[M + H]^+$ with *m/z* 523.2211, corresponding to an addition of an oxygen atom. This indicated replacement of the phenylalanine by a tyrosine in the

asperphenamate skeleton, corroborated by the fragment ions at *m/z* 268.0975 $[C_{16}H_{14}NO_3]^+$ and 240.1014 $[C_{15}H_{14}NO_2]^+$ (Figure 6III) as opposed to 252.1062 $[C_{16}H_{14}NO_2]^+$ and 224.1070 $[C_{15}H_{14}NO]^+$ in asperhenamate (Figure 6F). These fragments matched the left side of the molecule formed from the ester cleavage followed by the loss of CO. The fragment 105.0334 $[C_7H_5O]^+$ corresponding to the benzoyl part was present in both asperphenamate and the analogues, and the lack of an ion at *m/z* 121.0287 also supported the presence of the tyrosine (Figure 6III).

Figure 6. BPC chromatogram of the crude *P. bialowiezense* extract (**A**); EIC from MS/HRMS showing fragment ions (**B**) *m/z* 256.1333 and (**C**) 238.123; EIC full scan showing (**D**) *m/z* 508.2232 ± 0.005 and (**E**) 523.2211 ± 0.005; (**F**) MS/HRMS spectrum at 20 eV of asperphenemate. (**I**) to (**IV**) show the tentatively assigned isomers of asperphenamate and their positions in the chromatogram.

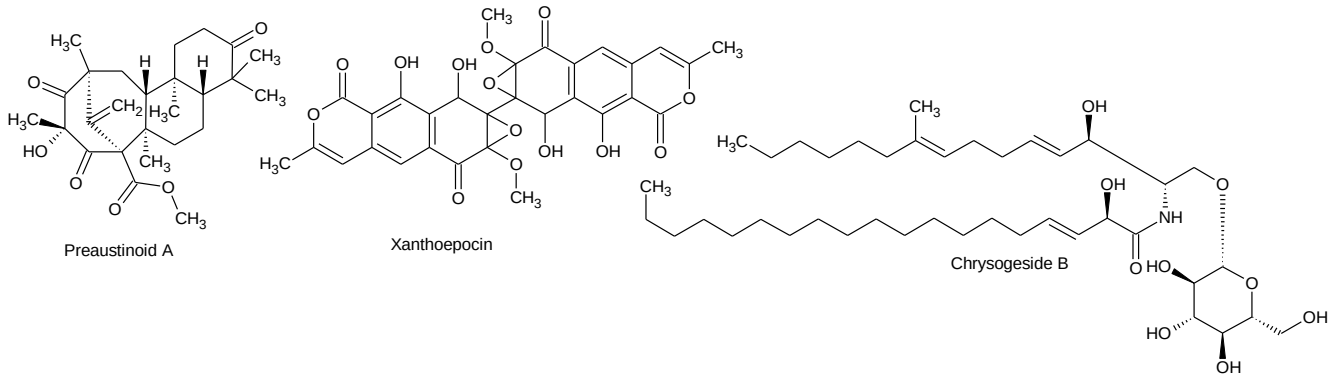


The other analogue IV with the same accurate mass as III had a similar fragmentation pattern with addition of the most prominent fragment ion 40 eV at *m/z* 121.0287 $[C_7H_5O_2]^+$. This fragment could match the presence of an extra oxygen atom in the benzoyl part instead of the phenylalanine part. The last two analogues (I and II) had $[M + H]^+$ *m/z* 508.2232 with similar fragmentation patterns to asperphenamate. Analogue II had *m/z* 239.1176 $[C_{15}H_{17}N_2O_2]^+$ and *m/z* 257.1283 $[C_{15}H_{15}N_2O]^+$ as major fragment ions not present in the asperphenamate MS/HRMS spectrum (Figure 6F), showing a replacement of a CH with an N atom, presumably in the phenylalanine moiety to the right of the ester bond, as a fragment ion corresponding to change in the benzoyl part was not observed. For Analogue I, the two ions differentiating it from asperphenemate were *m/z* 253.0964 $[C_{15}H_{13}N_2O_2]^+$ and 225.1010 $[C_{14}H_{13}N_2O]^+$ (Figure 6I), which also corresponded to the replacement of a CH with a nitrogen atom,

in this case to the left of the ester bond (Figure 6I) and, as in the example with II, showed a lack of extra fragments.

As the MS/HRMS library only covers about 5% of the compounds reported from fungi in AntiMarine (2012), though with a higher coverage of *Pencilllium* and *Aspergillus* compounds (~20%), we compared the MS/HRMS-based results with those obtained with: (i) aggressive dereplication based on extracted ion chromatograms and isotope patterns, using a search list of all metabolites known from *Penicillium* [20]; and (ii) an unbiased approach based on the Agilent Molecular Feature Extraction (MFE) algorithm which finds all chromatographic peaks and collects adduct, dimeric, and trimeric ions into one feature [62]. The peaks and matching candidates that were identified by the aggressive dereplication approach were evaluated by manually assessing the fragmentation pattern and by using the MassHunter Molecular Structure Correlator program which uses a systematic bond disconnection approach [27]. Likewise, the retention time was compared to the calculated LogD [43], and if possible the UV/Vis data evaluated. This further identified the known compounds chrysogesides B (Figure 7), C, D and E (characteristic loss of glucose and other specific fragments) [63] and three preaustinoids (fragmentations not very specific). Xanthoepocin (Figure 7) [64] was identified and verified from the very specific UV/Vis spectrum and MS/HRMS fragmentations. In full scan positive mode only $[M + Na]^+$ and $[M + H - H_2O]^+$ were observed.

Figure 7. Structures of preaustinoid A, xanthoepocin, and chrysogeside B.



The aggressive dereplication approach also identified fellutamides and breviones which are expected from the species [55]; this could, however, not be supported by the MS/HRMS. Most false positive results originated from fragments or adducts of other compounds in the extract. Examples of these were: (i) the loss of acetate from the andrastin A in ESI^+ matching andibenin B; (ii) quinolactacin A producing $[2M + Na]^+$ and $[2M + H]^+$ ions matching the $[M + Na]^+$ and $[M + H]^+$ of fellutanine D, respectively. Close inspection of adduct pattern and retention times, however, showed that andibenin B and fellutanine D were false positives. This underlines the importance of the MS/HRMS dimension for improved confidence in dereplication. False positives are eliminated and compounds that are missed because they are not part of the library can still be verified based on the MS/HRMS data. The unbiased minimum free energy (MFE) algorithm did, as expected, find many more peaks (50%–100%) than the two targeted approaches (data not shown); however, all major peaks in the chromatograms were detected by the targeted approaches, and all major biological activities could be accounted for by compounds in the MS/HRMS library.

2.2.2. Ophiobolins from a Marine-Derived *Aspergillus insuetus*

The extract of a *Aspergillus insuetus* strain (IBT 28443) derived from a sea water sample collected near Greenland was found to have activity in an anticancer assay (unpublished assay). The most potent fractions were found to be enriched in compounds belonging to the ophiobolin family. They are fungal sesterterpenoids with more than 35 known, closely related analogues [1,65]. Of these analogues, eight were available as standards and included in the library. The ophiobolins are known to exhibit a broad spectrum of bioactivities including antifungal and anticancer [1,65].

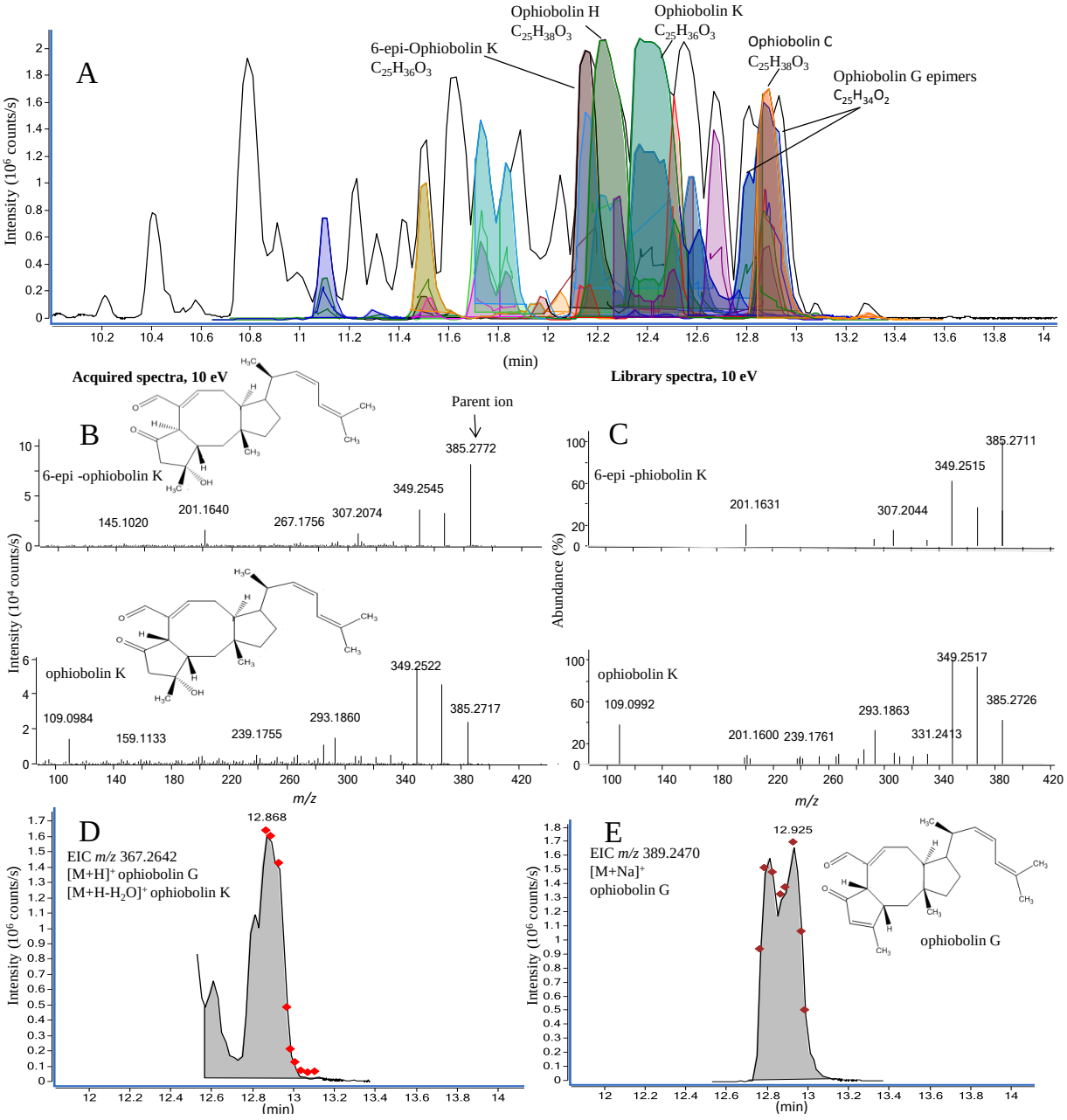
The analysis of a potent fraction is seen in Figure 8A, depicting MS/HRMS library-identified ophiobolins. The identification of four ophiobolins, namely 6-epi-ophiobolin K, ophiobolin H, ophiobolin K, and ophiobolin C, was further corroborated by matching HRMS, retention time, and UV/Vis. Several unidentified ophiobolin analogues seemed to be present in the fraction based on the HRMS and MS/HRMS data.

To illustrate the value of the MS/HRMS library approach, the MassHunter scoring and matching results for the two epimers, 6-epi-ophiobolin K and ophiobolin K (reference standards included in the LC-MS sequence) were compared to demonstrate if compounds varying at only one stereocenter would be unambiguously assigned by the library search. Both reverse and especially forward scoring showed that the epimers could be differentiated based on the intensity for the fragment ions as illustrated for 10 eV in Figure 8 B and C. The forward score for the MS/HRMS of the $[M + H]^+$ ion for the 6-epi-ophiobolin K peak was 71% 6-epi-ophiobolin K and 52% ophiobolin K, while it was 71% ophiobolin K and 58% 6-epi-ophiobolin K for the ophiobolin K peak.

For closely related analogues like the ophiobolins, the number of scans and the integration by auto MS/MS highly influence the outcome from the algorithm. This can be seen in Figure 8 for the series of overlapping peaks (between 12.8 and 13.0 min). From the EIC of the MS/HRMS scans of the *m*/*z* 367.2642 ion (Figure 8D) four peaks at 12.60, 12.78, 12.87, and 12.94 integrated as one peak and the average spectrum was matched to ophiobolin K (forward 81%) as the best match which is incorrect, while the likely correct match ophiobolin G (forward 53%) was the second best match. The reason for the incorrect match was both (i) poor peak integration mixing spectra from several compounds, and (ii) that the water loss ion of ophiobolin K was included in the library as it loses water in the ion source. Looking at the structures of ophiobolins K and G, it is apparent that ophiobolin K reacts into ophiobolin G losing water and forming a double bond. The subsequent MS/HRMS spectra of $[M + H]^+$ ophiobolin G and $[M + H - H_2O]^+$ ophiobolin K will thus be identical.

This underlines the difficulty of differentiation of isomers based on library matches. Fortunately investigating the $[M + Na]^+$ ions (EIC shown in Figure 8E) solves the problem and shows the likely ophiobolin G and 6-epi- ophiobolin G peaks at 12.81 and 12.93 min, respectively. Thus it would strengthen the validity of a compound identification if the matches from the different adducts could be combined and forced to include e.g., the match of the $[M + Na]^+$ ion.

Figure 8. (**A**) Active fraction enriched with compounds from the ophiobolin family in positive ESI mode. More than one color shading of the same peak is either due to different EIC and ECC for same match but different adducts or for other matches that had scored less; (**B**) MS/HRMS acquired spectra (10 eV) for library match of 6-epi-ophiobolin K and ophiobolin K; (**C**) MS/HRMS library spectra (10 eV) of 6-epi-ophiobolin K and ophiobolin K; (**D**) The EIC for the parent *m/z* 367.2642 with ophiobolin K as the best library match; (**E**) The EIC for the parent *m/z* 389.2470 with ophiobolin G as the library match. The diamond markers indicate number of scans across the peak.



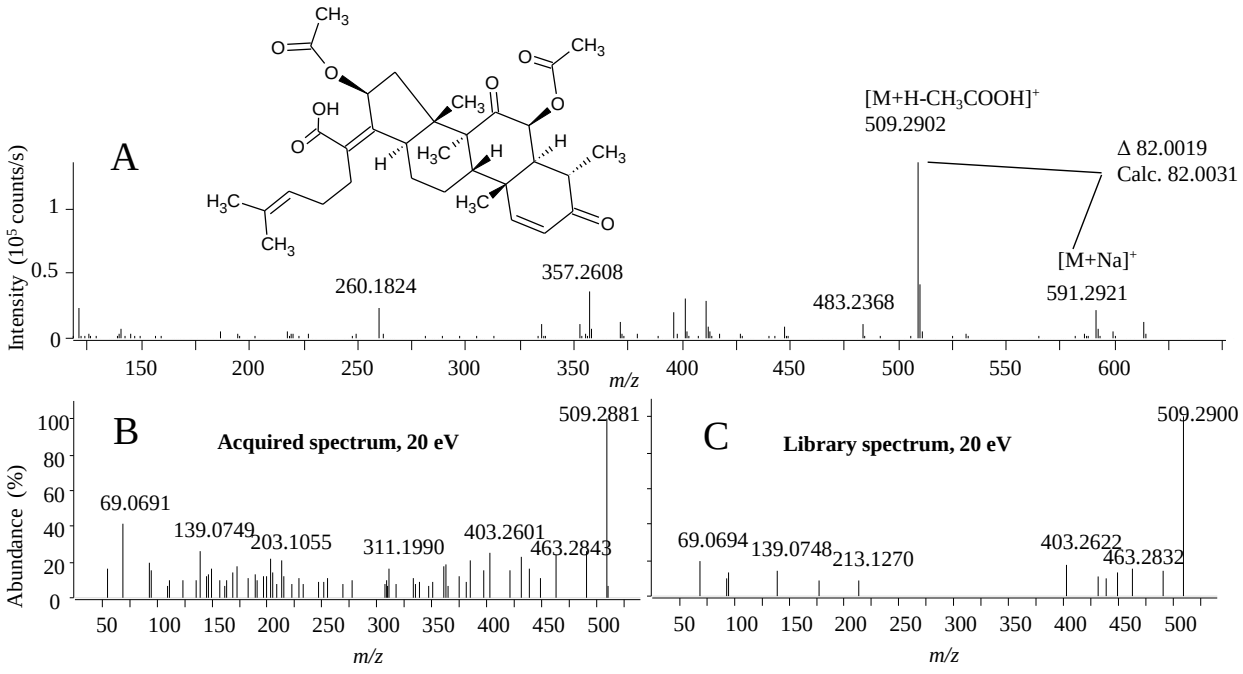
2.2.3. Helvolic Acid as the Anti-Microbial Compound in a Marine-Derived *Emericellopsis* sp.

Emericellopsis sp. strain (IBT 28361), a possibly new species, was isolated from a sea water sample collected off the coast of the Danish island Fanoe. *Emericellopsis* include both terrestrial and

marine species with *E. maritima* being associated with the seaweed Fucus (*Phaeophyceae*) [66]. A potent fraction of the crude extract displayed antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) [67].

Emericellopsis has not been extensively studied for its chemical potential and thus is it likely that it is not well represented by the compounds in the MS/HRMS library. This was the reason for the very few peaks identified by the MS/HRMS approach compared to the previous cases. Nonetheless, the known antibacterial nortriterpenoid, helvolic acid was identified by MS/HRMS (Figure 9) [68,69] consistent with the biological activity of the fraction. In full scan, ESI^+ identification was not based on the $[M + H]^+$ but rather the accuracy of the fragment at *m/z* 509.2902 $[C_{31}H_{41}O_6]^+$ which was the most abundant peak in the spectrum (Figure 9A). This fragment corresponds to the loss of an O-acetyl group that can be easily lost from the structure of helvolic acid which was verified by the presence of the $[M + Na]^+$ at *m/z* 591.2921 ion also showing that it was not a deacetyl-helvolic acid. As for the ophiobolins, automated use of both the MS/HRMS spectrum and $[M + Na]^+$ from full scan would increase validity of spectral matches. Helvolic acid has formerly been found in related fungal species such as *Emericellopsis terricola* [70] and *Sarocladium oryzae* [71].

Figure 9. (**A**) The structure of helvolic acid and the ESI^+ MS spectrum; (**B**) MS/HRMS spectrum at 20 eV from ESI^+ of helvolic acid and (**C**) the corresponding library spectrum (parent ion *m/z* 509).



The aggressive dereplication approach based on compounds known from *Emericellopsis* and five related genera (*Acremonium*, *Verticillium*, *Chaetomium*, *Sarocladium*, and *Cephalosporium*) likewise only returned few candidates. The total number of annotated peaks was roughly similar for the two methods. Helvolic acid was annotated by both methods, but apart from that there was almost no overlap between the candidates suggested, underlining the inherent bias of the compound library. The MS/HRMS library is biased by mainly containing metabolites from *Penicillium* and *Aspergillus*, while the targeted is generally biased towards the compounds from the most examined genera. The

aggressive dereplication matched the anti-protozoal compound, antiamebin I [72], which was originally not included in the MS/HRMS library. However, this compound could be verified later from a reference standard added to the MS/HRMS library. Using the MFE, a series of another five peptaibiotics in the same mass range as antiamebin was detected. These were not detected by the aggressive dereplication approach, as they were not indexed in AntiMarin 2012. However, searching the monoisotopic masses in The Comprehensive Peptaibiotics Database [73] tentatively identified them as different antiamebins (XIII, XIV, XV, III/IV/IX/VII/VIII, and XVI).

3. Experimental Section

3.1. Strains and Cultivation

All fungal strains used were from the IBT culture collection at the Department of Systems Biology, DTU. The strains described here were *Penicillium antarticum* (IBT 20733 and IBT 27985), *Penicillium bialowiezense* (IBT 28294), *Aspergillus insuetus* (IBT 28443) and *Emericellopsis* sp. (IBT 28361). The marine-derived fungi were cultivated on Czapek yeast extract agar (CYA) and Yeast extract sucrose agar (YES) media for 9 days in the dark at 25 ℃ [43].

3.2. Sample Preparation

Eight plates in total (four CYA and four YES) were extracted with 150 mL ethyl acetate containing 1% formic acid. The crude extracts were fractionated on a reversed phase C_{18} flash column (Sepra ZT, Isolute, 10 g) using an Isolera One automated flash system (Biotage, Uppsala, Sweden). The gradient used was 15%–100% acetonitrile buffered with 20 mM formic acid over 28 min (12 mL/min). Fractions were automatically collected based on UV signal (210 nm and 254 nm). A total of 126 crude, fractions, and blanks were submitted for bioassays antifungal (*A. fumigatus*, *C. albicans*) [74,75], and antibacterial MRSA [67], anticancer (unpublished assay), CNS in zebra fish larvae [51], and anti-inflammatory activity [49].

3.3. Standard Metabolites

Secondary metabolite standards have been collected over the past 30 years, either from commercial sources, as gifts from other research groups, or purified from our own projects [43,76], hence their quantity and purity varies (micro- to milligram quantity, ≥50% purity). The collection contains approximately 1600 standards with 95% of them being of fungal origin (5% of bacterial origin). Commercial sources of purchased standards include Sigma-Aldrich (Steinheim, Germany), Axxora (Bingham, UK), Cayman (Ann Arbor, MI, USA), TebuBio (Le-Perray-en-Yvelines, France), Biopure (Tulln, Austria), Calbiochem (San Diego, CA, USA), ICN (Irvine, CA, USA), Bachem GmbH (Weil am Rhein, Germany), and AnalytiCon Discovery GmbH (Potsdam, Germany). All standards were kept dry at −20 °C and, unless stated otherwise, were dissolved in 140 µL acetonitrile prior to analysis. If not soluble in pure acetonitrile, 50% acetonitrile in MilliQ water was used. Prepared standard solutions were also preserved on −20 ℃.

3.4. UHPLC-DAD-QTOFMS Analysis

Ultra-high performance liquid chromatography-diode array detection-quadruple time of flight mass spectrometry (UHPLC-DAD-QTOFMS) was performed on an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a diode array detector. Separation was obtained on an Agilent Poroshell 120 phenyl-hexyl column (2.1 × 150 mm, 2.7 μm) with a linear gradient consisting of water (A) and acetonitrile (B) both buffered with 20 mM formic acid, starting at 10% B and increased to 100% in 15 min where it was held for 2 min, returned to 10% in 0.1 min and keeping it for 3 min (0.35 mL/min, 60 °C). Injection volume, depending on sample concentration, typically varied between 0.1 and 1 μL. To avoid carry-over, the auto-sampler was operated in the flow-through-needle mode and further coupled to an Agilent Flex Cube which was used to back flush the needle seat for 15 s. at a flow of 4 mL/min with each of: (i) isopropanol: 0.2% ammonium hydroxide in water (1:1 *v/v*); (ii) acetonitrile with 2% formic acid; (iii) water with 2% formic acid.

MS detection was done on an Agilent 6550 iFunnel QTOF MS equipped with Agilent Dual Jet Stream electrospray ion source with the drying gas temperature of 160 °C and gas flow of 13 L/min and sheath gas temperature of 300 °C and flow of 16 L/min. Capillary voltage was set to 4000 V and nozzle voltage to 500 V. Ion-source parameters were the same for ESI^+ and ESI^- mode. Mass spectra were recorded as centroid data for *m*/*z* 85–1700 in MS mode and *m*/*z* 30–1700 in MS/MS mode, with an acquisition rate of 10 spectra/s. Automated data-dependent acquisition MS/HRMS (auto-MS/HRMS) analysis was commonly done for ions detected in the full scan above 50,000 counts (may be adjusted for low/high concentration samples) with a cycle time of 0.5 s, the quadrupole isolation width in narrow (*m*/*z* ±0.65), using fixed CID energies of 10, 20, and 40 eV and maximum three selected precursor ions per cycle. A narrow exclusion time of 0.04 min was used to get MS/MS of less abundant ions when compounds co-eluted.

