Development, validation and application of mass spectrometric methods for indoor environmental quality evaluation with special emphasis on microbial metabolites

Submitted by

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Tulln, January 9th, 2012 **Vinay Vishwanath**

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Aims and structure of the thesis

The critical aspect of microbes is their ability to influence their surroundings both as viable cells and dead entities. This is mainly because of their bioactive substances that are released into immediate surroundings or concealed within mycelial fragments or spores (inhalation toxicity). On the basis of elaborated literature survey we realized that, there is only handful of validated analytical methods available for evaluation of microbial toxins in indoor environments. Also prevailing knowledge on the quantitative occurrence, exposure, response relationships for biological agents are inadequate, with exception to allergens e.g. pollens, dust mite, animal dander and very few microbes.

Another major challenge for indoor evaluation is the non-availability of control or blank samples and baseline values for toxic substances. In the present context, the working definition for baseline value is defined as a numerical range of analyte concentration or spectrum of chemical entities that can be found in an indoor environment without any (reported or observed) building damage and health problems among inhabitants. This issue is partly addressed by us with an emphasis to indoor relevant microbial non volatile bioactive compounds. This could be useful differentiating moisture damaged houses from houses without any forms of water damage.

This thesis is prepared within the frame work of the project the IFUMEP and the ongoing European Commission funded FP7 project HITEA. The objectives of the thesis (and also of the projects mentioned) are to recognize accumulating biological agents/bio-actives of microbial origin in indoor environments of Austria, Slovakia, Finland, The Netherlands and Spain. In order to achieve the set goals and bridge methodological gaps multi target liquid chromatography/tandem mass spectrometry (LC-MS/MS) and HS-SPME-GC-MS methods were developed at the CAC (Center for Analytical Chemistry). Additionally, the LC-MS/MS method was also validated for indoor settled floor dust (SFD). However, the majority of the dissertation duration was invested on elucidation of biological pollutants accumulating indoor environments. Similarly we demonstrated the feasibility of using settled floor dust (SFD) as representative matrix for indoor quality evaluation.

The thesis is structured into an overall introduction, laying out the background, various chapters describing the performed research and a conclusion section. The background is with an intention to give glimpses about indoor safety standardization bodies, existing guidelines and indoor associated problems. The chapters 1, 2 and 3 give an account of indoor micro flora (respective metabolites and their bioactive properties), aspects of liquid chromatography-tandem mass spectrometry and gas chromatography-mass spectrometry respectively. The conclusion is to enable reader have a bird eye view of research activity and major findings of the dissertation research which are more detailed in publications.

The results pertaining LC-MS/MS method development, validation (publication 1) and application are given in publications 1, 2 and 3 and in the conference proceedings. The results of GC-MS parameter optimization and method application to settled floor dust are summarized in publication 3. In chapter 2, the ~~"u~~npublished data" pertaining to microbial specificity of indoor matrices and comparison of swab and vacuum sampling methods are discussed in combination with Welch's significance test.

Moreover we are optimistic about the outcome of the ongoing research project HITEA (till 2013), which is intends to provide a comprehensive picture of microbes and their metabolites, relating to (observed) health effects among pupils and staff in schools. Similarly another aspect of the project, i.e. renovation monitoring of water damaged buildings, can be useful in formulating criteria for building restoration purposes.

"Vision without action is a dream. Action without vision is simply passing the time. Action with Vision is making a positive difference."
-Joel Barker

Glossary

AMDIS	Automated Mass Spectral Deconvolution and Identification System
AME	Alternariol monomethyl ether
Amu	Atomic mass unit
APCI	Atmospheric-pressure chemical ionization
ASHRE	American Society of Heating, Refrigerating and Air Conditioning Engineers
a_w	Availability of water or Water activity
BRI	Building related illness
CAR/PDMS	Carboxene/Polydimethylsiloxane
CF-FAB	Continuous flow FAB
CI	Chemical ionization
CID	Collision induced dissociation
CRM	Certified reference material
cSRM	Classical selected reaction monitoring
CV	Coefficients of variation
DLI	Direct liquid injection
DVB/CAR/PDMS	Divinylbenzene/Carboxene/Polydimethylsiloxane
EC	European commission
EF	Exfoliative toxins
EI	Electron ionization
EMEA	European medicines agency
EMT	Electron multiplier tube
Enn	Enniatin
EPI	Enhanced product ion
ESI	Electrospray ionization
EU	European union
eV	Electron volt
FAB	Fast atom bombardment
FAMEs	Fatty acid methyl esters
HCA	Hierarchical Cluster Analysis
HETP	Height equivalent to a theoretical plate
HPLC	High performance liquid chromatography
HVAC	Heating, ventilation, and air conditioning
IgG	Immunoglobulin G
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/z	mass/charge
MRM	Multiple reactions monitoring
MRSA	Methicillin resistant Staphylococcus aureus
MS	Mass spectrometry
MVA	Multivariate statistical analyses
MVOCs	Microbial volatile organic compounds

MWE	Microwave extraction
n-alkanes	Linear alkanes
NCI	Negative chemical ionization
NIFIES	Non-infectious Fungal Indoor environmental Syndrome
NIST	National Institute of Standards & Technology
ODTS	Organic Dust Toxic Syndrome
OSE	Orbital shaker extraction
Pa	Pascal
PA	Polyacrylate
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal Component Analysis
PCB	Polychlorinated biphenyl
PDMS	Polydimethylsiloxane
PDMS/DVB	Polydimethylsiloxane/Divinylbenzene
PMT	Photon multiplier tube
PPB	Parts per billion
PPM	Parts per million
qPCR	Quantitative real time polymerase chain reaction
qTOF	Quadrupole time of flight
R_A	Apparent recovery
R_E	Recovery extraction
RI	Retention index
RM	Reference material
RSD	Relative standard deviation
RSP	Respirable suspended particles
RT	Retention time
S/N	Signal-to-Noise ratio
SBS	Sick building syndrome
SD	Standard deviation
SE	Soxhlet extraction
SIM	Selected ion monitoring
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
SSE	Signal suppression enhancement
sSRM	Scheduled reaction monitoring
TIC	Total ion current /chromatogram
TOF	Time of flight
TPGC	Temperature programmed gas chromatographic
UPLC	Ultra Performance Liquid Chromatography
USE	Ultrasonic extraction
USP 25	United States pharmacopeia 25
VOC	Volatile organic compound
XIC	Extracted ion chromatogram

Abstract

The mold growth in indoors may be the result of moisture accumulation during natural calamities (flood, hurricane and tsunami) and/or also due to defective plumbing and ventilation systems. Working and living in such environments is regarded as risk factor for human health. However, exact causes for the clinical symptoms are not known. This is particularly true for mycotoxins and co-occurring bacterial metabolites in these environments. Hence, a set of non specific or less specific clinical symptoms observed in inhabitants of these buildings are classified as sick building syndrome (SBS) and building related illnesses (BRI) respectively. Though prevalence of SBS and BRI are known for decades, exact causes could not be determined so far. The reasons for this could be attributed to limitations of current methods dedicated for indoor safety evaluation and multidimensionality of the problem itself in water damaged interiors. The goal of the FFG-funded project "Determination of mold and fungal metabolite prevalence in buildings" is ensuring the availability of competent methods for determination of microbial bioactive substances in indoor environments. This includes both development and validation of analytical methods as well as the screening of fungal strains for the production of analytical calibrants.