Lock mass solution in 95% acetonitrile was infused in the second sprayer using an extra LC pump at a flow of 10–50 μL/min, the solution contained 1 μM tributyle amine (Sigma-Aldrich), 10 μM Hexakis(2,2,3,3-tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd., Cheshire, UK), and 1 μM trifluoroacetic acid (Sigma-Aldrich) as lock masses. The $[M + H]^+$ ions of first two (*m*/*z* 186.2216 and 922.0098 respectively) were used in positive mode, while $[M + HCOO]^-$ and $[M - H]^-$ of the latter two were used in negative mode (*m*/*z* 966.0007 and 112.9856).

3.5. Library Setup and Auto-MS/MS Data Analysis

The MS/HRMS library was constructed from our internal ChemFolder library (Advanced Chemical Developments, Toronto, ON, Canada) of 7400 compounds of which 1600 were available as reference standards [20]. For reference standards and tentatively identified compounds, name, structure, and CAS no. were transferred to the Agilent Masshunter PCDL manager 4.00 (Service release 1), and linked to the retention time and MS/HRMS spectra of 10, 20, and 40 eV, either by manually pasting from MassHunter or imported via a cef file. All major pseudomolecular ions ($[M + H]^+$, $[M + Na]^+$, $[M + NH_4]^+$, $[M - H]^-$, $[M + HCOO]^-$), and simple fragment ions (mainly $[M + H - (H_2O)_n]^+$, $[M + H - CH_3COOH]^+$, $[M - H - CO_2]^-$) which provided characteristic MS/MS spectra were included.

Data files were processed by the Find by Auto MS/MS function in Masshunter, usually without any intensity threshold but often with a limit to the 200 largest peaks, mass match tolerance *m*/*z* 0.05. Unless otherwise stated the MS/HRMS library was searched using precursor and product ion expansion of 50 ppm + 2 mDa as well as minimal reverse and forward scores of 50 each.

3.6. Aggressive Dereplication and Molecular Feature Extraction

For analysis of compounds described in the literature and not necessarily available as reference standards, Aggressive dereplication (Klitgaard *et al.* 2014 [20]) was performed on the ESI^+ and ESI^- full scan data using the *Find by Formulae* function in Agilent Masshunter Qualitative analysis B06.00 software. The following adducts and common fragments were included: ESI^+, $[M + H]^+$ and $[M + Na]^+$; ESI^-, $[M - H]^-$, $[M + HCOO]^-$. All ions analyzed were treated as being singularly charged. The area cut-off was set to 10,000, and the mass spectrum was recorded below 10% of the height of the peak to avoid detector overload. A minimum score of 70 was used to ensure that only compounds with fitting isotope patterns were annotated.

The search lists were constructed from the AntiMarin2012 which was converted into an sdf-database and then imported into ChemFolder and from here to Excel (Klitgaard *et al.* 2014 [20]) where it was formatted to the Agilent search list format. All this work was made on an AntiMarin-licensed computer.

The MFE screening was performed in the Agilent Masshunter Qualitative analysis B06.00. The following adducts and common fragments were included: ESI^+, $[M + H]^+$ and $[M + Na]^+$; ESI^-, $[M - H]^-$, $[M + HCOO]^-$. All ions analyzed were treated as being singly charged. The area cut-off was set to 10,000, and the mass spectrum was recorded below 10% of the height of the peak to avoid detector overload. A minimum quality score of 99 was used to ensure that only compounds with fitting mass, isotope patterns, and peak shape were annotated.

4. Conclusions

In this work we demonstrate that MS/HRMS search in a library is a robust and reliable way of tentatively identifying known bioactive compounds on a single instrument. With spectra reproducibility across Agilent instruments [26,77] the library should be directly usable on these, while others instruments presumably need adjustment against collision energies (e.g., 10 eV on the Agilent may correspond to 15 eV on a Bruker QTOF). Furthermore MS/HRMS aided the tentative identification of novel isomers, e.g., to be used in bioactivity optimization. Many highly bioactive compounds are found across the fungal kingdom, and even when exploring specialized marine environments where it is likely to find novel bioactive compounds it is of outmost importance to identify known nuisance compounds in the first screen. To aid drug discovery dereplication we thus suggest that it is required to deposition MS/MS spectra of all novel published compounds in Massbank, MetLin and/or GNPS [31], although for all mentioned an easy interface for depositing spectra is needed.

Aggressive dereplication of full scan data supplemented by auto MS/HRMS to strengthen the correct match and elimination of false positives proved efficient and could in many cases be strengthened even further by UV/Vis data.

Both described strategies can handle extracts produced months in-between which is a problem for the unbiased peak picking and adduct pattern algorithms which in general requires samples to be run within a sequence and with replicated and blank samples to handle variations in chromatographic separation, mass spectra, sample preparation, and growth media. Nonetheless, an unbiased peak picking strategy was the only way to detect a series of non-data based compounds as demonstrated in the last case, proving the need to integrate many data-analysis strategies and tools to obtain comprehensive compound coverage.

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Author Contributions

S.K., I.D., and K.F.N. designed the research; S.K. and I.D. performed the experimental work; S.K., I.D., J.C.F. and A.K. analysed the data; T.O.L. and J.C.F. provided strains and samples as well as assisted in compound identification; S.K., M.M., I.D., and K.F.N. wrote the paper. All authors read and corrected the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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5.3 Paper 3 - Potentially Harmful Secondary Metabolites Produced by Indoor *Chaetomium* species on Artificially and Naturally Contaminated Building Materials

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

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 well-being. 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 to establish differences between metabolite production *in vitro* and on building materials as well as in comparison with non-indoor reference strains. On building materials, six different chaetoglobosins were detected in total concentrations of up to 950 mg/m^2 from *Chaetomium 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 *Chaetomium elatum* and are thus candidates as *Chaetomium* biomarkers. No sterigmatocystin was produced by *Chaetomium* spp. from indoor environment.

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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 *Chaetomium globosum,* as well as a common biomarker for *C. globosum* and *Chaetomium elatum* for their identification. The presence of the common biomarker, cochliodone, in, for example, 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 molds (WHO Regional Office for Europe, 2009). Otherwise, healthy people may also experience negative health effects, such as skin rashes, headaches, dizziness, and chronic fatigue (Miller and McMullin, 2014; Täubel et al., 2011). 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 (Brewer et al., 2013; Rand and Miller, 2011). There are several routes of exposure to fungal metabolites in moldy 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 with 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 mold-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 molds (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 (B. Andersen, 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 microparticles (Gorny et al., 2002; Kildesø et al., 2003; Madsen et al., 2012) are likely to be the vehicle of exposure (Green et al., 2006) in moldy buildings.

More than 200 biological active metabolites are known from different species of *Chaetomium* (Zhang et al., 2012), and these include compounds such as chaetomugilins, cochliodinol, cochliodones, and chaetoglobin A (Chen et al., 2012; Ge et al., 2008; Jerram et al., 1975; Phonkerd et al., 2008). 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* (Andersen et al., 2011; McGregor et al., 2008; Samson et al., 2010; Wang et al., 2016). Both *C. globosum* and *C. elatum* have been reported to produce chaetoglobosins (Thohinung et al., 2010; Udagawa et al., 1979), and chaetoglobosins A and C (Figure 1) have been detected on building materials (Nielsen, 2003; Nielsen et al., 1999). 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).

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 (Polizzi et al., 2012; Van Lanker et al., 2008). Several targeted multianalyte 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 (Polizzi et al., 2009; Vishwanath 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., 2013a,b), the authors analyzed 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 from different *Chaetomium* species produced in pure agar cultures and determine which of those can be produced on artificially inoculated building materials and on 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 MilliQ system (Millipore, Bedford, MA, USA) and autoclaved.

Standards of secondary metabolites included in library have been collected over the past 30 years, from commercial sources, as gifts from other research groups or purified from different in-house projects (Kildgaard 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, USA), 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

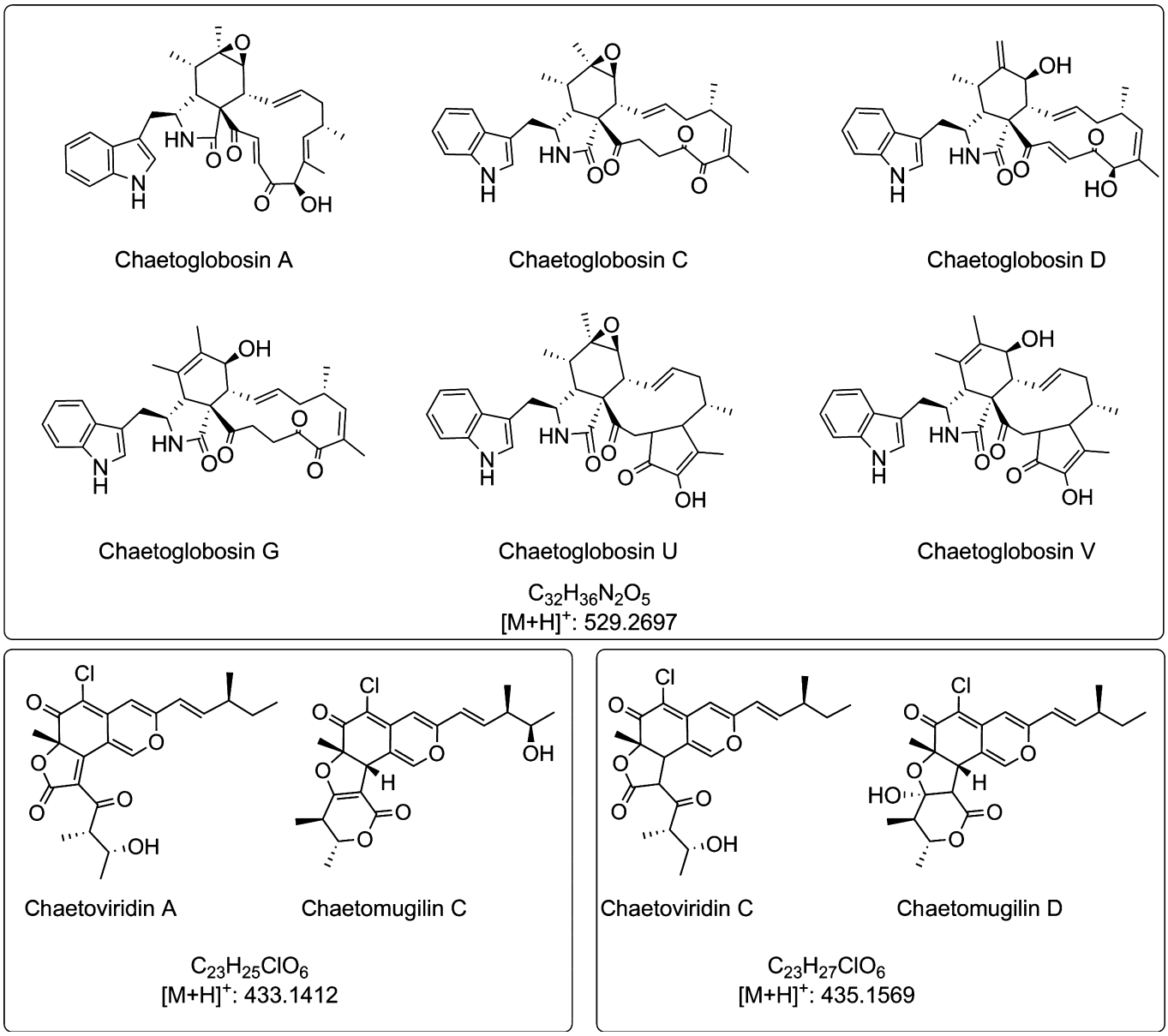

Fig. 1 Chemical structures of chaetoglobosins, chaetoviridins, and chaetomugilins with the same elemental composition found in *Chaetomium globosum*

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 and species identification and were consequently included in our IBT Culture Collection. Five different agar media were used for inoculation: yeast extract sucrose agar, Oatmeal agar, malt extract agar (MEA), V8 juice agar (V8), and potato dextrose agar (PDA). 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 fiberboard), and medium density fiberboard (MDF). New, unblemished panels (90 × 240 cm) of each material were cut into sample blocks (13 × 18 cm), and each block was further divided into four equal oblong sections (13 × 4.5 cm) (Figure 2). Each section represented one of four different conditions: (i) wallpaper

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 photograph 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.

adhesive + nonwoven woodchip wallpaper (wp1); (ii) only wallpaper adhesive; (iii) wallpaper adhesive + nonwoven wallcovering with pattern (wp2), and (iv) non-treated material (no surface treatment), giving four inoculation sites per block. Wallpaper adhesive used was Pattex Direct Control Universal (Henkel, Düsseldorf, Germany).

Each sample block was placed in a plastic box (22.5 × 17.5 × 4.5 cm) with a lid, sealed, and sterilized using 1 × 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 (Figure 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 reweighed and incubated at room temperature in darkness. The blocks were inspected and growth progress recorded once a week. Sampling for metabolite extraction was carried out 4 weeks after inoculation for *C. globosum* and *C. elatum,* while material inoculated with *C. virescens* was not analyzed due to the absence of growth.

Naturally contaminated building materials

Ten samples, naturally contaminated with *Chaetomium* spp., were analyzed. 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 h 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). To verify *Chaetomium* contamination on the ten materials, tape preparations for phase contrast microscopy (200×, and 400×) were taken directly from the mold-infected area. This was carried out 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 laboratory or when discovered in the water-damaged building.

Metabolite extraction of building materials

Sampling from both artificially inoculated and naturally contaminated materials was carried out 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, redissolved 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 analyzed.

Metabolite extraction of pure cultures

The metabolite profiling was carried out on the 15-day-old MEA and PDA cultures using a microscale extraction method modified for *Chaetomium*

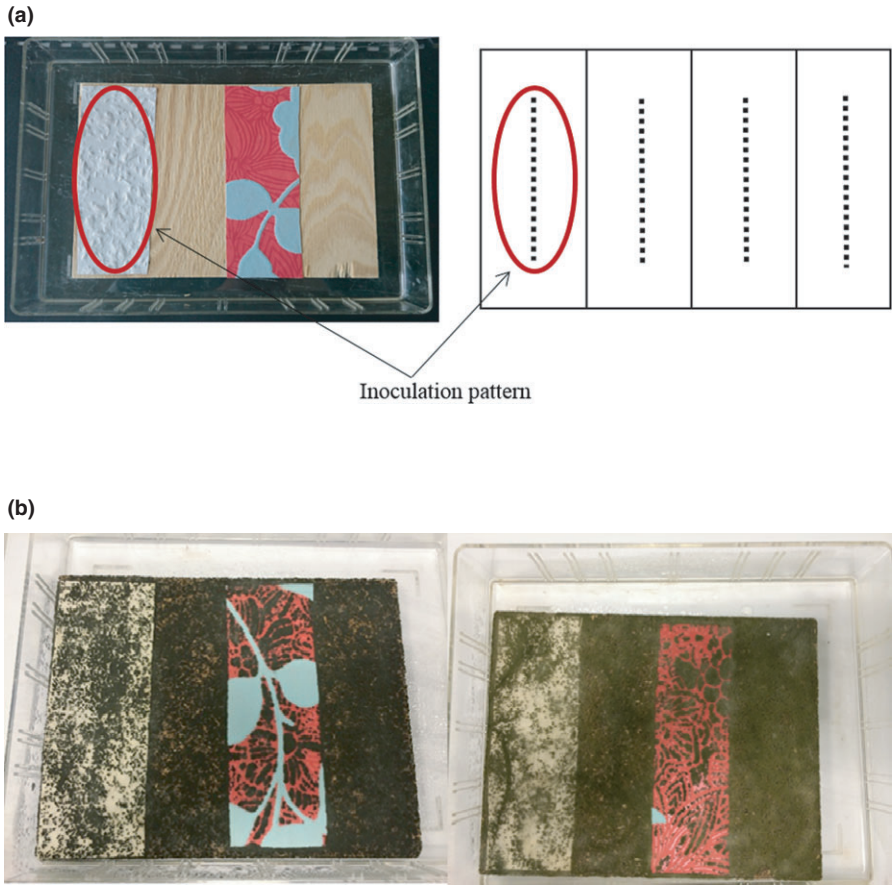

Fig. 2 Artificially inoculated building blocks with inoculation pattern and specific growth in different sections. (a) Sections from left to right: wallpaper adhesive + nonwoven woodchip wallpaper (wp1), wallpaper adhesive, wallpaper adhesive + nonwoven wall-covering with pattern (wp2), non-treated material. (b) From left to right: *Chaetomium elatum* and *C. globosum* on chipboard showing characteristic absence of fungal growth on pattern of wallpaper type two (red wallpaper with blue leaves)

metabolites. 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

Ultrahigh-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, CA, 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 (Kildgaard 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 Kildgaard et al. (2014). All samples were analyzed only in ESI^+ mode, due to the nature of analyzed compounds.

Identification of secondary metabolites was performed using a combination of the following approaches: (i) direct search and matching of MS/HRMS data in MS/HRMS library containing all fungal secondary metabolites (~1500), (Kildgaard et al., 2014); (ii) 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 (iii) UV/Vis detection of poorly ionizing compounds.

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

The search list used in the aggressive dereplication approach (Klitgaard et al., 2013) 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 as follows: $[M + H]^+$, $[M + Na]^+$, $[M + H - H_2O]^+$, and $[M + NH_4]^+$. All analyzed ions were treated as being single charged; the area cutoff was set to 10,000, and the mass spectrum was recorded below 10% of the peak height to avoid overloading the detector (Kildgaard et al., 2014).

For fast screening of larger number of samples and for semiquantification purposes, MassHunter 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 (RT) 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 2 weeks. Once started, the growth and production of perithecia on plywood and chipboard were rapid, resulting in densely overgrown material after 4 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 4 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 unfavorable 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 (nonwoven 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 (Figure 2). In addition to that, a recent research paper reports wallpapers with flatter or irregular surface structures to be advantageous in limiting mold germination and growth (Ryu and Moon, 2014).

Table 2 Growth evaluation of different *Chaetomium* spp. artificially inoculated on different building materials with two types of wallpapers (wp1 and wp2) and adhesive, adhesive alone, and blank with no treatment (see Figure 1)

		Growth			
Materials	Water content (% w/w)	*C. globosum* IBT 7029	*C. globosum* IBT 41801	*C. elatum* IBT 41944	*C. virescens* IBT 26237
Chipboard					
wp1	20	+++	+++	+++	NG
Adhesive		++++	++++	++++	NG
wp2		++	+++	+++	NG
Blank		++++	++++	++++	NG
Plywood					
wp1	15	+++[a]	+++	++	NG
Adhesive		++++	+++	+	NG
wp2		++	++	+	NG
Blank		+++	+++	+	NG
Gypsum					
wp1	17	++	++	+	NG
Adhesive		++	+	NG	NG
wp2		+	+	NG	NG
Blank		++	++	++	NG
Masonite					
wp1	17	NG	NG	NG	NG
Adhesive		NG	NG	NG	NG
wp2		NG	NG	NG	NG
Blank		NG	NG	NG	NG
MDF					
wp1	5	NG	NG	NG	NG
Adhesive		NG	NG	NG	NG
wp2		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.

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 (Figure 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

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

	Chipboard								Plywood								Gypsum							
	wp1		Adhesive		wp2		Blank		wp1		Adhesive		wp2		Blank		wp1		Adhesive		wp2		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 analyzed, 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, seven strains isolated in total.

Metabolites in bold are found in the highest amounts.

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 (Figure 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 RT 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 (Figure 1). Because 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* (Tables 4 and 5).

Chaetoviridins/chaetomugilins. This is another example of a metabolite group consisting of compounds with

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 (photograph 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.

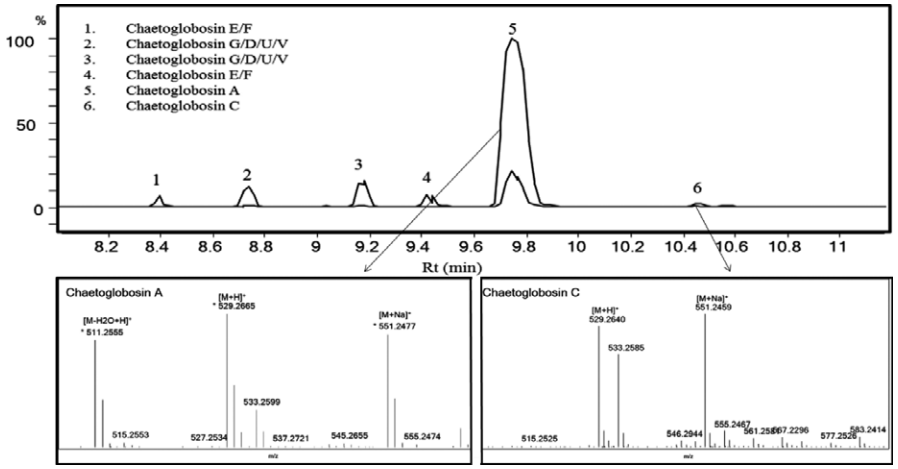

Fig. 3 Combined extracted ion chromatograms of m/z 529.2697 corresponding to pseudomolecular ion of chaetoglobosins A, C, G, D, U, and/or V resulting in four peaks: 2, 3, 5, and 6 and of m/z 531.2853 corresponding to pseudomolecular ion of chaetoglobosins E and F (peaks 1 and 4). MS spectra of identified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

the same elemental composition and hence, due to the unavailability of standards, needed to be identified as members of specific group (Figure 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, while 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 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* (Tables 4 and 5). Chaetoviridins and chaetomugilins were also not detected from any materials artificially inoculated with *C. elatum* (Table 3).

Cochliodones. Chaetomium globosum and *C. elatum* seemed to have a few metabolites in common, of which none has 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 RTs of 7.74, 9.53, and 11.39 min, sharing the same $[M + H]^+$ m/z of 639.2436 (Figure 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 the presence of three peaks with m/z matching cochliodone A/B. Inconsistency in the available literature [several compounds with the same elemental composition, for example, chaetospirone (Bitzer, 2005)] and limited knowledge of the biosynthetic pathways of these compounds suggests that NMR data are neces-

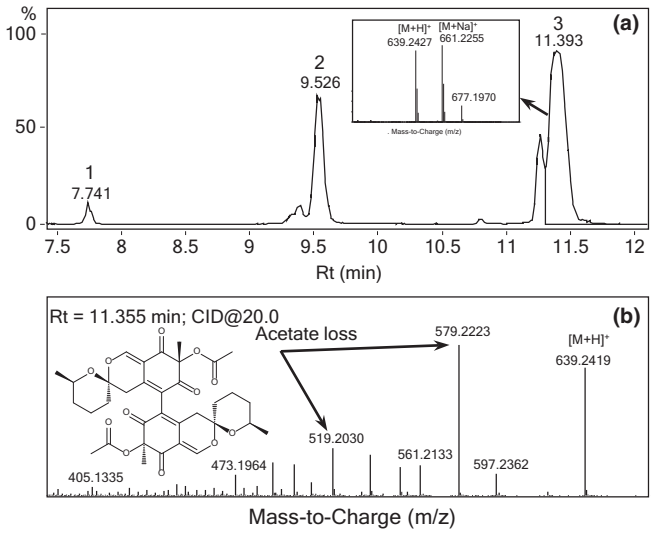

Fig. 4 Chromatogram (a) of three peaks identified as 1 – cochliodone 1 (Rt 7.74 min), 2 – cochliodone 2 (Rt 9.52 min), and 3 – cochliodone 3 (Rt 11.39 min) together with MS/MS spectrum of cochliodone 3 at 20 eV (b)

sary for their unequivocal identification. However, the presence of all proposed fragments (Figure 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, while 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* (Tables 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 Figure 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 (Figure 5) except in the case of *C. globosum* reference strain (IBT 7029).

The chemical profiles of all 14 *C. globosum* strains were very similar. Several chaetoglobosins, chaetoviridins, and chaetomugilins were identified in all analyzed strains (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 with 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,* while 3-*O*-methyl sterigmatocystin was found only in *C. virescens* extracts. The identity of these metabolites was confirmed by characteristic UV spectra and RT (Figure 5). *Chaetomium virescens, C. longicolleum,* and *C. malaysiense* also produced several versicolorins (Figure S3) (Kingston et al., 1976). Analysis of the two *C. homopilatum* strains indicated the presence of longirosterones (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 the presence of sterigmatocystin in indoor environment can be linked only to *Aspergillus versicolor* and not to *C. globosum* and *C. elatum*.

Table 6 Estimation of concentration range ($\mu g/cm^2$) for four major metabolite groups found on artificially inoculated building materials

	Concentration range ($\mu g/cm^2$)							
	Chaetoglobosins		Chaetoviridins		Cochliodones		Chaetoglobin A	
Materials	*Chaetomium globosum*	*Chaetomium elatum*	*C. globosum*	*C. elatum*	*C. globosum*	*C. elatum*	*C. globosum*	*C. elatum*
CHIPBOARD								
wp1	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
wp2	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								
wp1	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
wp2	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								
wp1	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
wp2	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

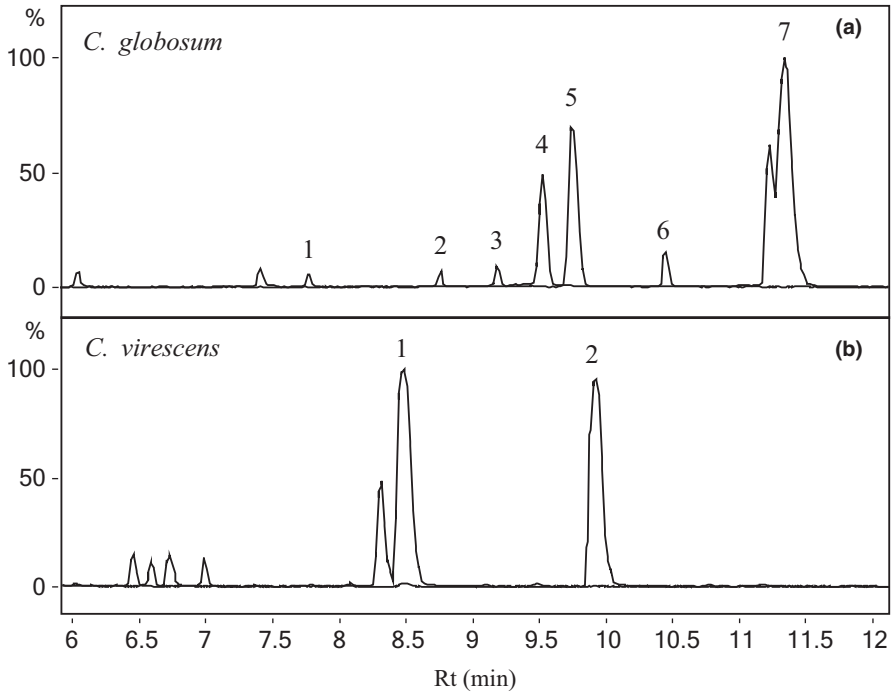

Fig. 5 Comparison of combined extracted ion chromatograms (6–12 min) of indoor (*Chaetomium globosum*, IBT 41801) and reference strain (*C. virescens*, IBT 26237) MEA extracts: (a) *C. globosum* 1 – cochliodone 1, 2 – chaetoglobosin G/D/U/V, 3 – chaetoglobosin G/D/U/V, 4 – cochliodone 2, 5 – chaetoglobosin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

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 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 $\mu g/cm^2$) being on chipboard and lowest on gypsum (up to 10 $\mu g/cm^2$). These concentrations were in accordance with what has previously been found for chaetoglobosin A [up to 13.8 $\mu g/cm^2$ on various sub-

strates (Polizzi et al., 2009)]. Same trend was seen with two other groups of metabolites: The highest amount of chaetoviridins (19 $\mu g/cm^2$) and cochliodones (21 $\mu g/cm^2$ for *C. globosum* and 5 $\mu g/cm^2$ for *C. elatum*) were also found on non-treated (no surface treatment) chipboard.

Concentration estimates for individual metabolites (Table S2) pointed toward high concentration (up to 100 $\mu g/cm^2$) 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 $\mu g/cm^2$, 1.7 $\mu g/cm^2$ for chaetoviridin C1 and C2, and 6.8 $\mu g/cm^2$ 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 metabolite 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 the 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, the absence of any visible growth or viable biomass and the 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 (Polizzi et al., 2009; Vishwanath 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, because 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 the presence of spores, microparticles, 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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nologies for the Thought Leader Donation of the UHPLC-QTOF system.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Proposed fragmentation pattern for cochliodone A.

Figure S2. Proposed fragmentation pattern for chaetoglobin A.

Figure S3. Versicolorin type of compounds in pure culture extracts of *C. longicolleum*

Table S1. Metabolite production for different *Chaetomium* species in pure culture.

Table S2. Amounts in area/cm^2 for secondary metabolites produced on four different sections on three different building materials by two strains of *C. globosum* and one strain of *C. elatum*.

Table S3. Evaluated signal enhancement/suppression (SSE %) for five different materials used in artificial inoculation experiment.

Table S4 Retention time, ion species and mas to charge ratio (m/z) of all metabolites screened for in this study.

Appendix S1. Supplementary information on method design, method performance and quantification on building materials.

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Supporting information

Figure S1. Proposed fragmentation pattern for cochliodone A

Figure S2. Proposed fragmentation pattern for chaetoglobin A

Figure S3. Versicolorin type of compounds in pure culture extracts of *C. longicolleum*

Table S1. Metabolite production for different *Chaetomium* species in pure culture

Table S2. Amounts in area/cm^2 for secondary metabolites produced on four different sections on three different building materials by two strains of *C. globosum* and one strain of *C. elatum*

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Table S4 Retention time, ion species and mas to charge ratio (*m/z*) of all metabolites screened for in this study

Appendix S1. Supplementary information on method design, method performance and quantification on building materials

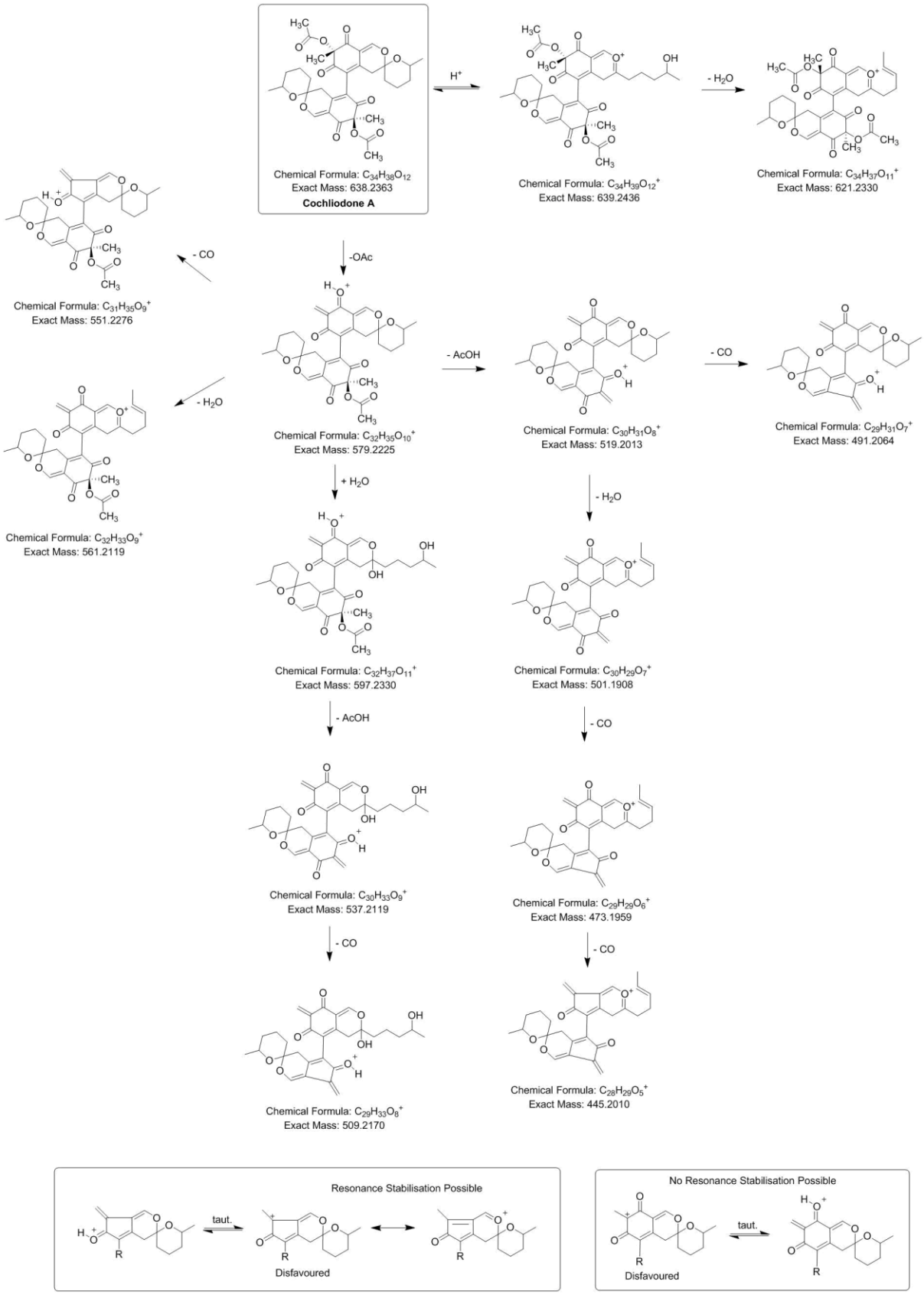


Fig. S1 Proposed fragmentation pattern for cochliodone A

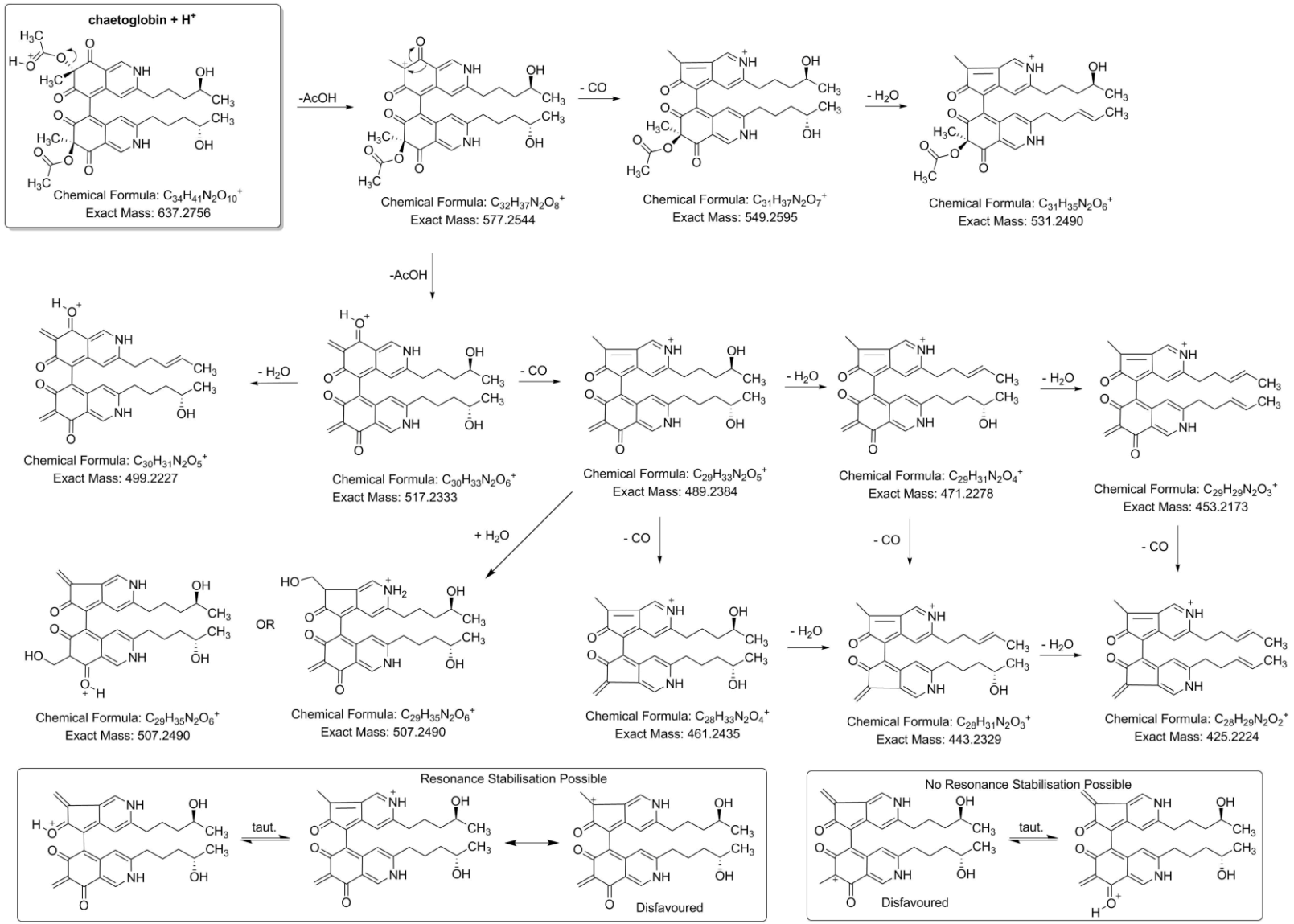
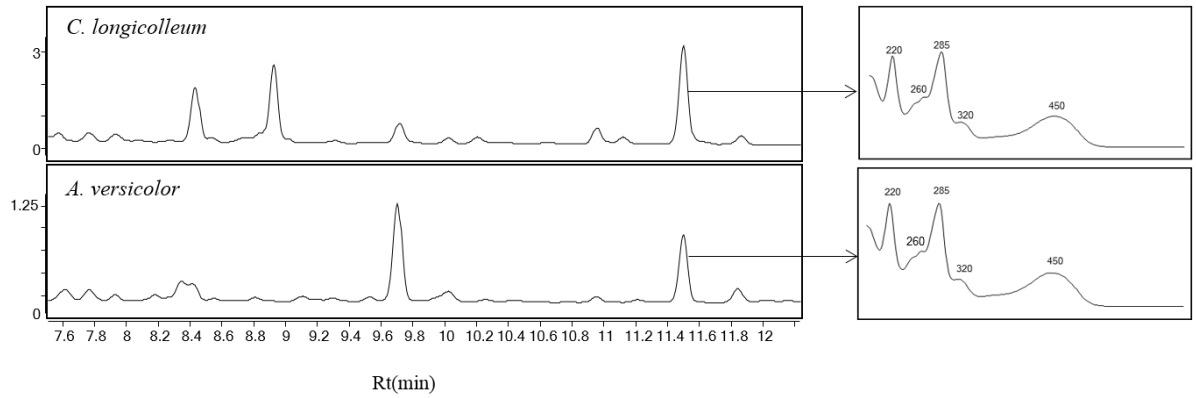
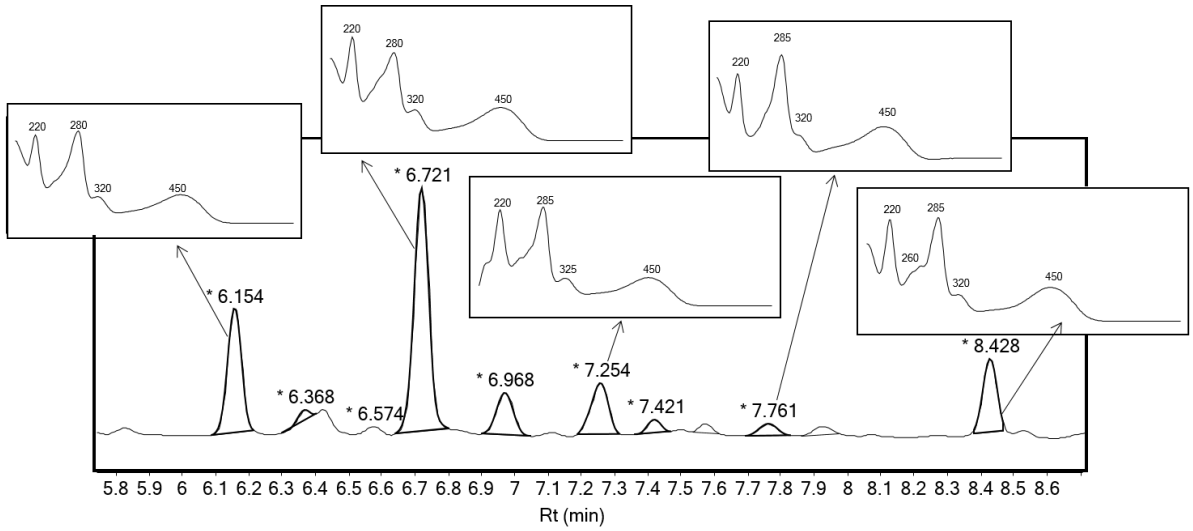


Fig. S2 Proposed fragmentation pattern for chaetoglobin A



Verification of versicolorin type of compound in extract of *C. longicolleum* (IBT 41566) by comparison with *A. versicolor* extract (IBT 29547): same compound found at 11.5 min with identical UV spectrum



Compounds belonging to versicolorin group eluting between 6-8.5 min with their characteristic UV spectra

Fig. S3 Versicolorin type of compounds in MEA extract of *C. longicolleum* (IBT 41566)

Table S1. Metabolite production for different *Chaetomium* species in pure culture (MEA and PDA).

Metabolite	*C. elatum* (2)	*C. erectum* (1)	*C. globosum* (14)	*C. homopilatum* (2)	*C. longicolleum* (2)	*C.malaysiense* (1)	*C. virescens* (1)
Chaetoglobosin A[1, 3]	-	-	+	-	-	-	-
Chaetoglobosin C[1, 3]	-	-	+	-	-	-	-
Chaetoglobosin G/D/U/V[1]	-	-	+	-	-	-	-
Chaetoglobosin E/F[2]	-	-	+	-	-	-	-
Prochaetoglobosin I[2]	-	-	+	-	-	-	-
Prochaetoglobosin II[2]	-	-	+	-	-	-	-
Prochaetoglobosin III[2]	-	-	+	-	-	-	-
Prochaetoglobosin IV[2]	-	-	+/-	-	-	-	-
Chaetoviridin C/ Chaetomugilin D/S[2]	-	-	+	-	-	-	-
Chaetoviridin A/ Chaetomugilin C[1, 3]	-	-	+	-	-	-	-
Chaetoviridin E[2]	-	-	+	-	-	-	-
Chaetoviridin H[2]	-	-	+	-	-	-	-
Cochliodone 1[2, 4]	+	-	+	-	-	-	-
Cochliodone 2[2, 4]	+	-	+	-	-	-	-
Cochliodone 3[2, 4]	+	-	+	-	-	-	-
Chaetoglobin A[2, 4]	+	-	+	-	-	-	-
Cochliodinol[2]	+	-	+/-	-	-	-	-
Longirosterone A[2]	-	-	-	+	-	-	-
Longirosterone B[2]	-	-	-	+	-	-	-
Longirosterone C[2]	-	-	-	+	-	-	-
Versicolorin**[3]	-	-	-	-	+	+	+
Sterigmatocystin[1, 3]	-	-	-	-	+	+	+
3-O-methyl sterigmatocystin[1, 3]	-	-	-	-	-	-	+
Dihydro-sterigmatocystin[1, 3]	-	-	-	-	+	+	-

[1] identified as MS/HRMS library;
[2] identified by aggressive dereplication;
[3] identified by comparison to reference standard and/or by specific UV spectrum;
[4] MS spectrum fitting proposed fragmentation pattern (supplementary)
[5] several compounds from versicolorin group present

Table S2 Amounts in area/cm^2 for secondary metabolites produced on four different sections on three different building materials by : A) *C. globosum* IBT 7029; B) *C. globosum* IBT 41801; C) *C. elatum* IBT 41944

		Conc µg/cm^2											
A) *C. globosum* IBT 7029		CHIPBOARD				PLYWOOD				GYPSUM			
METABOLITES	Rt (min)	wp type 1	adhesive	wp type 2	blank	wp type 1	adhesive	wp type 2	blank	wp type 1	adhesive	wp type 2	blank
Chaetoglobosin A	9.75	2.88	51.6	2.72	48.56	10.64	39.84	2.40	42.40	2.32	9.92	2.24	2.72
Chaetoglobosin C	10.40	0.56	1.29	6.52	6.64	0.49	1.81	2.16	8.45	NA*	NA*	NA*	NA*
Chaetoglobosin G/D/ U/V 1	8.75	NA	NA*	NA*	0.7	1.83	NA*	NA	1.22	NA	NA	NA	NA*
Chaetoglobosin G/D/U/V 2	9.20	NA	NA*	NA*	0.69	4.47	NA*	NA*	1.31	NA	NA	NA*	NA*
Chaetoglobosin E/F 1	8.40	NA*	1.23	1.07	2.71	NA*	0.92	NA*	1.63	NA	NA	NA	NA
Chaetoglobosin E/F 2	9.45	NA*	NA*	NA*	NA*	NA	0.67	NA*	NA*	NA	NA	NA	NA
Chaetoviridin C 1	10.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoviridin C 2	11.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoviridin A	11.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cochliodon 1	7.70	0.17	0.04	0.02	NA*	1.44	0.21	NA	0.20	0.88	1.18	0.34	0.71
Cochliodon 2	9.50	1.72	1.76	1.34	1.90	2.02	1.12	0.54	1.70	1.42	1.69	1.38	1.84
Cochliodon 3	11.40	4.36	4.46	3.68	4.78	2.37	3.39	1.87	4.41	2.41	3.97	2.24	4.01
Chaetoglobin A	4.20	0.97	2.33	2.02	3.00	NA	0.11	NA	NA	NA	NA	NA	NA

B) *C. globosum* IBT 41801		Conc µg/cm^2											
		CHIPBOARD				PLYWOOD				GYPSUM			
METABOLITES	Rt (min)	wp type 1	adhesive	wp type 2	blank	wp type 1	adhesive	wp type 2	blank	wp type 1	adhesive	wp type 2	blank
Chaetoglobosin A	9.75	2.56	95.43	2.50	81.72	8.80	36.80	36.32	62.40	2.64	6.00	2.56	2.24
Chaetoglobosin C	10.40	0.33	4.31	5.26	3.84	0.20	1.46	0.99	5.36	NA*	NA*	NA	NA*
Chaetoglobosin G/D/ U/V 1	8.75	1.23	1.02	NA*	1.30	NA*	NA*	NA*	1.60	NA*	NA	NA*	NA*
Chaetoglobosin G/D/U/V 2	9.20	2.55	NA*	NA	NA*	NA	NA	NA	1.15	NA	NA	NA*	NA
Chaetoglobosin E/F 1	8.40	NA*	4.58	1.60	4.46	NA*	0.96	0.78	3.90	NA	NA	NA	NA*
Chaetoglobosin E/F 2	9.45	NA*	NA*	NA*	NA*	NA	NA*	NA	NA*	NA	NA	NA	NA
Chaetoviridin C 1	10.70	0.79	1.77	0.75	1.59	0.29	0.74	0.39	1.56	0.18	0.40	0.43	0.55
Chaetoviridin C 2	11.05	0.64	1.44	0.47	1.77	0.19	0.76	0.36	1.26	0.10	0.20	0.29	0.54
Chaetoviridin A	11.45	2.75	NA	8.49	19.19	9.32	13.38	14.11	15.26	6.00	8.85	3.88	NA
Cochliodon 1	7.70	NA	NA	NA	NA	NA	NA	0.02	NA	0.09	0.07	0.46	NA
Cochliodon 2	9.50	0.09	NA	6.88	6.89	2.14	3.05	5.37	5.04	2.95	1.69	1.38	1.84
Cochliodon 3	11.40	0.87	NA	18.67	21.04	7.05	11.74	14.57	12.89	4.75	6.75	3.46	NA
Chaetoglobin A	4.20	NA	NA	6.99	3.19	NA	0.25	NA	0.02	NA	NA	NA	NA