The aim of this dissertation research was the qualitative and quantitative determination of mold and bacterial metabolite prevalence in moisture damaged buildings. The methodological limitations for evaluation of indoor relevant microbial metabolites were addressed by developing dedicated mass spectrometric methods. The developed multi target LC-MS/MS method was validated using certified settled floor dust reference material. The validated method is capable of detection and quantification of 186 microbial secondary metabolites. Concerning method performance, the signal suppression and extraction losses were relatively higher in floor dust matrix compared to other indoor matrices. The method had a limit of detection of 0.25-11000 µg/kg for house dust depending on the analyte. The apparent recoveries of half of the investigated analytes were above 50%. One third of analytes experienced severe matrix suppression, with analytical signal of reduction to below 50%. With certain exceptions, coefficients of variation of the whole procedure was lower than 10%. A wide range of indoor relevant samples from moisture damaged, renovated and also from newly constructed buildings were investigated (~2000 samples). Samples for the study were procured from Austria, Slovakia, Spain and the Scandinavian region of Europe. Moreover, supplementary microbiological

investigations were performed in collaboration. In one of the collaborative studies (University of Nitra, Slovakia), we detected the prevalence of species *Engyodontium album* in water damaged buildings of Slovakia. This was an important finding as *Engyodontium album* is rarely reported in indoor evaluation studies. The multi-site and multi- matrix analysis revealed the prevalence of more than 30 different microbial metabolites in indoor environments. Moreover the co-prevalence of bacterial and fungal metabolites was detected and reported for the first time ever. The study confirmed predominant prevalence of beauvericin, equisetin, alternariol and alternariol monomethyl ether (AME) in indoor environments (houses and schools). In all severely water damaged buildings the black mold signature metabolite stachybotrylactam was a detected. However, in our study satra toxins were rarely detected.

Similarly, volatiles organic compound (both of anthropogenic and microbial origin) adsorbed on surface of settled dust samples from widely differing indoor environments were also analyzed by gas chromatography coupled to mass spectrometry. Principal component analysis (PCA) of peak areas of 18 microbial volatile organic compounds (MVOCs) resulted in identification of nonanal as potential MVOC marker.

The multi dimensional approach used in this study was highly advantageous. The limitation of one technique was compensated by strengths of other techniques thereby facilitating a comprehensive evaluation of indoor quality regarding microbial burden.

“An ounce of practice is worth more than tons of preaching.”
-Mahatma Gandhi

Kurzfassung

Das Auftreten von Schimmelpilzen in Innenräumen kann durch Überflutungen, Rohrgebrechen und ungenügende Durchlüftung verursacht werden. Das Arbeiten und Wohnen in solchen Räumen ist ein anerkannter Risikofaktor für die menschliche Gesundheit. Die genauen Ursachen für die auftretenden Symptome sind jedoch ungeklärt, insbesonders die Rolle von toxischen Sekundärmetaboliten der beteiligten Schimmelpilze. Das Ziel des vom FFG geförderten Projekts "Determination of mold and fungal metabolite prevalence in buildings" ist es, Lösungen für die verschiedenen methodischen Einschränkungen der derzeitigen Methoden zur Analyse von innenraum-relevanten Pilzmetaboliten zu erarbeiten. Dazu zählt sowohl die Entwicklung qualitativer und validierter quantitativer Analysenmethoden als auch die Identifizierung und Kultivierung der beteiligten Pilzstämme zur Produktion von Referenzsubstanzen.

Ziel dieser Arbeit war es, das Vorkommen von mikrobiellen Metaboliten in durch Feuchtigkeit geschädigten Innenräumen quantitativ zu untersuchen. Um die methodischen Lücken hinsichtlich einer entsprechenden Analytik zu schließen, wurden massenspektrometrische Methoden entwickelt. Eine Methode für die simultane Bestimmung von 186 Sekundärmetaboliten, die auf Flüssigkeitschromatographie gekoppelt mit Tandem-Massenspektrometrie basiert, wurde mittels dotiertem Hausstaub validiert. Die entsprechenden Detektionslimits lagen zwischen 0.25 und 11000 µg/kg, abhängig vom Analyten. Die Wiederfindungen über das Gesantverfahren lagen für etwa die Hälfte aller Analyten unter 50%, was sowohl durch Signalsuppression als auch durch Extraktionsverluste verursacht wurde. Trotzdem lagen die Variationskoeffizienten der Gesamtbestimmung mit einigen wenigen Ausnahmen unter 10%.