C) *C. elatum* IBT 41944		Conc µg/cm^2											
		CHIPBOARD				PLYWOOD				GYPSUM			
METABOLITES	Rt (min)	wp type 1	adhesive	wp type 2	blank	wp type 1	adhesive	wp type 2	blank	wp type 1	adhesive	wp type 2	blank
Chaetoglobosin A	9.75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoglobosin C	10.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoglobosin G/D/ U/V 1	8.75	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoglobosin G/D/U/V 2	9.20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoglobosin E/F 1	8.40	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoglobosin E/F 2	9.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoviridin C 1	10.70	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoviridin C 2	11.05	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Chaetoviridin A	11.45	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Cochliodon 1	7.70	0.32	0.07	NA	0.03	0.07	0.04	NA	NA	0.05	NA	NA	0.19
Cochliodon 2	9.50	2.13	1.41	0.86	1.17	1.39	0.90	0.55	0.62	0.80	NA	NA	1.59
Cochliodon 3	11.40	4.81	3.72	2.65	2.62	3.50	2.99	1.88	2.03	1.68	NA	NA	2.85
Chaetoglobin A	4.20	1.26	2.97	2.17	2.54	NA	NA	NA	NA	NA	NA	NA	NA

NA: not analysed ; either metabolite was not detected at all

NA*: metabolite only detected (above LOD but below LOQ levels)

Table S3 Signal enhancement/suppression (%) in 5 different materials on two different levels: L_{low} = 0.1 µg/mL and L_{high} = 10 µg/mL

	SSE (%)			
	Chaetoglobosin A		Chaetoviridin A	
Materials	L_{low}	L_{high}	L_{low}	L_{high}
Chipboard	152	84	135	102
Plywood	92	87	94	98
Gypsum	102	99	104	101
Wallpaper type 1	89	98	99	94
Wallpaper type 2	98	97	104	94

Table S4 Retention time, ion species and mas to charge ratio (*m/z*) of all metabolites screened for in this study

Metabolite	Rt (min) [a]	Ion species[b]	*m/z* of ion species[b]
Chaetoglobosin A	9.8	**$[M+H]^+$** $[M+Na]^+$	**529.2697** 551.2517
Chaetoglobosin C	10.5	$[M+H]^+$ **$[M+Na]^+$**	529.2697 **551.2517**
Chaetoglobosin G/D/U/V	8.7, 9.2	$[M+H]^+$ **$[M+Na]^+$**	529.2697 **551.2517**
Chaetoglobosin E/F	8.4, 9.4	**$[M+H]^+$** $[M+Na]^+$	**531.2853** 553.2673
Prochaetoglobosin I	12.9	$[M+H]^+$ **$[M+Na]^+$**	483.3006 **505.2817**
Prochaetoglobosin II	12.6	$[M+H]^+$ **$[M+Na]^+$**	497.2799 **519.2610**
Prochaetoglobosin III	11.7	**$[M+H]^+$** $[M+Na]^+$	**513.2739** 535.2559
Prochaetoglobosin IV	10.9	$[M+H]^+$ **$[M+Na]^+$**	499.2955 **521.2760**
Chaetoviridin C/ Chaetomugilin D/S	10.7, 11.05	$[M+H]^+$ **$[M+Na]^+$**	435.1569 **457.1389**
Chaetoviridin A/ Chaetomugilin C	11.4	**$[M+H]^+$** $[M+Na]^+$	**433.1412** 455.1232
Chaetoviridin E	12.8	**$[M+H]^+$** $[M+Na]^+$	**415.1307** 437.1127
Chaetoviridin H	11.3	$[M+H]^+$ **$[M+Na]^+$**	399.1802 **421.1622**
Cochliodone 1	7.7	**$[M+H]^+$** $[M+Na]^+$	**639.2436** 661.2256
Cochliodone 2	9.5	**$[M+H]^+$** $[M+Na]^+$	**639.2436** 661.2256
Cochliodone 3	11.4	**$[M+H]^+$** $[M+Na]^+$	**639.2436** 661.2256
Chaetoglobin A	4.2	**$[M+H]^+$** $[M+Na]^+$	**637.2756** 659.2576
Cochliodinol	12.4	**$[M+H]^+$** $[M+Na]^+$	**507.2278** 539.2098
Longirosterone A	11.8	**$[M+H]^+$** $[M+Na]^+$	**537.2847** 559.2667
Longirosterone B	11.7	$[M+H]^+$ **$[M+Na]^+$**	511.3054 **533.2874**
Longirosterone C	13.2	$[M+H]^+$ **$[M+Na]^+$**	519.2741 **541.2561**
Sterigmatocystin	9.9	**$[M+H]^+$** $[M+Na]^+$	**325.0707** 347.0527
3-O-methyl sterigmatocystin	8.5	**$[M+H]^+$** $[M+Na]^+$	**339.0863** 361.0683
Dihydro- sterigmatocystin	10.3	**$[M+H]^+$** $[M+Na]^+$	**327.0863** 349.0683

[a]If there are two or more compounds with same elemental composition, retention times of all peaks with the corresponding *m/z* are listed

[b]Most abundant ion species and their m/z are presented in bold

Appendix S1

Method: external calibration and matrix effect

For external calibration, mixture of two available reference standards (chaetoglobosin A and chaetoviridin A) was prepared in triplicates at six different levels with relative concentrations 1:10:30:100:300:1000. Matrix effect was evaluated for four representative materials included in artificial inoculation experiment: non-treated plywood, non-treated chipboard, wallpaper type 1 and wallpaper type 2. For this purposes, 100 mL of autoclaved, double-distilled water was added to clean sterile building blocks. Materials were left to absorb water for 24 h, after what the excessive water was decanted and blocks were incubated for seven days. Each of four chosen materials was subsequently extracted as described in ‘’Sample preparation’’ in ratio 1:8 cm^2/mL. In order to extract specified surface, wallpapers were cut and entirely extracted while in case of chipboard and plywood, surface layer was cut from the desired area and extracted. Blank extracts were spiked with mixture of two standards in triplicates on two concentration levels: second lowest and highest, and analysed along with the blank extract as a control. This approach enabled direct determination of matrix effect as the result of signal enhancement/suppression (SSE), when results were compared with spiked solvent at the same concentration levels. For the same concentration levels, the coefficients of variation (CVs) were calculated by dividing standard deviation with average concentration multiplied by 100. Linear non-weighed calibration curves were calculated by plotting the peak area of analyte signal against the analyte concentration using Agilent MassHunter Quantitative Analysis for QTOF (version B.06.00). All quantitative data analysis were performed using the same software. Matrix effect due to the signal enhancement/suppression at the given concentration was

calculated as ratio of standard spiked matrix extract signal (peak area) and standard spiked solvent signal multiplied by 100. The CVs were calculated using Excel .

Quantification of secondary metabolites on artificially infested building materials

Table S2 represents calculated concentrations in area/cm^2 for all metabolites in different sections on all materials where growth was obtained. In the lack of reference standards for all identified secondary metabolites, the calculation for all chaetoglobosins was based on external calibration prepared for chaetoglobosin A while for all other identified metabolites calibration curve for chaetoviridin A was used.

Using calibration curve of chaetoglobosin A to calculate concentration of all identified chaetoglobosins was based on the same elemental composition for chaetoglobosin A, C, G, U and V therefore providing the same molecular ion which was used as quantifier, or in case of hydrogenated analogues, molecular ion with two *m/z* units more (chaetoglobosin E and F). Furthermore, all chaetoglobosins have very similar fragmentation patterns and UV spectra, due to the minor differences in their structures. Based on all those facts, the assumption of similar ionization properties for all chaetoglobosins was made. Since area under the peak in case of MS analysis is not directly proportional to the concentration and is highly dependent on the ionisation properties of the compound of interest, relative comparison of the peaks for these metabolites needed to be confirmed by UV.

Concentrations of metabolites other than chaetoglobosins were calculated based on calibration curve for chaetoviridin A, although these metabolites showed less similarity in elemental composition and chemical structure to chaetoviridin A than in case of chaetoglobosins. Assuming similar ionisation properties for chaetoviridin A and C seemed plausible, considering

the only structural difference between them being the reduced furanone in case of chaetoviridin C (Fig. 1). On the other hand, the only similarity between chaetoviridin A on one side and cochliodone and chaetoglobin A on the other was azaphilone moiety, making prediction of ionisation relative to each other almost impossible. Clearly, calculated concentrations, especially in cases where structural differences between metabolites were more prominent, are not exact. However, the purpose of the study was not to determine exact concentrations of each metabolite but to make rough estimation of their amounts on building materials, taking into consideration that these amounts are always much lower in comparison to chaetoglobosin A and chaetoviridin A. Therefore, this approach showed to fit its intended purpose and it was the closest we could come to explain true situation and the amounts of secondary metabolites produced on wet building materials.

Method performance and matrix effect

Due to the type of the samples, our method performance testing was limited and far from full analytical method validation. However, our method was utilized only to estimate levels (nanograms, micrograms) rather than accurate amounts of produced metabolites, therefore it was found to be suitable for its intended purpose.

Peak area to concentration response for chaetoglobosin A was linear across all levels with limit of detection being 0.09 µg/cm^2 and limit of quantification of 0.3 µg/cm^2. Chaetoviridin A did not exhibit linearity across entire calibration range, but good linearity was observed across first four levels, which were used for calibration curve with limit of detection being 0.008 µg/cm^2 and limit of quantification 0.02 µg/cm^2.

Table S3 gives an overview of observed signal suppression/enhancement (SSE %) on low and high concentration level, for three different materials and two wallpapers. The results show signal suppression within allowed limits (± 20 %), except in the case of low level spiked chipboard where signal enhancement outside of the allowed limits were noted for both metabolites. Signal enhancement for chaetoglobosin A on low level spiked chipboard can be explained by the fact that this level for metabolite in question lies below level of quantification, and very close to the level of detection. Therefore all obtained values for chaetoglobosin A on low level are questionable and obtained values on high levels should be used in matrix assessment. The least signal alteration caused by matrix was observed in gypsum. Generally, no material showed significant signal enhancement/suppression abilities; similar results for some of the materials (gypsum, wood) being also observed in previous studies (Vishwanath et al., 2009). It should also be taken into consideration that the effect of matrix in our experiment was on purpose largely overestimated. Although our samples probably contained small amounts of background due to the wetness of sampled material, they contained mostly scraped biomass as *Chaetomium* is known to provide substantial amounts of biomass on very wet materials (Nielsen, et al. 1999). Therefore, it can be said with certainty that our spiked matrix samples contained more matrix than any of our samples of *Chaetomium* grown on building materials. On the other hand, in the cases of real life fungal infected building materials , there are other factors that can contribute to the matrix effect, such as presence of co-extracted constituents of possibly present bacteria and other microorganisms, as well as dust, which was previously reported to alter signal heavily (Vishwanath et al., 2009). Hence, all of this needs to be taken into consideration when analysing real life samples from mouldy buildings.

5.4 Paper 4 - *Stachybotrys* secondary metabolites and mycotoxins on artificially and naturally contaminated building materials

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Manuscript in preparation

Stachybotrys mycotoxins and bioactive metabolites produced on artificially and naturally contaminated building materials

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Abstract

In progress

Introduction

The presence of fungi in indoor environment is inevitable, regardless of existing conditions in buildings. Fungal mycobiota in indoor environment under normal conditions does not seem to have an impact on occupants of the buildings. On contrary, fungal growth associated with damp indoor environment can lead to adverse health effects in sensitive individuals (WHO Regional Office for Europe, 2009) as well as in otherwise healthy individuals (Täubel et al., 2011; Miller and McMullin, 2014). It is suggested that several components produced during fungal growth in indoor environment could be either individually and/or combined responsible for adverse health effects, although causality has not been documented yet. The component of a fungal cell wall β-glucan as well as bioactive fungal metabolites are some of the suggested causal factors (Rand and Miller, 2011; Brewer et al., 2013). The literature reports inhalation as the most common and most toxic route of exposure inhalation (Craesia et al., 1987), whilst other routes of exposure include dermal absorption (Beko et al., 2013) and oral ingestion, especially in cases of small children.

The presence of *Stachybotrys* spp. in the indoor environment has been so far the most commonly associated to the occurrence of negative health effects. The first reported case of isolated *Stachybotrys* spp. from indoor environment dates back to 19th century (Miller et al., 2003). Since then, several cases reporting occurrence of negative health effects in occupants of the buildings contaminated with *Stachybotrys* spp. were reported (Johanning et al., 1993; Johanning et al.,1995; Johanning et al., 1996, Dearborn et al., 1997; Flappan et al., 1999). *S. chartarum* has also been possibly connected with occurrence of idiopathic pulmonary haemosiderosis in babies (Jarvis et al., 1998; Etzel, 2007). Literature also reports several cases of mycotoxicoses in animals and humans caused by *S. chartarum* contaminated hay (Forgacs and Carll, 1962; Jarvis et al., 1986).

S. chartarum and *S. chlorohalonata* are most frequently found species in the indoor environment (Andersen et al., 2011; Flannigan and Miller, 2011; Miller and McMullin, 2014). *S. chartarum* species exists in two chemotypes, S and A, that differentiate only by type of metabolites they produce (Andersen et al., 2002). Chemotype S produces macrocyclic trichothecenes (satratoxins and roridins) which are some of the most cytotoxic compounds currently known (Miller et al., 2003; Nielsen, PhD thesis, 2002). Chemotype A produces atranones and their precursors dolabellanes, as well as the simple non-macrocyclic trichothecene trichodermin (Andersen et al., 2002 and 2003). Atranones and dolabellanes are also produced by *S. chlorohalonata*, which also can be differentiated from *S. chartarum* morphologically. Both *S. chlorohalonata* and *S. chartarum* (regardless of chemotype) also produce many metabolites belonging to spirocyclic drimanes (KFN 2001). Spirocyclic drimanes are normally produced in much higher amounts than the trichothecenes (Hinkley and Jarvis, 2000; Andersen et al 2011, Nielsen 2003).

Currently, there are around 140 bioactive *Stachybotrys* metabolites reported (Antibase 2012; Hinkley and Jarvis, 2000; Nielsen, 2002; Li et al., 2013; Ma et al., 2013; Wu et al., 2014). Only few of those are commercially available as standards. Due to the lack of analytical reference standards, already existing analytical methods dedicated to *Stachybotrys* toxins are mostly focusing on detection/semi-quantification of some of the macrocyclic trichothecens (satratoxin H and G) (Nielsen et al., 2001; Bloom et al., 2007; Gottschalk et al., 2008) and stachybotrylactam (Vishwanath et al., 2009; Polizzi et al., 2009). In addition, literature reports immunochemical methods also focusing only on macrocyclic trichothecens (Brasel et al., 2005). Although a lot of focus has been drawn to presence of *Stachybotrys* mycotoxins in the indoor environment, the existing methods dedicated to detection of macrocyclic trichothecens are bound to fail in cases where contaminant is *S. chartarum* chemotype A, as existing methods do not even screen for mycotoxins produced by this chemotype.

The purpose of this study was therefore to identify and quantify as many *Stachybotrys* metabolites as possible using previously presented methods (Došen et al., 2016; Dust paper) for purposes of finding

relevant *Stachybotrys* compounds for future exposure/health risk studies and detection of biomarkers. The relevant metabolite profiles of three different *Stachybotrys* spp. were first obtained in pure cultures on agar media. Subsequently the same metabolites were screened for on artificially inoculated building materials as well as on building materials in damp and water-damaged buildings. Finally, the previously published method (Dust paper) was used to estimate concentrations of macrocyclic trichothecens and spirocyclic drimanes on building materials from damp indoor environments.

Materials and methods

Chemicals and standards

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

Collection of standards of secondary metabolites included in library included commercially available standards, gifts from other research groups or compounds 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.

Artificially inoculated building materials

Types of building materials used in this study were: 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). Each block was further divided in four equal oblong sections (13×4.5 cm) (Fig. 1) where 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). 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 *Stachybotrys* strains (see Table 1) prepared from 14-day-old V8 cultures were used for streak inoculation of the 5 materials. Boxes with sample blocks were weighed individually and streak-inoculated with two different *Stachybotrys* strain along the horizontal lines at the top and bottom of

each block across all segments (10 samples blocks in total) (Fig.1). Combination of two strains inoculated on the same sample block represented different species within same genus (*S. chartarum* IBT 9631/*S. chlorohalonata* IBT 40285, *S. chartarum* IBT 9466/*S. nephrospora* IBT 9458) with different metabolite profiles when applicable (satratoxin producer/atranone producer, 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 six weeks after inoculation.

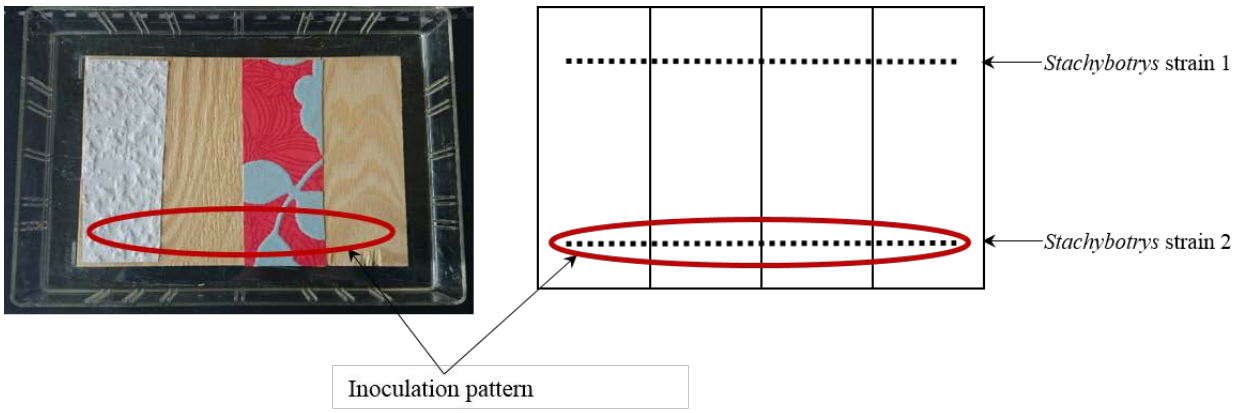

Fig. 1 Artificially inoculated building blocks with inoculation pattern and distribution of strains across sections. Sections from left to right: wallpaper adhesive + nonwoven woodchip wallpaper (WP1), wallpaper adhesive, wallpaper adhesive + nonwoven wall-covering with pattern (wp2), non-treated material

Naturally contaminated building materials

Samples, naturally contaminated with *Stachybotrys* spp., were collected from March 2013 to March 2015. Samples originated from both private dwellings (apartments, houses and basements) and public buildings (kindergarten) from different parts of Zealand, Denmark. Sampling was performed in buildings with water damage and/or fungal problems either reported by building inspectors or self-reported by occupants. Seven individual sampling cases from water-damaged buildings include

samples taken from several contaminated gypsum board, insulation material, cardboard, and chipboard. In order to verify *Stachybotrys* contamination on collected 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.

Fungal strains and cultivation

All four *Stachybotrys* strains used for artificial inoculation were from the IBT Culture Collection at the Department of Systems Biology, Technical University of Denmark (Table 1). These four strains were: *S. chartarum* (IBT 9631, chemotype S), *S. chartarum* (IBT 9466, chemotype A), *S. chlorohalonata* (IBT 40825) and *S. nephrospora* (IBT 9458). Furthermore, xx *Stachybotrys* strains isolated from indoor samples were inoculated on agar plates for metabolite profiling and included in the study. Two different agar media were used for metabolite profiling: Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) (Samson, et al. 2010). Once inoculated, all strains were incubated in darkness at 25 °C for 2 weeks. The identity of fungal strains and isolates was determined using Ames (1963), von Arx et al. (1986) and Wang et al. (2014). Four *Stachybotrys* strains used in artificial inoculation experiment were also inoculated on V8 juice agar (V8) for spore suspension preparation.

Metabolite extraction

Sampling from contaminated building materials (artificially and naturally) was done either by scraping off fungal biomass from an area of approximately 1 cm^2 or by cutting pieces (1 cm^2) of

infected material surface with a disposable scalpel, if scraping was not possible. Sampling from artificially inoculated building blocks was performed separately for each strain with sampling site for one strain being as far as possible (on the other side of the inoculation line) to the other strain inoculated on the same building block (Fig 1). 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. Extraction of pure cultures was done on the 15-days-old MEA and PDA cultures using a micro-scale extraction method modified for *Stachybotrys* 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. After completed sonication the extracts were 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.

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 (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) including 25 tentatively identified *Stachybotrys* compounds,, 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 library was extracted for secondary metabolites produced by *Stachybotrys* spp. and combined with tentatively identified compounds creating *Stachybotrys* library.

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 *Stachybotrys* compounds with known retention time selecting the most abundant ion.

Results

Fungal growth on building materials

Artificially inoculated building materials: First fungal growth was observed on gypsum board (drywall) two weeks after inoculation. In the next four weeks, growth was spread across all four sections, with the highest density on clean (non-treated) section and with no visual difference in growth between two strains inoculated on the same building block. Generally, the growth appeared as irregularly distributed black patches with sparse biomass and highest growth density around inoculation lines, regardless of section (Fig 2.). Growth on wooden based materials (plywood and chipboard) was first noted three weeks after inoculation. Neither plywood nor chipboard were fully overgrown by the time materials were sampled (six weeks). The biomass distribution was sporadic, appearing as dark green to black discolouring patches concentrated around inoculation line. Masonite supported very scarce growth for all used *Stachybotrys* strains, whilst MDF did not support any growth, which was further confirmed with microscopy. As also observed earlier with *Chaetomium* spp. (Dosen et al., 2016) comparing growth on wallpaper type 1 (nonwoven woodchip wallpaper, WP 1) and wallpaper type 2 (non-woven wall covering with a polystyrene pattern, WP2) revealed more prominent growth on WP1. Growth on WP2 was inhomogeneous, appearing only on the flat surfaces in between polystyrene pattern and never on pattern itself (Fig. 2, Dosen et al., 2016)

Naturally contaminated building materials: All collected samples were visibly heavily contaminated with *Stachybotrys* spp.. *Stachybotrys* on these materials (gypsum, chipboard, cardboard) appeared as black dust, largely covering materials/infected surface. In one of the cases, collected whole pieces of gypsum board were contaminated to that extent, that biomass completely overgrew the material's surface, appearing as a thick black layer sitting on top of the material (Fig. 3, Table 4. gypsum board 1).

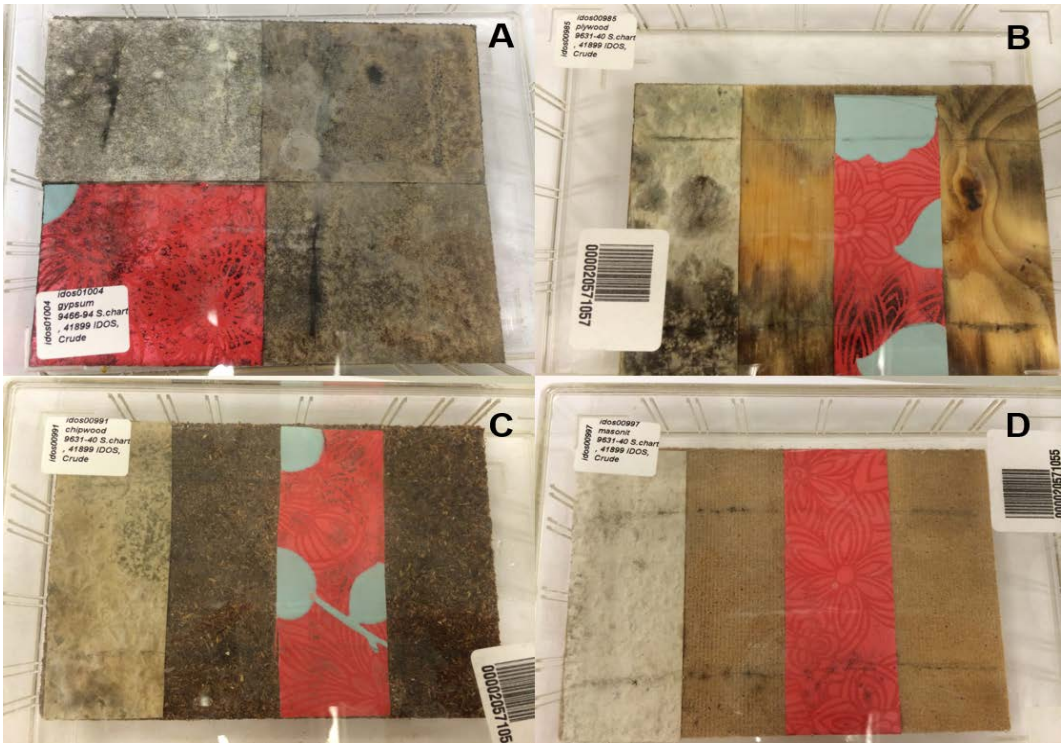

Fig. 2 *Stachybotrys* growth on artificially inoculated building materials: A – gypsum board/drywall, B – plywood, C – chipboard, D – masonite

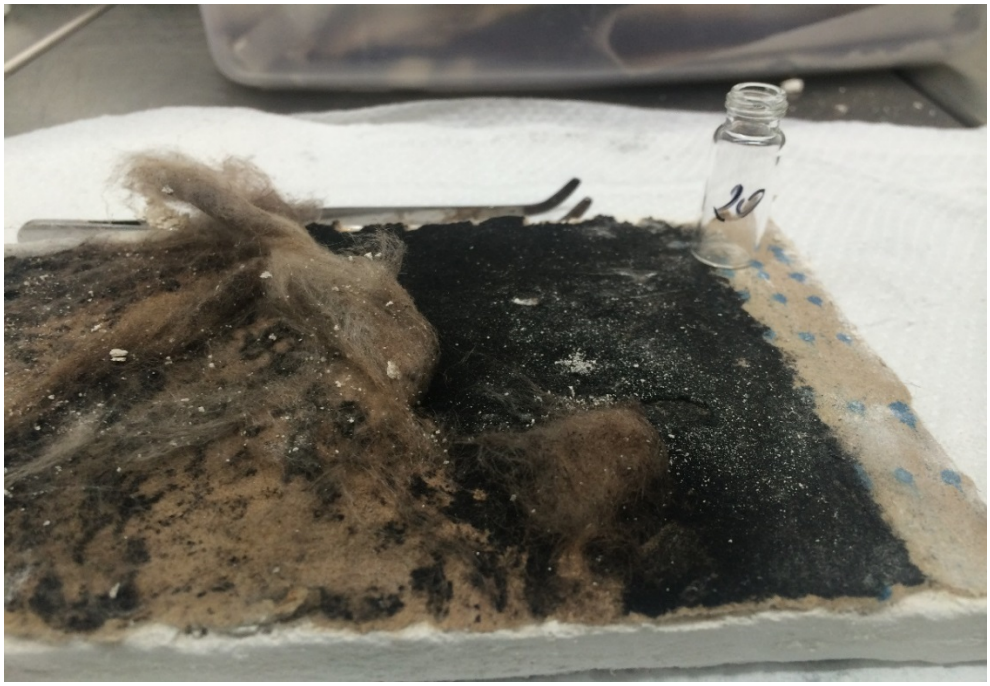

Fig. 3 Naturally contaminated gypsum board/drywall: thick layer of *Stachybotrys* biomass found on the material's surface

Metabolite production

Metabolite profiles from pure cultures of different *Stachybotrys* species, including strains used for artificial inoculations, are presented in Table 1. The metabolite production of all individual strains on artificially inoculated materials is presented in Table 2, whilst detected secondary metabolites from naturally infected building materials are presented in Table 3.

Metabolite production in pure agar cultures

Analysis of pure cultures extracts showed that all metabolites detected in this study belong to one of the following chemical groups of compounds: spirocyclic drimanes, macrocyclic trichothecens and atranones and their precursors dolabellanes.

Table 1 Metabolite production for different *Stachybotrys* species in pure culture (MEA and PDA).

Metabolite	*S. chartarum* chemotype S (2)	*S. chartarum* chemotype A (2)	*S. chlorohalonata* (2)	*S. nephrospora* (1)
Atranone A[c]	-	-	+	+
Atranone B[c]	-	-	+	-
Atranone G[b]	-	-	+	-
Dolabellane 1[c]	-	+	+	-
Dolabellane 2[c]	-	+	+	-
Roridine E[a]	+	-	-	-
epi/iso-Roridine E[b]	+	-	-	-
Roridin H[c]	+	-	-	-
Roridin L2[a]	+	-	-	-
Satratoxin H[c]	+	-	-	-
Isosatratoxin H[b]	-	-	-	-
Isosatratoxin F[c]	-	-	-	-
satratoxin G[c]	+	-	-	-
Chartarlactam C[b]	+	+	-	-
Chartarlactam D[b]	+	+	+	-
Chartarlactam K[b]	+	+	+	+
Chartarlactam M/O[b]	+	+	+	+
Stachybotrylactam[a]	+	+	+	+
Stachybotryamide[c]	+	+	+	+
Mer-NF-5003-B[a]	+	+	-	+
Stachybotrydial[b, d]	+	+	+	+
SDHBFL III/Chartarlactam B[b]	+	+	+	+
SDHBFL IV/K 76 compound 1[a]	+	+	+	+
K 76 compound 2[b, d]	+	+	+	+
L-611776[b, d]	+	+	+	+
F-1839-I[b, d]	+	+	+	+
F-1839-J[b, d]	+	+	+	+
Compound 2[b, d]	+	+	+	+

[a] identified as MS/HRMS library;
[b] identified by aggressive dereplication;
[c] identified by comparison to reference standard and/or by specific UV spectrum;
[d] MS spectrum fitting proposed fragmentation pattern

All analysed species and strains produced number of spirocyclic drimanes. Library matching yielded hits for stachybotrylactam, stachybotryamide (Ayer et al., 1993), Mer-NF-5003-B (Kaneto et al.,

1994) as well as stachybotrydial (Ayer et al., 1993). At least two peaks with *m/z* of 386.2326 and retention time difference of 0.7 min were constantly identified in extracts as stachybotrylactam. The peaks showed no difference in fragmentation pattern at 10 or 20 eV or in UV spectra (Dust paper). The identity of stachybotrylactam was confirmed by comparison with authentic standard. The other peak was identified as chartarlactam M/O (Li et al., 2013), based on the same elemental composition and structural similarities (Fig). Aggressive dereplication further identified chartarlactam C, D and K in analysed extracts as well as chartarlactam B (Li et al., 2013), a compound that due to the same elemental composition and high structural similarity could not be distinguished from spirodihydrobenzofuranlactam III (Roggo et al., 1996; Fig. 4). L-611776 (Roggo et al., 1996) and compound 2 (Jarvis, 2003), two compounds structurally related to stachybotrydial and Mer-NF-5003-B were also identified, and later confirmed by comparison to structurally related stachybotrydial and Mer-NF-5003-B (Fig. 4). F-1839-I and F-1839-J, structurally related to stachybotrylactam and stachybotryamide were identified and confirmed based on the same principle (Fig. 4).

Several macrocyclic trichothecens (MRT) were detected in pure culture extracts of two analysed *S. chartarum* chemotype S strains (IBT 9631 and 7709), whilst two precursors to atranones 3,4-epoxy-6-hydroxy-dolabella-7E,12-dien-14-one (dolabellane 1) and 6-hydroxydolabella-3E,7E,12-trien-14-one (dolabellane 2) were detected in pure culture extracts of two *S. chartarum* chemotype A strains (IBT 9466 and 7617). Three different atranones (A, B and G) as well as both dolabellanes were detected in pure culture extracts of two analysed *S. chlorohalonata* strains (IBT 40285 and 40294), whilst only atranone A was found in extracts of *S. nephrospora* (IBT 9458).

Metabolite production on artificially inoculated building materials

As follows from results in Table 2, the best metabolite production on the materials (expressed as number of metabolites found on material) was observed on gypsum board/drywall. Among wood-based materials, the highest number of metabolites was found on plywood, whilst chipboard yielded

lowest number of metabolites. On gypsum board/drywall the best production was observed on blank (no surface treatment) section which was not the case for wood based materials. Comparison of metabolite production in pure agar cultures with metabolite production on materials showed inconsistencies in atranone production for *S. chartarum* but not for *S. chlorohalonata*. Although *S. chartarum* chemotype A (IBT 9466) was not able to produce atranones in pure cultures, atranone A was found on all materials inoculated by the same strain. The opposite situation was observed for some of the spirocyclic drimanes. Namely both chemotypes (S and A) of *S. chartarum* (IBT 9631 and 9466) were able to produce chartarlactame C and D in pure agar cultures but not on any of inoculated building materials. Furthermore, Mer-NF-5003-B was produced by both *S. chartarum* (IBT 9631 and 9466) strains in pure cultures, but *S. chartarum* chemotype S (IBT 9631) was not able to produce it on building materials. On contrary Mer-NF-5003-B was found in gypsum extracts inoculated by *S. clorohalonata* (IBT 40285), although this compound was not produced by the same strain in pure agar cultures. Atranones and dolabellanes were detected in gypsum extracts of *S. chartarum* chemotype S (IBT 9631), whilst macrocyclic trichothecens were detected in extracts of *S. chlorohalonata* (atranone producer, IBT 40285), both in extracts from gypsum and plywood.

Table 2 Secondary metabolites found in artificially wetted building materials artificially contaminated with *S. chartarum*, *S. chlorohalonata* and *S. neprospora*

	S. chartarum IBT 9631															
	Gypsum				Chipboard				Masonite				Plywood			
Metabolite	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank
Atranone A	+*	+*	+*	+*	-	-	-	-	-	NA	NA	NA	-	-	-	-
Atranone B	+*	+*	+*	+*	-	-	-	-	-	NA	NA	NA	-	-	-	-
Atranone G	+*	+*	+*	+*	-	-	-	-	-	NA	NA	NA	-	-	-	-
Dolabellane 1	+*	+*	+*	+*	-	-	-	-	-	NA	NA	NA	-	-	-	-
Dolabellane 2	+*	+*	+*	+*	-	-	-	-	-	NA	NA	NA	-	-	-	-
Roridine E	+	+	+	+	+	-	+	-	+	NA	NA	NA	+	+	+	+
epi/iso-Roridine E	+	+	+	+	+	-	+	-	+	NA	NA	NA	+	+	+	+
Roridin H	+	+	+	+	+	-	+	-	+	NA	NA	NA	+	-	+	+
Roridin L2	-	-	-	+	+	-	-	-	+	NA	NA	NA	+	-	-	-
Satratoxin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	-
Isosatratoxin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	-
Isosatratoxin F	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	-
satratoxin G	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	-	+	+
Chartarlactam C	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	-
Chartarlactam D	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	-
Chartarlactam K	+	+	+	+	-	-	-	-	-	NA	NA	NA	+	-	+	-
Chartarlactam M/O	-	-	-	-	+	-	+	-	-	NA	NA	NA	-	-	-	-
Stachybotrylactam	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	+	+	+
Stachybotryamide	+	+	+	+	+	-	+	-	+	NA	NA	NA	+	+	+	+
Mer-NF-5003-B	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	-
Stachybotrydial	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	-	+	+
SDHBFL III/Chartarlactam B	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	-	+	-
SDHBFL IV/K 76 compound 1	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	+	+	+
K 76 compound 2	+	+	+	+	+	-	+	-	+	NA	NA	NA	+	+	+	+
L-611776	+	+	+	+	-	-	+	-	+	NA	NA	NA	+	+	+	+
F-1839-I	+	+	+	+	-	+	+	+	-	NA	NA	NA	+	-	-	+
F-1839-J	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	+	+	+
Compound 2	+	+	+	+	-	-	+	+	+	NA	NA	NA	+	+	+	+

	S. chlorohalonata IBT 40285															
	Gypsum				Chipboard				Masonite				Plywood			
Metabolite	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank
Atranone A	+	+	+	+	-	-	+	-	+	+	+	NA	+	+	+	+
Atranone B	+	+	+	+	+	-	+	-	+	+	+	NA	+	+	+	+
Atranone G	+	+	+	+	-	-	-	-	+	+	+	NA	+	+	+	+
Dolabellane 1	+	+	+	+	-	-	-	-	+	-	+	NA	+	+	+	+
Dolabellane 2	+	+	+	+	+	-	+	-	+	+	+	NA	+	+	+	+
Roridine E	-	-	-	+*	-	-	-	-	+	-	+	NA	-	-	-	-
epi/iso-Roridine E	-	-	-	+*	-	-	-	-	-	-	+	NA	-	-	-	-
Roridin H	-	-	-	+*	-	-	-	-	-	-	-	NA	-	-	-	-
Roridin L2	-	-	-	+*	-	-	-	-	-	-	-	NA	-	-	-	-
Satratoxin H	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-
Isosatratoxin H	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-
Isosatratoxin F	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-
satratoxin G	-	-	-	+*	-	-	-	-	-	-	+	NA	-	-	-	+*
Chartarlactam C	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-
Chartarlactam D	-	-	-	-	-	-	-	-	-	-	-	NA	-	-	-	-
Chartarlactam K	+	+	+	+	+	-	+	-	+	+	+	NA	+	+	+	+
Chartarlactam M/O	-	-	-	-	+	+	+	+	-	-	-	NA	-	-	-	-
Stachybotrylactam	+	+	+	+	+	+	+	+	+	+	+	NA	+	+	+	+
Stachybotryamide	+	+	+	+	+	+	+	+	+	+	+	NA	+	+	+	+
Mer-NF-5003-B	+	+	+	+	-	-	-	-	-	-	-	NA	-	-	-	-
Stachybotrydial	+	+	+	+	-	-	-	-	+	+	+	NA	+	+	+	+
SDHBFL III/Chartarlactam B	+	+	+	+	-	-	-	-	+	+	+	NA	+	+	+	-
SDHBFL IV/K 76 compound 1	+	+	+	+	+	-	-	-	+	+	+	NA	+	+	+	+
K 76 compound 2	-	+	+	+	+	-	+	-	+	+	+	NA	+	+	+	+
L-611776	+	+	+	+	+	-	-	-	+	+	+	NA	+	+	+	+
F-1839-I	+	+	+	+	+	-	+	-	-	-	-	NA	+	+	+	+
F-1839-J	+	+	+	+	+	-	+	-	+	+	+	NA	+	+	+	+
Compound 2	+	+	+	+	-	-	-	-	+	+	+	NA	+	+	+	+

	S. chartarum IBT 9466															
	Gypsum				Chipboard				Masonite				Plywood			
Metabolite	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank
Atranone A	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
Atranone B	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Atranone G	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Dolabellane 1	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
Dolabellane 2	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
Roridine E	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
epi/iso-Roridine E	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Roridin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Roridin L2	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Satratoxin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Isosatratoxin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Isosatratoxin F	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
satratoxin G	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Chartarlactam C	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Chartarlactam D	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
Chartarlactam K	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	NA	+	NA
Chartarlactam M/O	-	-	-	-	+	+	+	+	-	NA	NA	NA	-	NA	-	NA
Stachybotrylactam	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
Stachybotryamide	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
Mer-NF-5003-B	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
Stachybotrydial	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	NA	+	NA
SDHBFL III/Chartarlactam B	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
SDHBFL IV/K 76 compound 1	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
K 76 compound 2	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	NA	-	NA
L-611776	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
F-1839-I	-	+	+	+	+	+	+	+	-	NA	NA	NA	+	NA	+	NA
F-1839-J	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA
Compound 2	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	NA	+	NA

	S. nephrospora IBT 9458															
	Gypsum				Chipboard				Masonite				Plywood			
Metabolite	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank	wp 1	adhesive	wp 2	blank
Atranone A	+	+	+	+	-	+	+	+	+	NA	NA	NA	+	+	+	NA
Atranone B	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Atranone G	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Dolabellane 1	+	+	+	+	-	+	+	+	-	NA	NA	NA	-	-	-	NA
Dolabellane 2	+	+	+	+	-	+	+	+	+	NA	NA	NA	+	+	+	NA
Roridine E	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
epi/iso-Roridine E	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Roridin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Roridin L2	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Satratoxin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Isosatratoxin H	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Isosatratoxin F	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
satratoxin G	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Chartarlactam C	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Chartarlactam D	-	-	-	-	-	-	-	-	-	NA	NA	NA	-	-	-	NA
Chartarlactam K	+	+	+	+	-	+	+	+	+	NA	NA	NA	+	+	+	NA
Chartarlactam M/O	-	-	-	-	+	+	+	+	-	NA	NA	NA	-	-	-	NA
Stachybotrylactam	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	+	+	NA
Stachybotryamide	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	+	+	NA
Mer-NF-5003-B	+	+	+	+	+	-	+	-	-	NA	NA	NA	+	-	-	NA
Stachybotrydial	+	+	+	+	-	-	-	-	+	NA	NA	NA	+	+	+	NA
SDHBFL III/Chartarlactam B	+	+	+	+	-	-	+	+	-	NA	NA	NA	+	+	+	NA
SDHBFL IV/K 76 compound 1	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	+	+	NA
K 76 compound 2	+	+	+	+	-	+	+	+	+	NA	NA	NA	+	+	+	NA
L-611776	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	+	+	NA
F-1839-I	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	-	-	NA
F-1839-J	+	+	+	+	+	+	+	+	+	NA	NA	NA	+	+	+	NA
Compound 2	+	+	+	+	+	+	+	+	+	NA	NA	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.
NA: not analysed, insufficient growth observed in these sections

Metabolite production on naturally infected building materials

In four out of seven cases, both atranones and macrocyclic trichothecens were present in the samples. In samples of two out of three remaining cases, an atranone producer was detected, whilst no known metabolites were detected in third case samples (photoalbum cardboard). All spirocyclic drimanes, detected on artificially inoculated building materials, were also found in six out of seven cases of naturally contaminated materials. In one case (gypsum board 1) samples also contained chartarlactam C, which was not found in any extract from artificially inoculated materials. No chartarlactam D was found on any naturally contaminated materials.

Table 3 Secondary metabolites found in water-damaged building materials naturally contaminated with *Stachybotrys* spp.

Metabolite	Gypsum board 1(8)	Gypsum board 2 (20)	Photoalbum cardboard (2)	Chipboard (3)	Gypsum board 3 (5)	Cardboard /insulation (7)	Gypsum 4 (3)
Atranone A	+	+	-	+	+	+	+
Atranone B	+	+	-	+	+	+	+
Atranone G	-	-	-	-	-	-	-
Dolabellane 1	+	+	-	+	+	+	+
Dolabellane 2	+	+	-	+	+	+	+
Roridine E	+	+	-	-	+	-	+
epi/iso-Roridine E	+	+	-	-	+	-	+
Roridin H	+	+	-	-	+	-	-
Roridin L2	+	-	-	-	+	-	-
Satratoxin H	-	-	-	-	+	-	-
Isosatratoxin H	-	-	-	-	-	-	-
Isosatratoxin F	-	-	-	-	-	-	-
satratoxin G	-	-	-	-	+	-	-
Chartarlactam C	-	+	-	-	-	-	-
Chartarlactam D	-	-	-	-	-	-	-
Chartarlactam K	+	+	-	+	+	+	+
Chartarlactam M/O	+	+	-	+	+	+	+
Stachybotrylactam	+	+	-	+	+	+	+
Stachybotryamide	+	+	-	+	+	+	+
Mer-NF-5003-B	+	+	-	+	+	+	+
Stachybotrydial	+	+	-	+	+	+	+
SDHBFL III/Chartarlactam B	+	+	-	+	+	+	+
SDHBFL IV/K 76 compound 1	+	+	-	+	+	+	+
K 76 compound 2	+	+	-	+	+	+	+
L-611776	+	+	-	+	+	+	+
F-1839-I	+	+	-	+	+	+	+
F-1839-J	+	+	-	+	+	+	+
Compound 2	+	+	-	+	+	+	+

Quantities of secondary metabolites on water-damaged building materials

In progress

Discussion

Growth

On both artificially and naturally infected materials gypsum board/drywall proved to be the best material to support *Stachybotrys* growth, which is in accordance with previously reported results (Andersen et al., 2011). Results from artificial inoculation experiment showed generally less preference of *Stachybotrys* spp. towards wood-based materials, especially towards chipboard and masonite, despite the high water absorption ability of the materials (20 % and 17 % *w/w* respectively, ref *Chaetomium* paper). Lack of any *Stachybotrys* growth on MDF could be explained, as in case of *Chaetomium* spp. (*Chaetomium* paper), by material's poor water absorption ability (5 % *w/w*). The type of material, collected in this study from residences naturally contaminated by *Stachybotrys* spp. confirmed preference of this fungal genus toward gypsum and insulation materials (Table 4.) as in only one out of seven cases, sampled material was wood-based (chipboard, Table 4). The less prominent *Stachybotrys* growth on WP2 in comparison to WP1, especially on the polystyrene pattern, could be explain by lack of nutrients (non-organic material) which creates generally unfavourable growth conditions.

Artificially inoculated building materials

Generally, metabolite production was in accordance with visual observation of the *Stachybotrys* growth on different materials. The higher number of metabolites on gypsum in comparison to other

materials used in this experiment, for all used strains, suggests general preference of *Stachybotrys* spp. towards gypsum. Poor metabolite production on wood-based materials could be expected, considering the occurrence of *Stachybotrys* spp. on the same type of materials in real life water-damaged environments. Most metabolites produced in pure agar cultures were found on at least one of the inoculated materials, suggesting used *Stachybotrys* spp. being well adapted to wet indoor environment.

Interestingly, the presence of atranones and their precursors dolabellanes was detected in gypsum extracts of chemotype S *S. chartarum* strain (IBT 9631). Furthermore, the same was observed in gypsum and plywood extracts of *S. chlorohalonata* (IBT 40285) where, besides atranones and dolabellanes, several MRTs were also detected. Earlier published results, where more than 200 isolates of *Stachybotrys* spp. were analysed for metabolite production (Andersen et al., 2002), showed that no tested isolate was able to produce both groups of metabolites. Considering the fact that both strains (IBT 9631 and 40285) were inoculated on the same material block, the only possible explanation of obtained results is cross-contamination of these two strains. These results showed no predomination or suppression of one strain towards the other and suggested scattered growth direction with no clear borderlines between areas of their contamination, as seen previously (ref dust paper). The interpretations of results was not as clear when two atranone producers were combined on the same building blocks (IBT 9466 and IBT 9458). *S. nephrospora* (IBT 9458) was able to produce atranone A in both pure cultures and on building blocks, whilst the same compound was found only in building material extracts for *S. chartarum* (IBT 9466). The question arising here is whether production of atranone A was boosted for *S. chartarum* on building materials in comparison to pure cultures or its presence is purely sign of cross-contamination with *S. nephrospora.*

Constant production of spirocyclic drimanes in all strains and species, in both pure cultures and on building blocks suggested this group as a good candidate for *Stachybotrys* indoor biomarkers. Besides

spirocyclic drimanes previously reported in indoor environment (stachybotrylactam, stachybotryamide, stachybotrydial) several other bioactive drimanes, known to be produced by soil derived *Stachybotrys* strains (ref) were present too. One of them is L-611776, a known inositol mono-phosphatase inhibitor, first isolated and described by Lam et al. (1996). Other two metabolites, F-1839-I and F-1839-J, known for its pancreatic cholesterol esterase inhibition activity, were first isolated also from soil derived *Stachybotrys sp.* F-1839 by Sakai et al. (1995). Not only that these compounds were constantly produced by indoor *Stachybotry*s spp, but they were also most abundant peaks found in analysed extracts (Fig). Probably the lack of reference standards and limitations of analytical techniques used, prevented their earlier detection on building materials. Nielsen (ref PhD thesis) reported several unknown spirocyclic drimanes based on observed UV spectra. The use of

Naturally contaminated building materials

In progress

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5.5 Paper 5 - *Stachybotrys* mycotoxins: from culture extracts to dust samples, *Anal Bioanal Chem*

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Stachybotrys mycotoxins: from culture extracts to dust samples

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Keywords: *Stachybotrys*, mycotoxin, dust, spirocyclic drimane, QTOF, QqQ

Abstract

The filamentous fungus *Stachybotrys chartarum* is known for its toxic metabolites and has been associated to serious health problems, including mycotoxicosis among occupants of contaminated buildings. Here we present results from a case-study, where an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was developed for known and tentatively identified compounds characterized via UHPLC-quadruple time of flight (QTOF) screening of fungal culture extracts, wall scrapings, and reference standards. The UHPLC-MS/MS method was able to identify twelve *Stachybotrys* metabolites, of which four could be quantified based on authentic standards, and a further six estimated based on similarity to authentic standards. Samples collected from walls contaminated by *S. chartarum* in a water damaged building showed that the two known chemotypes, S and A, coexisted. More importantly, a link between mycotoxin concentrations found on contaminated surfaces and in settled dust was made. One dust sample, collected from a water damaged room, contained 10 pg/cm^2 macrocyclic trichothecenes (roridin E). For the first time, more than one spirocyclic drimane was detected in dust. Spirocyclic drimanes were detected in all 11 analyzed dust samples and in total amounted to 600 pg/cm^2 in the water damaged room and 340 pg/cm^2 in rooms adjacent to the water damaged area. Their wide distribution in detectable amounts in dust suggested they could be good candidates for exposure biomarkers.