Mit der entwickelten Methode wurden ca. 2000 Proben auf Innenraum relevante Materialien aus feuchtegeschädigten renovierten und neu gebauten Häusern und Schulen vermessen. Die Proben stammten aus Österreich, der Slowakei, Spanien, den Niederlanden und Skandinavien. Proben aus der Slowakei (Universiät Nitra) zeigten *Engyodontium album* als primär vorkommenden Organismus auf. Dieser Schimmelpilz wurde in durch Feuchte geschädigten Bausubstanzen bislang selten beobachtet. Diese umfangreichen Analysen ergaben ein Spektrum von über 30 mikrobiellen Metaboliten, allen voran Beauvericin, Equisetin, Alternariol und

Alternariolmethylether. Zusätzlich wurde erstmals ein simultanes Auftreten bakterieller und fungaler Metabolite im Zuge dieser Dissertation beobachtet.

Eine Komplämentärmethode basierend auf Gaschromatographie gekoppelt mit Massenspektrometrie wurde für die Bestimmung von anthropogenen und mikrobiellen flüchtigen Metaboliten (MVOCs, microbial volatile organic compounds) in Hausstaub verwendet. Mittels Datenbanksuche und Hauptkomponentenanalyse wurden 18 mikrobielle flüchtige Verbindungen identifiziert und Nonanal als Markersubstanz für MVOCs bestimmt.

Durch die Verwendung komplementärer analytischer Methoden wurden die Anwendungsgrenzen einzelner Methoden kompensiert und somit ein größeres Gesamtbild über die Qualität von Innenräumen bezüglich mikrobieller Analyten erhalten.

List of publications & other presentations

International peer reviewed publications

1. **Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry**.
   Vishwanath V, Sulyok M, Labuda R, Bicker W, Krska R**. Anal Bioanal Chem**. 2009 Nov; 395(5):1355-72.
2. **Co-occurrence of bacterial toxins and mycotoxins in moisture damaged indoor environments.**
   Täubel M, Sulyok M,**Vishwanath V**, Bloom E, Turunen M, Järvi K, Kauhanen E, Krska R, Hyvärinen A, Larsson L and Nevalainen A. Täubel M, Sulyok M, **Vishwanath V**, Bloom E, Turunen M, Järvi K, Kauhanen E, Krska R, Hyvärinen A, Larsson L, Nevalainen A. **Indoor Air**. 2011 Oct;21(5):368-75.
3. **Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC-MS/MS and GC-MS methods.**
   Vishwanath V, Sulyok M, Weingart G, Kluger B, Täubel M, Mayer S, Schuhmacher R, Krska R. **Talanta**. 2011 Sep 30;85(4):2027-38.
4. **Differential protein levels and post-translational modifications in spinal cord injury of the rat.**
   Afjehi-Sadat L, Brejnikow M, Kang SU, **Vishwanath V**, Walder N, Herkner K, Redl H, Lubec G.J **Proteome Res**. 2010 Mar 5;9(3):1591-1597 (this work is not part of the Ph.D. thesis)

Peer reviewed conference proceedings

1. **Occurrence of toxic bacterial and fungal metabolites on mold damaged building materials.** Sulyok M, Täubel M, Vishwanath V, Peitzsch M, Kauhanen E, Krska R, Larsson L, and Nevalainen A, Healthy Buildings, Syracuse, USA, 14.09.2009.

2. **Microbial toxins in surface swab and dust samples from school buildings in three European countries.** Peitzsch M, Suylok M, Täubel M, Vishwanath V, Jacobs J, Borras A, Krop E, Vepsäläinen A, Zock JP, Hyvärinen A, Heederik D, Nevalainen A, and Larsson L. Indoor Air Conference, Austin, Texas, 09.06.2011

3. **An Intervention Study In Moisture Damaged Homes – preliminary results before renovation.** Hyvärinen A[*], Täubel M, Sulyok M, **Vishwanath V**, Turunen M, Järvi K, Patovirta RL, Tuomainen TP, Pirinen J, Krska R, Pekkanen J and Nevalainen A. Indoor Air Conference, Austin, Texas, 09.06.2011