Introduction

Fungal spores are ubiquitous in indoor environments, and the growth of mold in buildings can often lead to negative health effects such as skin rashes, headaches, dizziness and chronic fatigue of the occupants [1-3]. Although the presence of molds in the indoor environment is generally undesirable, some species, such as *Stachybotrys chartarum* and *Chaetomium globosum*, are considered more problematic [3]. *S. chartarum* is known for its production of toxic metabolites that have been connected to extensive occurrence of negative health effects [1, 4], and possible also linked to idiopathic pulmonary haemosiderosis in babies [5-6]. *Stachybotrys* has historically been shown to be responsible for severe toxicoses in farm animals fed with contaminated hay [7-8].

S. chartarum can be found in two chemotypes, S and A, both sharing the same morphology, but differing in some of the metabolites they produce [9]. Chemotype S produces macrocyclic trichothecenes (satratoxins, verrucarins, and roridins, Fig. 1), which are some of the most cytotoxic compounds currently known [10]. Chemotype A produces atranones and their precursors, dolabellanes together with the simple non-macrocyclic trichothecene, trichodermin [9]. Both chemotypes also produce many metabolites belonging to the spirocyclic drimane family [11], in much greater quantity than the trichothecenes and antranones [12-14]. Chemotype A induces highly inflammatory effects both *in vivo* and *in vitro* [10, 14-15], but the causative agents for this have not been revealed yet. It is possible that observed inflammatory effects are caused by the drimanes, atranones or other compounds. These effects may be concealed in the chemotype S by the highly toxic effects of macrocyclic trichotehcenes [14]. Furthermore, *S. chlorohalonata* is also found in buildings, but chemically it can currently not be differentiated from *S. chartarum* chemotype A [9].

S. chartarum requires high water activity (0.98) for optimal growth [16], which in practice for the indoor environment means water ingress rather than high levels of condensation. *S. chartarum* is mainly observed on materials with high cellulose content, such as gypsum wallboard, straw, wallpapers and insulation materials [13, 17]. Spores of *S. chartarum* are produced in sticky slime heads [18] and do not easily become airborne when they are moist, making detection in the air difficult. Hence, exposure mainly occurs from dry, disintegrating spores and mycelium as micro-particles [19-21].

Based on the database Antibase 2012, as well as other sources [10, 12, 22-24], there are currently around 140 compounds described from *Stachybotrys* spp. Only one of these compounds, stachybotrylactam, is commercially available whilst satratoxins H and G have also been partially available. A few authors have developed trace analytical methods including these three compounds, based on liquid chromatography-tandem mass spectrometric (LC-MS/MS) detection [25-27]. The macrocyclic trichothecenes can also be analysed by immunochemical methods [28-29] or via

hydrolysis, derivatization and GC-MS [11, 30]. Although a large amount of attention has been placed on the presence of *Stachybotrys* mycotoxins in the indoor environment, attempts to identify these toxics in the dust usually fail, as do attempts to correlate amounts found on contaminated surfaces with amounts found in dust. This failure is especially true in cases where the contaminant is *S. chartarum* chemotype A or *S. chlorohalonata*, as none of existing methods targets atranones or their precursors, the dolabellanes. Furthermore, all the major spirocyclic drimanes, except for stachybotrylactam, are not covered by the existing targeted LC-MS/MS methods.

Earlier analytical methods for *Stachybotrys* metabolites, mainly based on LC separation with UV/Vis diode array detection (DAD) [12, 9, 31], clearly showed that drimanes were the dominant compounds in the analysed extracts. In order to separate drimanes from atranones or macrocyclic trichothecens, samples were purified by normal phase solid phase extraction on PEI silica [12]. The introduction of LC in combination with high resolution mass spectrometry (HRMS) has allowed both Time of Flight (TOF) and Orbitrap instruments to be used routinely for fungal metabolite profiling [32-33]. One of the advantages of LC-HRMS over UV/Vis is that it is easier to distinguish co-eluting compounds using extracted ion chromatograms [34]. Despite this advantage, many fungal metabolites possess the same elemental composition and even more specific/accurate identification than HRMS is required to distinguish similar compounds. This greater specificity of identification can be obtained from MS/HRMS either by using standardized MS/HRMS libraries with three fixed fragmentation energies (e.g. 10, 20 and 40 eV as introduced by Agilent Technologies) [35-36], or more energies, as on ThermoFisher instruments [37].

Our recent work [36] presented the use of MS/HRMS libraries in fungal drug discovery, and we are currently expanding our existing library with *Stachybotrys* metabolites. Agilent Technologies state that fragmentation energies are standardized across their instruments, including triple quadrupole instruments (QqQ). Consequently, we hypothesize that multiple reaction monitoring (MRM) could be predicted directly from the MS/HRMS library, not only including fragment ions but also the collision energies. In culture extracts analyzed by UHPLC-DAD-HRMS, we observed that most of the spirocyclic drimanes ionized very well compared to their apparent intensity in the UV/Vis chromatogram. Considering that QqQ instruments are typically 5-50 fold more sensitive than HRMS instruments, we hypothesized that by mapping all the major spirocyclic drimanes from *Stachybotrys* cultures and infected material samples, and transferring method to QqQ instrument, we would stand a greater chance of detecting these in settled dust in *Stachybotrys* infected buildings than other metabolites such as the trichothecenes.

In this case-study we tested these hypotheses by identifying promising *S. chartarum* biomarkers using UHPLC-QTOF in extracts from pure fungal cultures and cotton tip swabs from infected gypsum wallboards, and further transferring

98 detection of these directly to a UHPLC-QqQ method. This method was combined with standards of several macrocyclic
99 trichothecens, several atranones, one commercially available spirocyclic drimane and the simple trichothecene
100 trichodermin.

Materials and Methods

Chemicals and standards

All solvents, including water for LC-MS analyses, were LC-MS grade; chemicals were analytical grade and were purchased from Sigma-Aldrich (Steinheim, Germany), if not otherwise stated. Standard of stachybotrylactam was purchased from Enzo (Exeter, UK). All other standards were available in-house, donated from other research groups or purified from different in-house projects [36]. Standards for quantification purposes were weighed and dissolved either in pure acetonitrile (trichodermin), acetonitrile/water (90:10, *v/v*) (roridin E) or pure methanol (stachybotrylactam, satratoxin H). Individual stock solutions were kept on -20 °C. Prior to analysis, stock solutions were brought to room temperature, thoroughly mixed and used to prepare multi-analyte standard solutions in triplicates, at five (satratoxin H, roridin E) or six (stachybotrylactame, trichodermin) different levels with concentrations of 10, 100, 300, 1000, 3000, and 10000 ng/mL.

Sampling of fungal biomass and settled dust

All samples in this case study were collected from a water-damaged kindergarten in the Greater Copenhagen area. A broken concealed water pipe in a ground floor bathroom had resulted in fungal contamination in the internal structures of the wall (> 80 % of the surface covered with fungal growth). The whole ground floor was closed off due to the high number of children (> 50 %) reporting sickness.

The wall was opened and fungal biomass samples were collected from the gypsum wallboards in the bathroom. Each sample was taken from an approximate surface area of 1 cm^2 using a sterile cotton tip in screw cap plastic tube (Q-tips) or by scraping off small pieces of infected surface materials. The collected samples were kept in tubes in a ventilated room at 10° C until analysis.

Settled dust samples were collected from all available surfaces (shelves, tables, fridge, tops of the hanging lamps) and other places (excluding the floor) that were regularly cleaned. Each sample was taken from an approximate surface area of 45 × 45 cm using a clean precision Kimwipes® Lite wipe (Kimberly-Clark, Georgia, US). Dust was collected both in the bathroom where the water damage occurred and in neighbouring rooms. The collected dust together with wipes were placed in 50 mL falcon tubes (VWR, Philadelphia, US), closed and kept in a ventilated room at 10° C until analysis.

Tape preparations for phase contrast microscopy (200× and 400×) 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 [18]. Scrapings were used for fungal identification by metabolite profiling [9] where obtained metabolite profiles from scraped contaminated material were compared to metabolite profiles of in-house indoor *Stachybotrys* strains. Four *S. chartarum* (two chemotype S and two chemotype A) and two *S. chlorohalonata* strains were used for metabolite profiling (Table 1). Agar media used for pure strain inoculation were Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA) [18]. After inoculation, strains were incubated in darkness at 25 °C for 1-2 weeks, after what sampling for metabolite profiling were performed (PDA and MEA extracts).

Table 1 *Stachybotrys* species and strains used in the study for metabolite profiling and comparison

Genus	Species	IBT no.	Chemotype	Origin
Stachybotrys	*chartarum*	7709	macrocyclic trichothecene producer (S)	Building material, DK
Stachybotrys	*chartarum*	9631	macrocyclic trichothecene producer (S)	Home, USA
Stachybotrys	*chartarum*	7617	atranone producer (A)	DK
Stachybotrys	*chartarum*	9466	atranone producer (A)	Gypsum, DK
Stachybotrys	*chlorohalonata*	40285	atranone producer (A)	USA
Stachybotrys	*chlorohalonata*	40295	atranone producer (A)	USA

IBT culture collection, author's address;

Extraction of pure cultures and biomass

Extraction of pure agar cultures was performed using a micro-scale extraction method modified for *Stachybotrys* metabolites [38]. Three agar plugs (6 mm ID) were cut from a 15 day old colony from each agar medium (PDA or MEA) and placed in a 2 ml screw top vial. 1.0 ml of extraction solvent (ethyl acetate/dichloromethane/methanol (3:2:1, *v/v/v*) 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 below for other type of samples.

For fungal biomass collected with Q-tips from the bathroom, the cotton tip of a swab was carefully cut using disposable scalpel and transferred to 15-mL falcon tube. For the settled dust collected on wipes, the wipes were transferred to 50-mL falcon tube.

To each Falcon tube was added either 15 mL (dust samples) or 2 mL (biomass samples) extraction solvent (acetonitrile:water (75:25 *v/v*) containing 1% formic acid). Tubes were placed in an ultrasonication bath for 60 min and then centrifuged at 4000g for 2 min after which the liquid was transferred to a clean tube and evaporated to dryness under a gentle stream of N_2. Thereafter samples were re-dissolved in 400 µL solvent (acetonitrile: MilliQ water (75:25 *v/v*) with 1% formic acid) and centrifuged (15 min, 15000 g). The supernatant was used directly for chemical analysis.

UHPLC-DAD-QTOF analysis

Metabolite profiling of extracts of pure cultures, biomass and dust samples collected in the water damaged kindergarten was performed using ultra-high performance liquid chromatography-diode array detection-quadrupole time of flight mass spectrometry (UHPLC-DAD-QTOFMS) consisting of an Agilent Infinity 1290 UHPLC system (Agilent Technologies, Santa Clara, California, USA) coupled to an Agilent 6545 QTOF MS equipped with Dual Jet Stream electro spray ion source. The system was equipped with a diode array detector scanning in range 200-640 nm 20 times/s [36]. Separation was performed on an Agilent Poroshell 120 Phenyl-Hexyl column (2.1 × 150 mm, 2.7 µm) at 60 °C at a flow of 0.35 mL/min. A linear solvent gradient, consisting of A: 20 mmol/L formic acid in water and B: 20 mmol/L formic acid in acetonitrile was used. Total analysis time of 20 min, graduating from 10% to 100% B within 15 min; held for 2 min, followed by returning to 10% in 0.1 min and remaining for 3 min [36]. Lock mass solution in 80/20 methanol/water (*v/v*) was infused in the second sprayer using an extra LC pump at a flow of 1.5 mL/min which was subsequently split 1:100, delivering 15 µL to the MS of 10 µM Hexakis(2,2,3,3-tetrafluoropropoxy)phosphazene (Apollo Scientific Ltd, Cheshire, UK) as lock mass. Other MS parameters, including information on automated data-dependant MS/HRMS (Auto-MS/HRMS) can be found in [36]. Samples were largely analysed in ESI^+ mode.

Identification of secondary metabolites was performed using a combination of the following approaches: i) direct search and matching of MS/HRMS data in MS/HRMS library and ii) 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. MS/HRMS library containing ~1500 compounds [36] included spectra from 25 reference standards originating from *Stachybotrys*, further five tentatively identified *Stachybotrys* compounds, and finally one tentatively identified in this study (stachybotrydial). A 300 compound version of this library containing all *Stachybotrys* compounds is available at our www site [39].

Aggressive dereplication of the full HRMS data was created by extracting Antibase2012 database for all compounds having *Stachybotrys* spp. as a source as well as recent references (~140 compounds) [10, 12, 22-24]. 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 [36].

For fast screening of larger number of samples MasHunter Quantitative Analysis for QTOF (version B.06.00) was used [40]. The method for screening included all *Stachybotrys* compounds with known retention time, selecting the most abundant ion.

Transfering the QTOF method to QqQ

Analysis of selected *Stachybotrys* metabolites was performed on Agilent Infinity 1290 UHPLC system coupled to an Agilent 6490 Triple Quadrupole (QqQ) mass spectrometer. Chromatographic separation was performed on an Agilent Poroshell 120 Phenyl-Hexyl column (2.1 × 100 mm, 2.7 µm) held at a temperature of 40 °C and at a flow of 0.4 mL/min. Both eluents contained 20 mM formic acid in water (eluent A) and in acetonitrile/2-propanol (80/20, *v/v*) (eluent B). Additionally 5 mM ammonium formate was added to eluent A. Total analysis time of 10 min, graduating from 20% to 80% B within 7 min, increasing to 100% B in 0.1 min, held for 1.1 min, returning to 20% B in 0.1 min and maintaining until the end of the run (10 min). To avoid carry-over, the auto sampler was operated in the flow-through-needle mode and further coupled to an Agilent Flex cube which was used to perform series of flushing switching between main-pass and bypass prior and after the injection at the flow of 4 mL/min. Precursor, product ion, fragmentor voltage and collision energy selection were based on the existing data previously obtained on the QTOF and entered in MassHunter Personal Compound Database and Library (PCDL) Manager. This library contained information on fragmentation using 10, 20 and 40 eV for each compound. After initial selection of precursor and product ion for each transition, the values for collision energies were further optimized around selected value in order to achieve maximum sensitivity.

Fragmentor voltage was set to 380 V for all compounds/transitions. MassHunter Data Acquisition software version B06.01 was used to control the instrument. All analyses were performed only in ESI^+ mode using multiple reaction monitoring (MRM) acquisition. For each compound two or three mass transitions were monitored with the dwell time set to 50 and cycle time of 1605 ms. General source settings were: gas temperature of 180°C; gas flow of 12 L/min; sheath gas temperature of 350°C; sheath gas flow of 12 L/min; nebulizer 20 psi (137.9 kPa); capillary voltage of 3500 V and nozzle voltage of 0 V in positive mode.

Screening method for metabolites produced by indoor species other than *Stachybotrys* spp. was based on work of Varga et al. [41]. Two mass transitions per compounds, for nine compounds in total were adopted and further adjusted for our instrument (supplementary information, Table S1).

QqQ Method validation

For evaluation of method performance, external calibration of four quantitatively available standards (satratoxin H, roridin E, stachybotrylactam and trichodermin) was performed as described in "Chemicals and standards". Linear non-weighed (satratoxin H, roridin E, stachybotrylactam) or 1/x weighed (trichodermin) calibration curves were calculated by plotting the peak area of analyte signal against the analyte concentration using Agilent MassHunter Quantitative Analysis for QqQ (version B.06.00). Limits of detection (LODs) and lowest limits of quantification (LLOQs) were calculated at the lowest concentration levels of liquid standards as concentrations corresponding to a signal-to-noise ratio (S/N) 3/1 for LOD and 10/1 for LLOQ. S/N was calculated using "Calculate S/N ratio" function in MassHunter Qualitative Analysis. The matrix effect was evaluated for dust samples, by extracting clean, unused Kimwipes® Lite wipes as described in "Extraction of samples". Blank extracts (solvents only) were spiked with mixture of four standards in triplicates on five (satratoxin H, roridin E) or six (stachybotrylactam, trichodermin) concentration levels and analysed together with the blank extract as a control. This approach enabled direct determination of matrix effect as the result of signal enhancement/suppression (SSE), by calculating slope ratios of the linear calibration functions SSE (%) = $[\text{slope}_{\text{matrix-matched standards}} / \text{slope}_{\text{liquid standards}}] \times 100$.

Results and discussion

Chemical analysis by UHPLC-QTOF

Chemical analysis of extracts of fungal biomass (supplementary information, Table S2) collected from the kindergarten's gypsum wallboard showed the presence of both atranones and macrocyclic trichothecens. Further comparison of these extracts to metabolite profiles from *Stachybotrys* pure agar cultures confirmed the presence of both chemotypes. Whilst these results clearly suggested *S. chartarum* chemotype S as macrocyclic trichothecene producing contaminant, identification of atranone producing contaminant was more difficult, as chemical analysis could not distinguish between *S. chartarum* chemotype A and *S. chlorohalonata.* Species level identification of atranone producing contaminant would require isolation of the strain and classical morphological methods [18, 9], which was not performed in this study due to the time limitations.

Analysis of gypsum wallboard extracts primarily revealed the presence of spirocyclic drimanes in all analysed samples. Stachybotrydial and its reduced analogue Mer-NF-5003-B (Fig.1) were predominant in all analyzed extracts. An example of stachybotrydial dereplication is presented in Fig. 2. Observed accurate mass (*m/z* 387.2170, 1 ppm deviation) of peak eluting at 10.1 min perfectly corresponded to theoretical *m/z* for stachybotrydial molecular ion (387.2166), whose identity was further confirmed with the known UV spectrum for this compound [31, 42]. Furthermore, full scan spectrum showed presence of both sodium adduct $[M+Na]^+$ and dehydrated fragment $[M–H_2O+H]^+$, which validated identification of the molecular ion. Finally, the presence of all proposed fragments (supplementary data, Fig S1) in MS/HRMS 20 eV spectrum served as a final confirmation of stachybotrydial's identity. After confirming its identity, stachybotrydial was added to the library.

Two peaks with *m/z* of 386.2326 (4 ppm deviation) and a retention time (RT) difference of 0.7 min were identified as stachybotrylactam in all extracts. No difference in fragmentation pattern at 10 and 20 eV was found between two compounds. Comparison with the stachybotrylactam standard identified the peak eluting at 8.35 min as the known compound, whilst a later eluting compound will henceforth be referred to as stachybotrylactam isomer. Library matching also identified a peak eluting at 8.1 min (*m/z* 405.227, 2.2 ppm deviation) as Mer-NF-5003-B, as well as a peak eluting at 8.3 min (*m/z* 430.2588, 1.6 ppm) as stachybotryamide (Fig. 3). Both matches were further confirmed using UV spectra from authentic standards (supplementary information, Fig S2).

In four out of seven wall scrapings (supplementary information, Table S2) from the water damaged bathroom, aggressive dereplication suggested the presence of atranone A and B, as well as (1S*,6S*,11S*)-6-hydroxydolabella-3E,7E,12-trien-14-one (Fig 3), which was further confirmed by authentic standards and UV/Vis data [14] (supplementary information, Fig. S3). Furthermore, analysis of wall board scrapings from the kindergarten, not only showed the presence of spirocyclic drimanes and atranones, but also yielded positive hit for satratoxin H, showing intense $[M+NH_4]^+$ and $[M+Na]^+$ as well as the MS/HRMS spectrum perfectly matching an authentic standard. Searching MS/HRMS data for *m/z* 231.1300 ± 0.0100, a characteristic fragment of trichothecens [14], not only confirmed the presence of satratoxin H, but also the presence of other trichothecens (roridin L2, satratoxin G, Roridin E, Fig S4, supplementary information), which were also verified by comparison to authentic standards. The compound with RT of 10.45 min, possessed the characteristic fragment with *m/z* 231.1384, and also showed presence of *m/z* 361.2003 characteristic for roridin E [14], as well as precursor ion of *m/z* 532.2904 (1.3 ppm deviation) matching $[M+NH_4]^+$ for roridin E. In this sample, the intensities of the trichothecenes with respect to the intensities of the spirocyclic drimanes were significantly (three to five fold) lower, increasing the risk of overlooking these compounds in

presence of drimanes. The problem of detection of atranones, dolabellanes and macrocyclic trichothecens in the presence of spirocyclic drimanes is well known [12, 31]. Jarvis et al. [12] developed a normal phase SPE method (PEI silica) for fractionation of the *S. chartarum* extracts in spirocyclic drimanes and macrocyclic trichothecens or atranones. Due to the possible loss of the target analyte and time associated with fractionation we decided to attempt to increase the sensitivity of our method for the low abundant compounds by transferring the method to the more sensitive and selective UHPLC-QqQ.

UHPLC-QTOF to UHPLC-QqQ method transfer

In contrast to the UHPLC-QTOF method, the UHPLC-QqQ gradient was started at 20% water (eluent A) instead of 10%, a shorter column was used and steeper increase to 100% acetonitrile/2-propanol (eluent B) to elute compounds faster and as sharper peaks. In order to provide sufficient precursor ion intensity and suppress formation of $[M+Na]^+$ (which gave poor daughter ions yields) for macrocyclic trichothecenes and promote $[M+NH_4]^+$ formation, ammonium formate was added to eluent A. For each macrocyclic trichothecene, both mass transition with molecular ion $[M+H]^+$ and ammonium adduct $[M+NH_4]^+$ as precursor ion were used, thus ensuring reliable identification (Table 2). Mass transition giving the most abundant signal were chosen as the quantifier ion, regardless of whether chosen precursor was the proton adduct $[M+H]^+$ or ammonium adduct $[M+NH_4]^+$. Since all compounds included in method eluted between 3 and 5.5 min, the retention time window for all transitions was set to 4 min (from 2 to 6 min). With the dwell time for each transition of 50 ms, the duty cycle of 1605 ms and the typical peak width of 20 s, we obtained minimum 12 data points across peak which was enough to ensure reliable peak integration [44].

Collision energies, chosen through comparison with the QTOF MS/HRMS spectra obtained at 10, 20 and 40 eV only required minimal optimization (changes of less than 5 eV) in most cases. This demonstrates the value of the information that can be obtained from full scan QTOF experiments, as well as that acceptable QqQ methods can be obtained by direct Library to QqQ transfer.

Transitions and starting collision energies for chosen metabolites included in the method detecting secondary metabolites produced by indoor fungi other than *Stachybotrys* were adopted from Varga et al. [41]. Further optimization of this method only included collision energy adjustment within a few volts (supplementary information, Table S1). Fragmentor voltage (380 V) cannot be altered between compounds as it is fixed by the ion-funnel instrument, but far less important than on a cone-based instrument.

297 **Table 2** List of *Stachybotrys* metabolites included in QqQ method with optimized ESI-MS/MS; precursor ion to
298 quantifier ion mass transition together with collision energy used are presented in bold.