Other publications

- **Determination of moulds and their metabolites in indoor environments**. **Vishwanath V**, Sulyok M, Krska R, Schuhmacher R, Proceedings, MOLD-MEETING, Mikrobiologie und Molekularbiologie, **ALVA-Mitteilungen**, Heft 7, 2009
- **Airborne workplace exposure to microbial metabolites in waste recycling plants.** Mayer S, Vishwanath V, Sulyok M, Proceedings, 6th International Scientific Conference on Bioaerosols, Fungi, Bacteria, Mycotoxins in Indoor and Outdoor Environments and Human Health. September 2011, Saratoga Springs, New York, United States (submitted).

Oral presentations

1. **Hyphenated technologies for occupational hazard assessment - Microbes friends and foes. Vinay Vishwanath;** Michael Sulyok; Stefan Mayer; Rudolf Krska **(Oral presentation**, at the 32. Mykotoxin Workshop, 14. - 16. June 2010, **Lyngby, Denmark)**
2. **Application of LC-MS/MS and GC-MS technologies for detection of active fungal growth indoors influencing air quality. Vinay Vishwanath;** Michael Sulyok; Georg Weingart; Rudolf Krska **(Oral presentation**, **at the** 13. Mold Meeting, 2 – 3 Dec 2010, **Linz, Austria)**

Poster presentations

1. **Development of an LC-MS/MS multi metabolite screening method and its application for the analysis of dust and water damaged building materials. Vinay Vishwanath;** Michael Sulyok; Roman Labuda; Rudolf Krska (poster, presented @ CBS symposium –***Fungi and Health***" 13–14 November 2008, Amsterdam, Netherlands).

2. **Microbial toxins in surface swab and dust samples from school buildings in three European countries.** Mirko Peitzsch, Michael Suylok, Martin Täubel, **Vinay Vishwanath;** José Jacobs; Alicia Borras; Esmeralda Krop; Asko Vepsäläinen; Jan-Paul Zock; Anne Hyvärinen; Dick Heederik; Aino Nevalainen; and Lennart Larsson **(poster, Indoor Air Conference, Austin, Texas, 09.06.2011).**
3. **Validation of an HPLC-MS/MS based multi-analyte method for the determination of fungal and bacterial metabolites in indoor samples**. **Vinay Vishwanath;** Michael Sulyok; Rudolf Krska **(**poster, presented @ **31. Mykotoxin Workshop**, 5. - 17. June 2009, Münster, Germany).

4. **A versatile HPLC-MS/MS method for the screening/detection of fungal and bacterial toxins in matrices of public health concern, Vinay Vishwanath**; Michael Sulyok; Rudolf Krska, (poster, presented @ ISM 2009, 9-11 September 2009, Tulln/Vienna, Austria).

5. **Monitoring of metabolite production by indoor microbial isolates using liquid and gas chromatography coupled to mass spectrometry**.**Vinay Vishwanath**; Michael Sulyok; Georg Weingart; Bernhard Kluger; Beata Gutarowska; Roman Labuda; Rudolf Krska(poster, presented @ 58th ASMS Conference on Mass Spectrometry, **May 23-27 2010,** Salt Lake City, Utah, USA**.).**
6. **Occurrence of microbial toxins in indoor environments.** Martin Täubel; Michael Sulyok; Mirko Peitzsch; **Vinay Vishwanath**; Erica Bloom; José Jacobs; Alicia Borras; Esmeralda Krop; Asko Vepsäläinen; Jan-Paul Zock; Anne Hyvärinen; Dick Heederik; Rudolf Krska; Lennart Larsson; and Aino Nevalainen (poster, Organic Dust Tromso Symposium, Tromso, Norway, 03.-06.04.2011).

Preface

The dissertation research was supported by research projects IFUMEP (Österreichische Forschungsförderungsgesellschaft FFG) and HITEA (EU-FP7). The national project consists of an industrial collaboration with Romer Labs GmbH, Tulln, Austria. The IFUMEP project - in addition to determination of mold and bacterial metabolite prevalence in moisture damaged buildings - also aims for the development and production of calibrants to be used in MS methods for indoor evaluation purposes.