Metabolite	Rt (min)	*m/z* precursor ion	Ion species	***m/z*** product ion (collision energy (V))
Satratoxin H[a]	3.60	546	$[M+NH_4]^+$	249(15)/231(15)
		529	$[M+H]^+$	**231(15)**
Satratoxin G[a]	3.50	562	$[M+NH_4]^+$	249(15)/231(15)
		545	$[M+H]^+$	**231(15)**
Roridin L2[a]	4.20	548	$[M+NH_4]^+$	249(15)/231(15)
		531	$[M+H]^+$	**231(15)**
Roridin E[a]	5.10	**532**	$[M+NH_4]^+$	**361(15)**/231(15)
		515	$[M+H]^+$	231(15)
Atranone A[b]	4.60	417	$[M+H]^+$	381(10)/357(15)
Atranone B[b]	5.40	447	$[M+H]^+$	387(10)/369(10)
Dolabellane[a,b,c]	3.90	303	$[M+H]^+$	219(15)
Stachybotrylactam[a]	4.10	**386**	$[M+H]^+$	256(20)/**178(35)**/150(40)
Stachybotrylactam isomer	3.70	**386**	$[M+H]^+$	256(20)/**178(35)**
Stachybotryamide	4.10	**430**	$[M+H]^+$	**260(30)**/222(20)
Stachybotrydial	4.90	**387**	$[M+H]^+$	207(20)/**179(20)**
Mer-NF-5003-B	3.65	**405**	$[M+H]^+$	387(15)/**369(20)**
Trichodermin[a]	3.85	**293**	$[M+H]^+$	143(10)/**109(15)**

299 [a] Reference standard available

300 [b] not quantified due to the unavailability of standard with the same/similar structure

301 [c] (1S*,3R*,4R*,6S*,11S*)-3,4-Epoxy-6-hydroxy-dolabella-7E,12-dien-14-one

302
303

304 **UHPLC-QqQ Method validation**

305 The matrix effect was calculated as the slope ratio of the linear functions for matrix-matched standards and liquid
306 standards and is presented in Table 3. The matrix effect was evaluated only for one matrix, clean Kimwipes® Lite wipes
307 used to collect dust. This matrix was chosen due to the fact that dust needed to be extracted together with the wipes.
308 Among evaluated metabolites, both satratoxin H and roridin E showed enhancement of the signal, which was minor for
309 roridin E (4 %) and significantly higher for satratoxin H (34 %). In contrast, signals for stachybotrylactam and
310 trichodermin were suppressed by 11 % and 21 % respectively. Evaluation of blank matrix extract showed no signal for
311 any of the tested analytes. Clearly, it can be expected that dust itself will further suppress/enhance the signals obtained.
312 Vishwanth et al. [26] reported severe matrix effect in dust for the majority of the target analytes, however, reported
313 enhancement for stachybotrylactam by 6 % was negligible, whilst matrix effect for roridin E, satratoxin H and
314 trichodermin was not reported. Undoubtedly, the observed enhancement and suppression in our study showed that
315 matrix–matched calibration is not adequate to account for the matrix effect and that the use of isotopic-labelled internal
316 standards would be preferable [26, 41].

The linear range covered three orders of magnitude for stachybotrylactam and trichodermin, but only two orders of magnitude for satratoxin H and roridin E, due to the limited quantities of these two standards. All constructed calibration curves showed good linearity across entire range, and given the expected amounts in dust this is considered sufficient. Calculated coefficients of variation (CV %) for both liquid and matrix-matched standards were generally low, with the highest for trichodermin (8 % for liquid standards and 4 % for matrix-matched standards). Non-weighed calibration curves were chosen for stachybotrylactam, roridin E and satratoxin H, due to the higher resulting linearity and the fact that the concentrations in analysed samples tended to shift towards middle and higher range of the constructed curves. Use of non-weighed calibration curves resulted in accuracy outside of the allowed limits (20 %) on the lowest level, however observed accuracy on all other levels was within limits (Table 3). It should be noted that dust was sampled and analysed on much larger surface (45 × 45 cm) and later recalculated to the amount per square centimetre, therefore obtained concentrations in all samples are comparable. In absence of quantitative standards for all mycotoxins included in the method, stachybotryamide, stachybotrydial and Mer-NF-5003-B were quantified based on calibration curve for stachybotrylactam. Similarily, roridin L2 was quantified based on calibration curve constructed for roridin E, whilst satratoxin G was quantified based on calibration curve for satratoxin H. This approach was obviously based on similarity in structures, although we are well aware that similar structures often show differences in fragmentation patterns and intensities of created fragments. Although possessing different elemental compositions and thereby different precursor ions, all macrocyclic trichothecens produce same fragments, namely *m/z* 231 and 249. Only roridin E exhibits specific mass transition (*m/z* 532 to 361), which was chosen for quantification.

The situation was more complicated in case of the spirocyclic drimanes, where the structural differences between compounds were greater, most notably in the lack of 2-pyrrolidone moiety in stachybotrydial and Mer-NF-5003-B in comparison to stachybotrylactam and stachybotryamide (Fig. 1). Clearly, calculated concentrations, especially in cases where structural differences between metabolites were more prominent, are likely to be more inaccurate however, since reference standards for all drimanes were not commercially available, we found that it was a better option to calibrate against stachybotrylactam which is commercially available.

Table 3 Matrix effect, signal suppression/enhancement (SSE), standard error of regression line (Sy), average observed accuracy (Acc) and maximum observed accuracy (Acc_{max}), coefficients of variation (CV), limit of detection (LOD) and lower limit of quantification (LLOQ) values for *Stachybotrys* metabolites.

Metabolite	SSE[d] (%)	Sy[e]	Acc (Acc_{max})[f](%)	CV[g] (%)	LOD[h] (ng/cm^2)	LLOQ[i](ng/cm^2)
Satratoxin H[a]	134	1.8	28 (79)	2.2	15	50
Satratoxin G[a]	134	1.8	28 (79)	2.2	15	50
Roridin E[b]	104	4.9	29 (114)	2.2	0.1	0.2

Roridin L2[b]	104	4.9	29 (114)	2.2	0.1	0.2
Stachybotrylactam[c]	89	36	21 (77)	2.5	2	6
Stachybotrylactam isomer[c]	89	36	21 (77)	2.5	2	6
Stachybotryamide[c]	89	36	21 (77)	2.5	2	6
Stachybotrydial[c]	89	36	21 (77)	2.5	2	6
Mer-NF-5003-B[c]	89	36	21 (77)	2.5	2	6
Trichodermin	79	40	13.4 (28)	4.4	5	17

[a]Compounds calibrated against satratoxin H standard

[b]Compounds calibrated against roridin E standard

[c]Compounds calibrated against stachybotrylactam standard

[d]SSE%: signal suppression/enhancement calculated as the slope ratio of the linear functions for matrix-matched standards and liquid standards multiplied by 100

[e]Standard error of regression line

[f]Average accuracy on all levels. Accuracy on each level was calculated as ratio of calculated concentration and expected concentration multiplied by 100. Acc_{max} represents maximum observed value regardless of level including the values outside allowed limits (20 %)

[g]CV%: calculated as the average of the ratios of standard deviation and average concentration multiplied by 100 for each level

[h]LOD: Limit of detection calculated at the lowest concentration levels as concentrations corresponding to a signal-to-noise ratio (S/N) 3/1

[i]LLOQ: Lower limit of quantification calculated at the lowest concentration levels as concentrations corresponding to a signal-to-noise ratio (S/N) 10/1

Wall swabs and dust wipe analyses by QqQ

The UHPLC-QqQ method was used to analyze dust wipes and wall swabs collected in the water-damaged kindergarten. The method was able to detect *Stachybotrys* compounds in settled dust, both in the infected bathroom but also in the adjacent rooms, and further differentiate two co-existing chemotypes. The results showing all detected metabolites are presented in Table 4.

The results confirmed the presence of both macrocyclic trichothecenes (chemotype S) and atranone producing contaminants (chemotype A) in both wall and dust samples. In two out of seven wall swab samples, both trichothecene mycotoxins and atranones/dolabellanes were detected in the same sample (wallboard swab 3 and 4). In other cases, there was either trichothecens or atranones/dollabelanes present in the wall swabs, depending on the sampling site on the wallboard. This shows the coexistence of two chemotypes on the same material, with no clear borderline between areas of their contamination. Among trichothecenes, the highest amounts (180 ng/cm^2) were estimated for satratoxin H, whilst atranones/dollabelanes could not be quantified due to the insufficient amounts of the standard. No trichodermin was found either in dust wipe or wall swab samples. Spirocyclic drimanes represented by stachybotrylactam, stachybotrydial and stachybotryamide were dominant in all samples as expected, based on results obtained on the QTOF. The exception was Mer-NF-5003-B, whose production on the materials was reduced compared to pure cultures.

Estimated concentrations for drimanes in samples collected with Q-tips were within one order of magnitude (135 – 1042 ng/cm^2) with the total amount for all drimanes collected with the Q-tip estimated to be 2.5 µg/cm^2. Screening for metabolites produced by species other than *Stachybotrys* revealed presence of roquefortine C and meleagrin. This suggests presence of *Penicillium chrysogenum* on sampled material, which is among species most commonly isolated from indoor environment [45].

For the dust wipe samples, all groups of metabolites (trichothecenes, atranones/dollabelanes, drimanes) were detected in settled dust collected in the bathroom where water damage occurred (Dust 1, Fig 4). Settled dust collected in adjacent rooms contained, in most cases, stachybotrylactam and stachybotrylactam isomer. Roridin E was found only in one sample in concentration of 6.4 pg/cm^2, whilst dollabelanes were found in two samples, however the peaks were of very low intesity. Neither meleagrin nor roquefortine C were found in dust wipe samples. The amounts of mycotoxins in dust wipes in comparison to the amounts found in wall swabs were for 3-4 orders of magnitude lower, both in case of drimanes and trichothecens (600 pg/cm^2 of total drimanes in the bathroom with the water damage and highest estimated total amount of drimanes of 340 pg/cm^2 in adjacent room). Although stachybotrylactam has been reported in dust samples previously, its presence has only been found in samples collected from waste management facilities [46] and in settled dust collected in Dutch schools (in only 1 % of the collected samples, [47]). In addition to this, attempts have been made to quantify macrocyclic trichothecens in dust, however, only the simple trichothecens verrucarol and trichodermol (hydrolysis products of the satratoxins) analyzed by GC-MS have been reported [30, 47-48]. Furthermore, Polizzi et al. [27] reported analysis of roridin E on contaminated material but not in dust. To the best of our knowledge, this is the first study where macrocyclic trichothecens have been found directly in settled dust collected from a water damaged building. It is also the first time that a comparison between the amounts of mycotoxins produced on contaminated wall surfaces and the amounts of mycotoxins that actually become airborne have been made. Moreover, presence of drimanes in dust samples collected in rooms adjacent to the room with water damage clearly demonstrates a wide distribution of mycotoxins in detectable amounts and this is promising for their use as biomarkers.

Table 4 Estimated amounts of *Stachybotrys* metabolites found on contaminated wall surface and in settled dust.

	Concentration (pg/cm^2)								Area		
Samples	Stachybotryamide	Stachybotrylactam	Stachybotrylactam Isomer	Stachybotrydial	Satratoxin H	Satratoxin G	Roridin L2	Roridin E	Atranone A[d]	Atranone B[d]	Dolabellanes[d]
Wall swab 1	18×10^4	32×10^4	35×10^4	37×10^4	ND	ND	ND	ND	31166	2578	590773
Wall swab 2	18×10^4	56×10^4	65×10^4	54×10^4	ND	ND	ND	ND	57702	3104	775107
Wall swab 3	18×10^4	36×10^4	55×10^4	23×10^4	180	ND	37×10^3	30×10^3	8695	1481	187816
Wall swab 4	18×10^4	104×10^4	91×10^4	38×10^4	ND	14×10^4	55×10^3	13×10^4	ND	ND	ND
Wall swab 5	18×10^4	38×10^4	40×10^4	32×10^4	ND	ND	ND	ND	33072	1096	607685
Wall swab 6	ND	14×10^4	14×10^4	ND	ND	ND	ND	ND	ND	ND	ND
Wall swab 7	17×10^4	16×10^4	18×10^4	17×10^4	140	ND	35×10^3	17×10^3	4926	380	5381
Dust wipe 1[a,b]	90	230	180	100	ND	ND	ND	10	18467	ND	495672
Dust wipe 2[a,c]	80	90	90	80	ND	ND	ND	ND	2718	ND	25308
Dust wipe 3[a,c]	ND	70	70	ND	ND	ND	ND	ND	ND	ND	ND
Dust wipe 4[a,c]	ND	70	70	ND	ND	ND	ND	ND	ND	ND	ND
Dust wipe 5[a,c]	ND	ND	70	ND	ND	ND	ND	ND	ND	ND	ND
Dust wipe 6[a,c]	ND	70	70	ND	ND	ND	ND	6.4	ND	ND	ND
Dust wipe 7[a,c]	ND	70	70	ND	ND	ND	ND	ND	ND	ND	ND
Dust wipe 8[a,c]	ND	70	70	ND	ND	ND	ND	ND	ND	ND	ND
Dust wipe 9[a,c]	ND	70	70	ND	ND	ND	ND	ND	ND	ND	ND
Dust wipe 10[a,c]	ND	70	70	ND	ND	ND	ND	ND	ND	ND	ND
Dust wipe 11[a,c]	ND	70	70	ND	ND	ND	ND	ND	ND	ND	ND

[a]Concentrations found in dust samples were analyzed for entire sampled surface (2025 cm^2) and recalculated to amounts per cm^2

[b] Dust sampled in the room where water damage occurred

[c]Dust sampled in rooms adjacent to the room where water damage occurred

[d]Metabolites only identified and not quantified due to the lack of quantitative standards with the same/similar structure.

Mycotoxin content of settled dust is important for overall exposure assessment. The presented method appeared to be a useful tool for identifying and estimating the amounts of indoor contaminants in dust. This indicates that the future focus could primarily be analysis of dust samples. Given that dust is the primary route of exposure to these mycotoxins, but the detection here has previously proven difficult, this method may provide an effective means to gather considerable chemical data and improve our understanding of mycotoxin toxicity in contaminated buildings.

Conclusion

This case-study presents a semi-quantitative UHPLC-QqQ method with several new exposure biomarkers, which was developed based on UHPLC-qQTOF screening of culture extracts. The method was able to identify twelve *Stachybotrys* metabolites of which four could be quantified based on authentic standards, and an additional six estimated based on similar compounds. The method was applied to samples collected in a water damage building contaminated by *S. chartarum* chemotype S, as well as with atranone producing contaminant (*S. chartarum* chemotype A or *S. chlorohalonata*). The obtained results represent a step forward in solving the problems of exposure to mycotoxins in damp indoor environments, detecting for the first time presence of same mycotoxins on contaminated gypsum wall board surfaces and in settled dust. Furthermore, the method enables fast estimation of mycotoxin's amounts in analyzed samples. Demonstrating that method transfer from UHPLC-qQTOF to UHPLC-QqQ instruments is facile means that this method can be supplemented with additional biomarkers in subsequent exposure studies. This methodology represents a significant advance in the detection of mycotoxins in dust samples, which is the main route of exposure leading to toxicity and illness. As such, this study could have an impact on our understanding of the relationship between mold exposure and sickness. Further work includes expanding this methodology to include mycotoxins and biomarkers produced by other species and genera of indoor fungi and further case studies to demonstrate the reliability and applicability of this method.

Acknowledgements

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Conflict of interest

The authors declare no conflict of interest.

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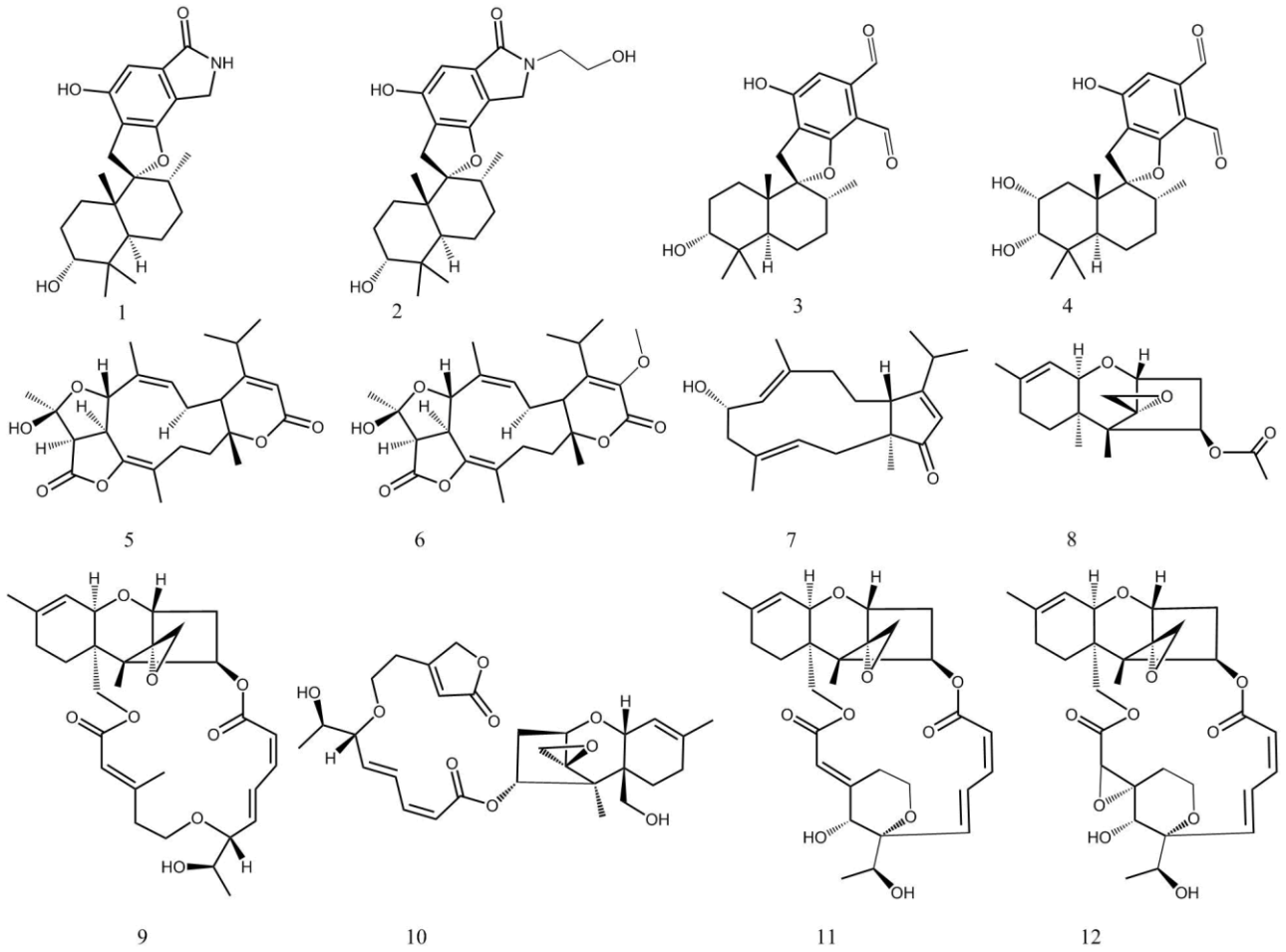

Fig. 1 Secondary metabolites and mycotoxins produced by *S. chartarum*: spirocyclic drimanes produced by both chemotypes (1 – stachybotrylactam, 2 – stachybotryamide, 3 – stachybotrydial, 4 – Mer-NF-5003-B); atranones and their precursors characteristic for chemotype A (5 – atranone A, 6 – atranone B, 7 – 6-hydroxydolabella-3E,7E,12-trien-14-one, 8 – simple trichothecene trichodermin); macrocyclic trichothecens characteristic for chemotype S (9 – roridin E, 10 – roridin L2, 11 – satratoxin H, 12 – satratoxin G)

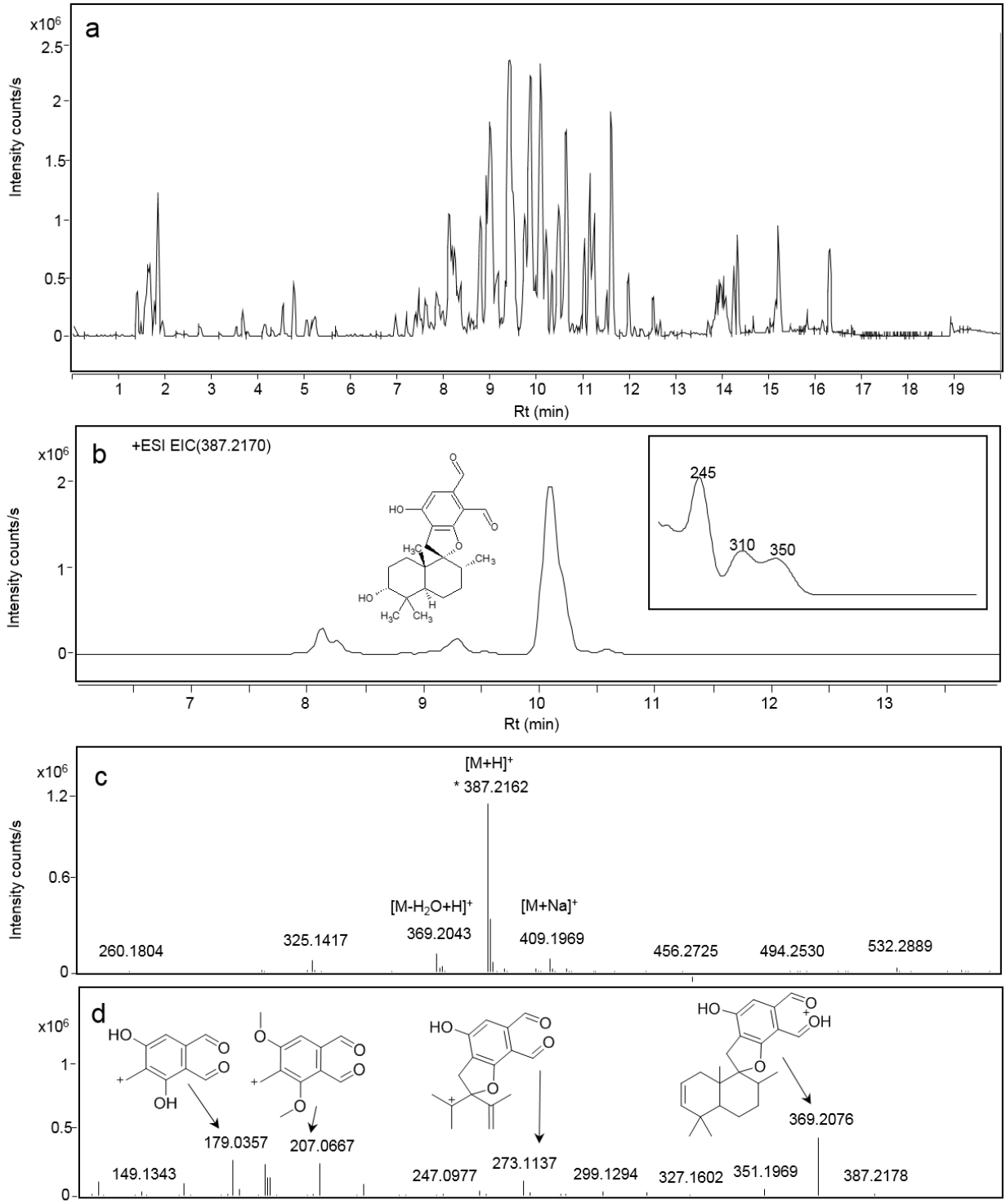

Fig. 2 Example of dereplication of stachybotrydial from data obtained on the QTOF: a – Base Peak Chromatogram (BPC) of *S. chartarum* pure culture (IBT 9631 on MEA) extract; b – Extracted Ion Chromatogram (EIC) for m/z of 387.2166 with UV spectrum characteristic for stachybotrydial (Andersen et al.,2002), c – full scan spectrum, d – MS/HRMS of stachybotrydial at 20 eV.

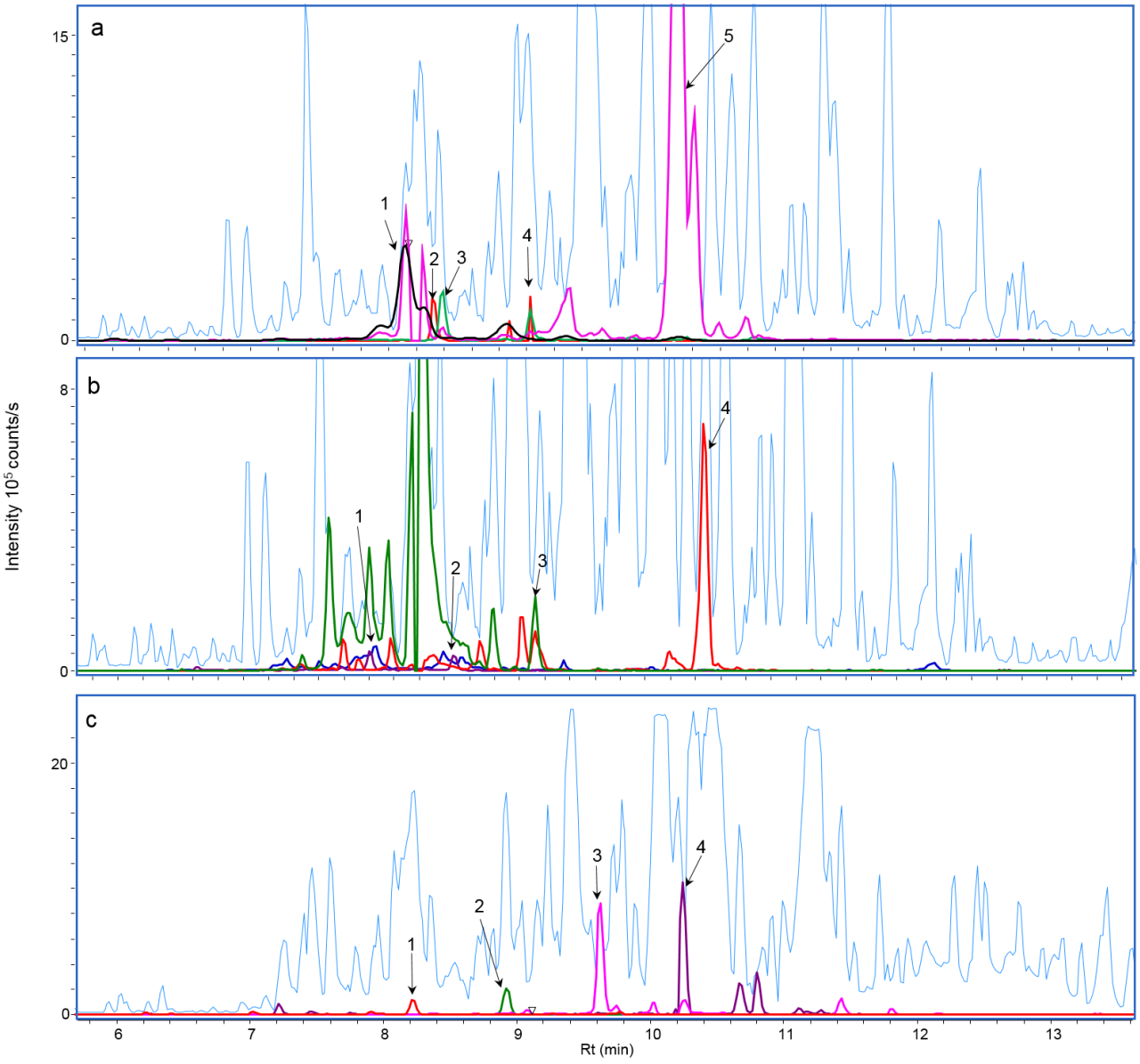

Fig. 3 Base Peak chromatograms (BPC) of wall scraping from contaminated wallboard analyzed on QTOF merged with combined extracted ion chromatograms for: **a** –spirocyclic drimanes: 1 – *m/z* 405.227 ($[M+H]^+$, Mer-NF-5003-B) [42], 2 – *m/z* 430.2588 ($[M+H]^+$, stachybotryamide) [43], 3 – *m/z* 386.2326 ($[M+H]^+$, stachybotrylactam) [42], 4 *m/z* 386.2326 ($[M+H]^+$, stachybotrylactam isomer, 5 – *m/z* 387.2166 ($[M+H]^+$, stachybotrydial) [42]; **b** – macrocyclic trichothecens**:** *m/z* 546. 2695 ($[M+NH_4]^+$, satratoxin H), 2 – *m/z* 562.2645 ($[M+NH_4]^+$ satratoxin G), 3 – *m/z* 548.2855 ($[M+NH_4]^+$ roridin L2), 4 – *m/z* 532.2904 ($[M+NH_4]^+$ roridin E); **c** - Diterpenoids and their precursors: 1 – *m/z* 319.227 ($[M+H]^+$,3,4-epoxy-6-hydroxy-dolabella-7E,12-dien-14-one), 2 – *m/z* 303.2319 ($[M+H]^+$, 6-hydroxydolabella-3E,7E,12-trien-14-one), 3 – *m/z* 417.227 ($[M+H]^+$, atranone A), 4 – *m/z* 447.238 ($[M+H]^+$, atranone B).

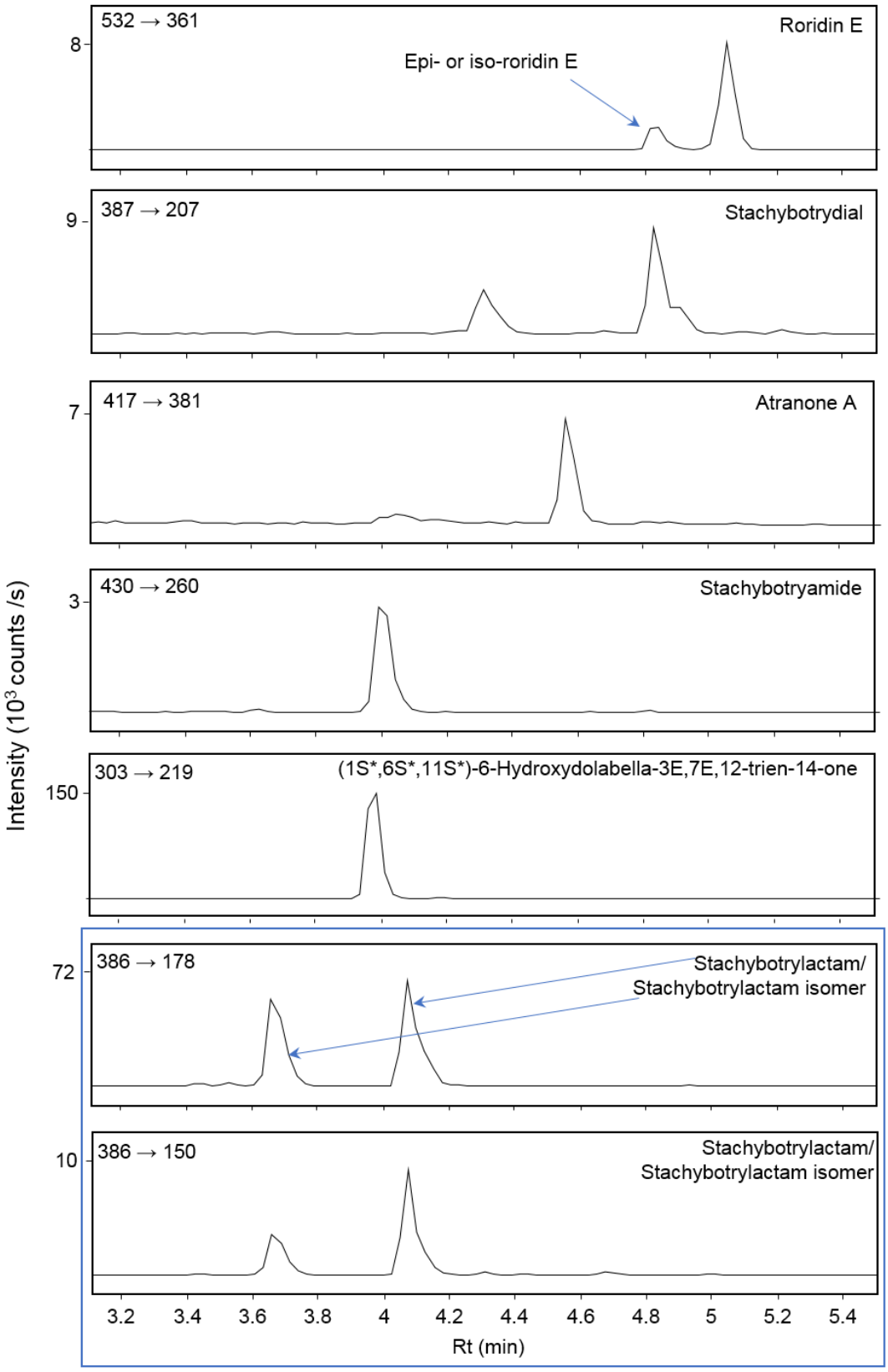

Fig. 4 MS/MS transitions for all mycotoxins identified in dust sample collected in the water-damaged room.

To ***Analytical and Bioanalytical Chemistry***

Stachybotrys mycotoxins: from culture extracts to dust samples

Electronic Supplementary Material

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Table S1 List of metabolites produced by species other than *Stachybotrys* spp. included in QqQ method with optimized ESI-MS/MS parameters

Metabolite	Rt (min)	*m/z* precursor ion	Ion species	m/z product ion (collision energy (V))
Altenuene	3.2	293	$[M+H]^+$	275(5)/257(10)
Austdiol	1.6	237	$[M+H]^+$	159(20)/117(25)
Chaetoglobosin A	4.4	529	$[M+H]^+$	511(5)/130(35)
Cytochalasin A		478	$[M+H]^+$	120(25)/91(40)
Meleagrin	3.4	434	$[M+H]^+$	403(10)/334(20)
Mycophenolic acid		338	$[M+NH_4]^+$	303(10)/207(25)
Penicillin G	3.3	335	$[M+H]^+$	176(10)/160(5)
Roquefortine C	4.3	390	$[M+H]^+$	322(15)/193(25)
Sterigmatocystin	4.6	325	$[M+H]^+$	281(40)/130(25)

Table S2 Overview of Stachybotrys metabolites identified on QTOF in pure agar cultures (MEA and PDA) and wallboard scrapings collected in water-damaged kindergarten

	Metabolites											
Samples	Stachybotryamide	Stachybotrylactam	Stachybotrylactam Isomer	Stachybotrydial	Mer-NF-5003-B	Satratoxin H	Satratoxin G	Roridin L2	Roridin E	Atranone A[d]	Atranone B[d]	Dolabellanes[d]
S. chartarum IBT 9631	+	+	+	+	+	+	+	+	+	-	-	-
S. chartarum IBT 7709	+	+	+	+	+	+	+	+	+	-	-	-
S. chartarum IBT 7617	+	+	+	+	+	-	-	-	-	-	-	+
S. chartarum IBT 9466	+	+	+	+	+	-	-	-	-	-	-	+
S. chartarum IBT 40285	+	+	+	+	-	-	-	-	-	+	+	+
S. chartarum IBT 40295	+	+	+	+	-	-	-	-	-	+	+	+
Wall scraping 1	+	+	+	-	+	-	-	-	-	+	+	+
Wall scraping 2	+	+	+	-	+	+	+	+	+	+	+	+
Wall scraping 3	+	+	+	+	+	-	-	-	+	+	+	+
Wall scraping 4	+	+	+	-	-	-	-	-	+	+	-	+
Wall scraping 5	+	+	+	-	-	-	-	-	+	-	-	-
Wall scraping 6	+	+	+	+	+	-	-	-	-	+	+	+
Wall scraping 7	+	+	+	-	+	+	-	+	+	-	-	+

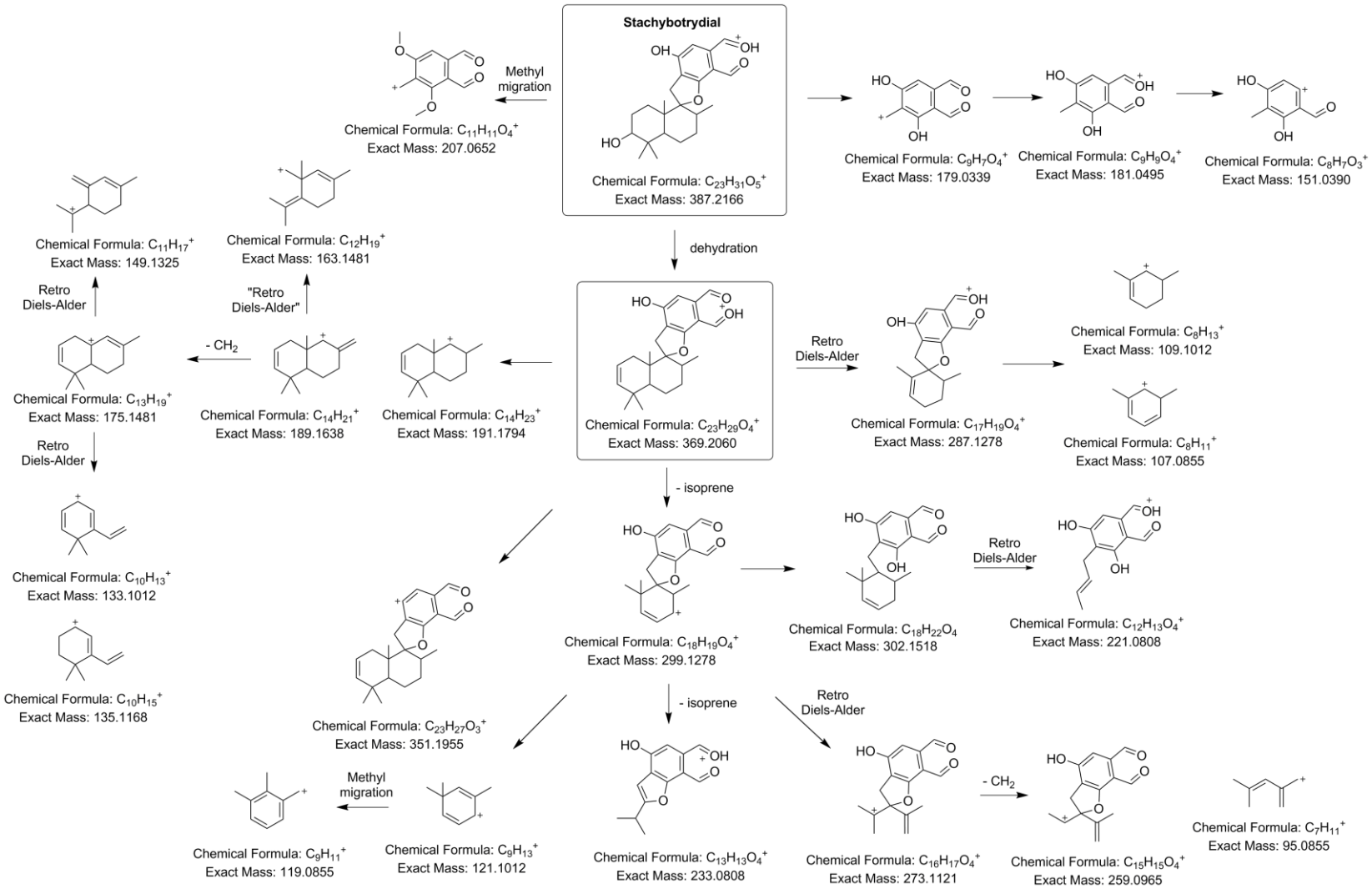

Fig. S1 Proposed fragmentation pattern for stachybotrydial

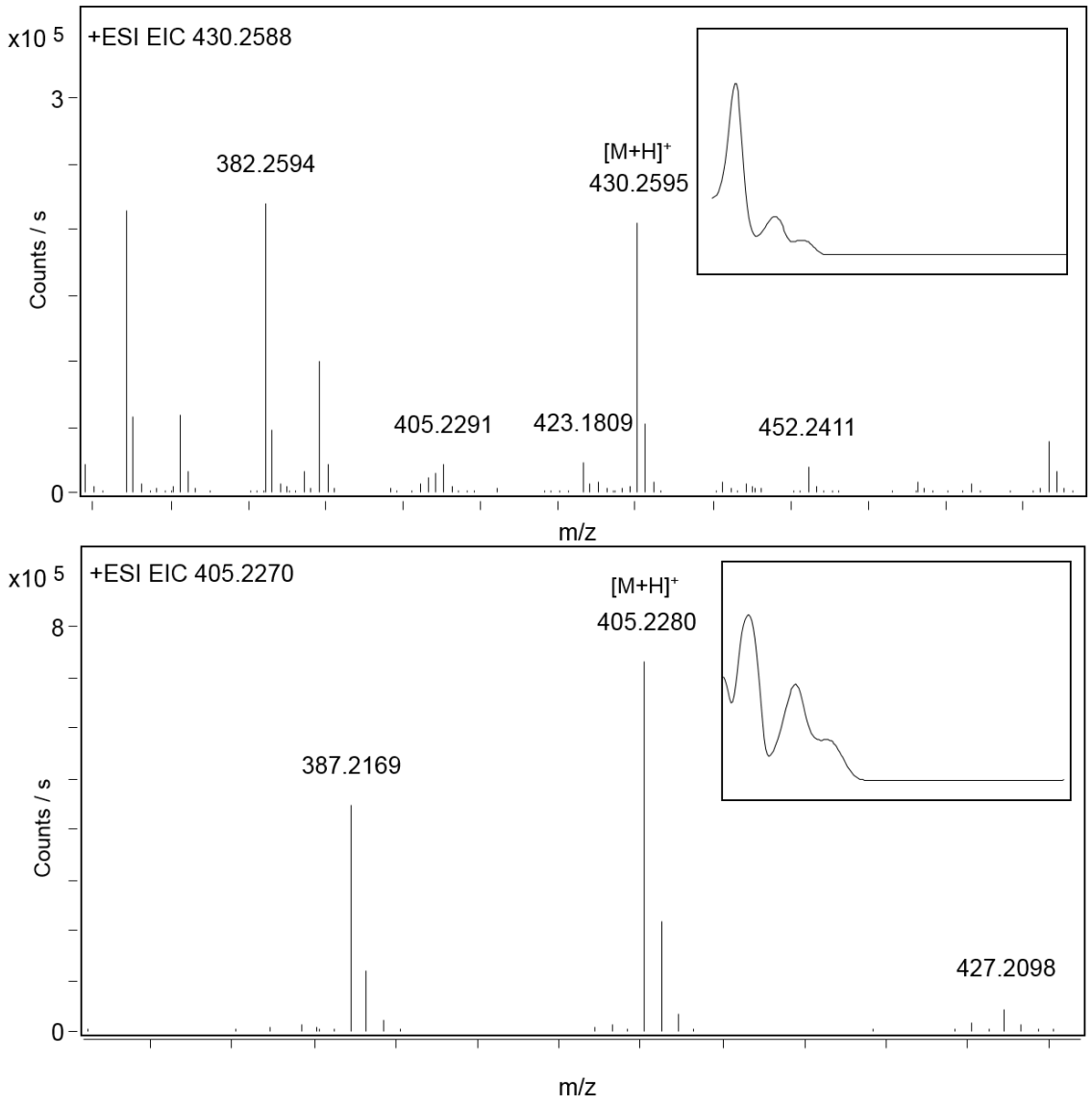

Fig. S2 Extracted Ion Chromatogram (EIC) and representative UV spectra for stachybotryamide (*m/z* 430.2588, 4.2 ppm) and Mer-NF-5003-B (*m/z* 405.2270, 2.5 ppm)

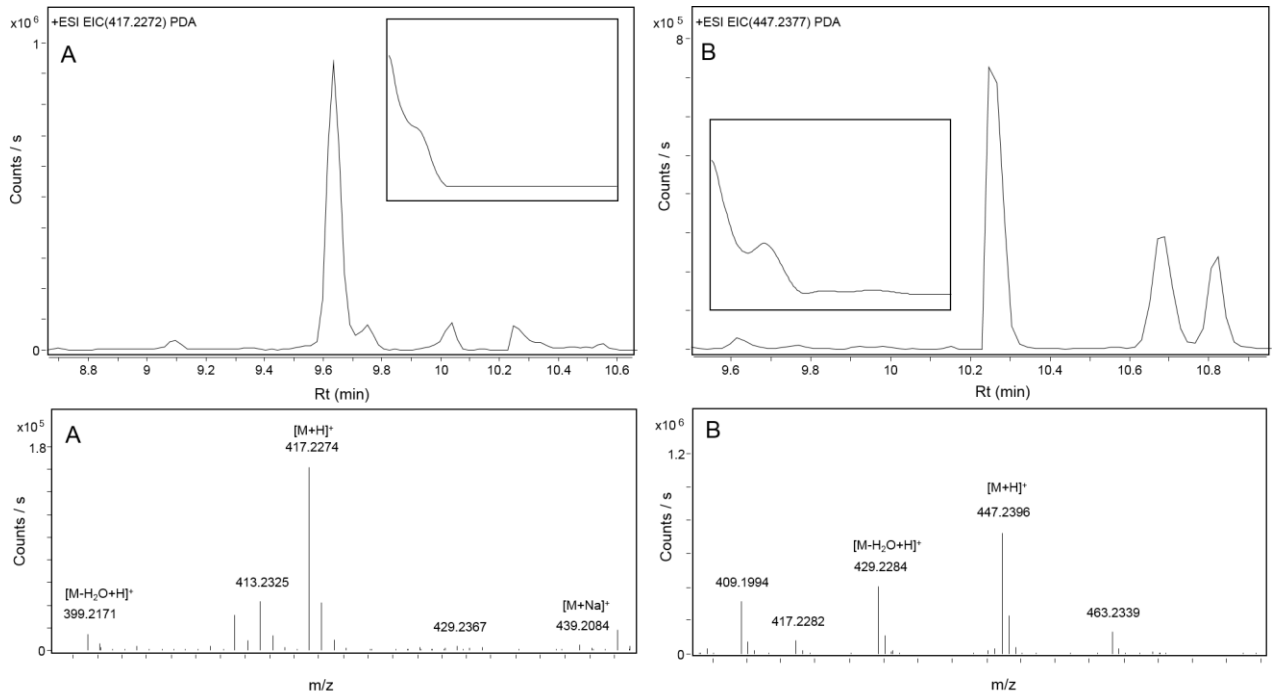

Fig. S3 Extracted ion chromatogram (EIC), full scan spectrum and UV confirmation for: A – atranone A (*m/z* 417.2272, 0.5 ppm), B – atranone B (*m/z* 447.2377, 4.5 ppm)

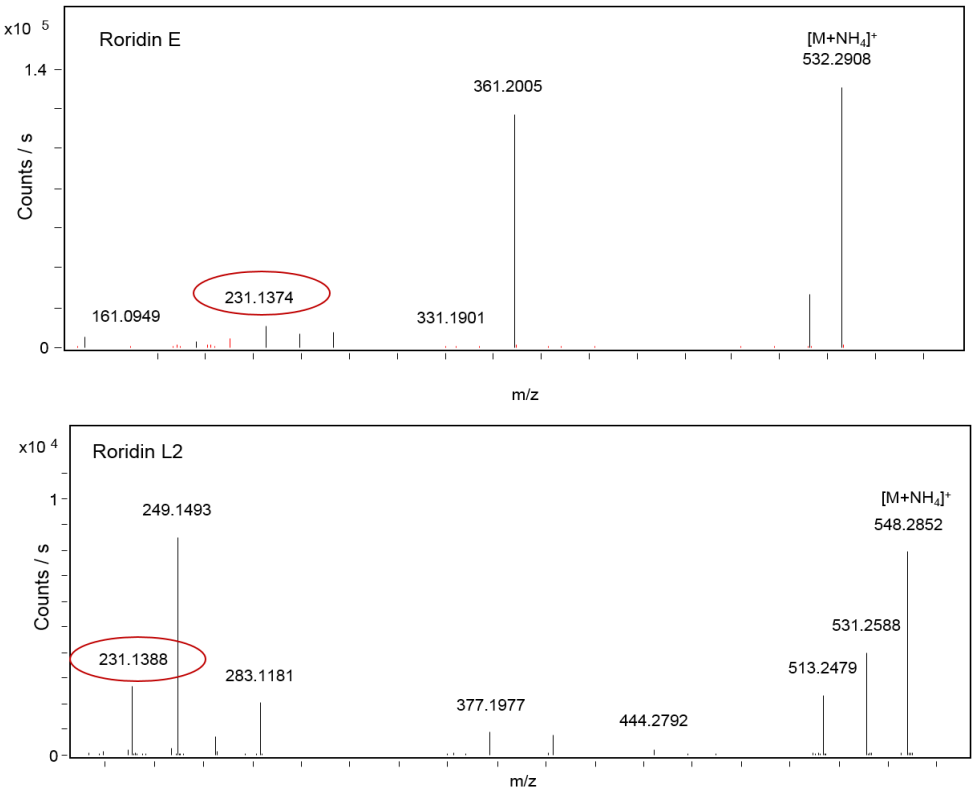

Fig. S4 MS/HRMS of macrocyclic trichothecens (roridin E and roridin L2) found by searching for 231.1300 ± 0.0100 fragment ion