The second project, HITEA is an EU-FP7 initiative with a similar objective. The project intends to investigate indoor environments in schools. This is a multi-center study with sites in Spain, Finland, The Netherlands and Germany. A salient feature of this initiative is that the health statuses of all subjects are regularly monitored in addition to the building investigation.

The core objective of our projects IFUMEP and HITEA is the estimation of probable direct exposure to microbes/secondary metabolites as a consequence of occupation or living in water damaged buildings. Hence we measured of toxins, bioactive molecules and other toxicants that are bound to indoor matrices. In this regard we relied on chromatography and mass spectrometry based techniques. The LC-MS/MS method was dedicated for detection of microbial non volatile compounds and GC-MS method for microbial and non microbial VOCs. The methods were applied for evaluation of various indoor relevant matrices.

The metabolites included in our multi method are listed in publication 1 (table 1). To a great extent, inclusion of an analyte into the multi method was determined by ionization properties of the analyte (see chapter 2) and commercial availability of the substance for the use as analytical standard. Similarly, in chapter 3 various GC parameters (as sample volume, sample preparation and introduction methods, phase polarity and GC column efficiencies) are discussed. This is in order to show the dependency of individual parameters determining the final efficiency of GC-MS method. The experimental aspects of GC parameter optimization can be found publication 3.

As a part of both research projects dedicated analytical methods for indoor safety evaluation were developed and extended. Three international peer reviewed publications are compiled based

on the results generated during the dissertation research (attached at the end of the thesis). Similarly, three peer reviewed conference proceeding are attached with list of publication where findings of our study are exhibited. One conference proceeding, as well as two oral and 8 poster presentations were made by the author to disseminate the knowledge generated during the dissertation research.

“You cannot believe in any supernatural until you believe in yourself.”
-swami Vivekananda

Introduction

Importance of indoor safety

“An expert is a man who has made all the mistakes which can be made, in a narrow field.”
-Niels Bohr

Background: Criteria for indoor safety

Since indoor air quality refers to the quality of air in relation to health of occupants, spectrum of indoors requiring safety standards are practically limitless, e.g., interiors of buildings to inner space of an automobiles or an airplane. Moreover in the present days people spend relatively longer time (≥ 90%) in environments isolated by artificially barriers (altered life style, long working hours, indoor playgrounds), as a result indoor safety issue naturally evolve as a prime common denominator determining well being of individuals.

With respect to ensuring indoor safety the roles played by technical societies and organizations regulating standards for HVAC (Heating, ventilation and air conditioning) and building standards are of immense importance. Some of the recognized organizations monitoring HVAC standards are HARDI (Heating, Air-conditioning & Refrigeration Distributors International), SMACNA (Sheet Metal and Air Conditioning Contractors' National Association), ACCA (Air Conditioning Contractors of America) and IBC (International Building Code). Regarding indoor air quality many technical guidelines are available from ASHRE (American Society of Heating, Refrigerating and Air Conditioning Engineers) and are accepted worldwide. The frequently referred ASHRE recommendations are ventilation for acceptable indoor air Quality (ASHRAE 62-2001) (*1*) and thermal environmental conditions for human occupancy (ASHRAE 55-2004) (*2*). They are accepted as benchmark guidelines for healthy or general population. Hence these guidelines may not be appropriate for dealing exceptional cases as intensive care units of hospitals, houses inhabited by people with respiratory disorders and individuals with hypersensitivity.

An extract from Illinois department of public health (IDPH), environmental health homepage describing standard for acceptable indoor environmental quality is given below.

Parameter	IDPH	ASHRE	OSHA *	ACGIH **
Humidity	20% - 60%	30% - 60%	N/A	N/A
Temperature	20°C - 23°C (winter)	20°C - 24°C (winter)	N/A	N/A
	23° – 27° (summer)	23° - 26° (summer)	N/A	N/A
Carbon dioxide	1000 ppm, (<800 ppm preferred)	1000 ppm	5000 ppm	5000 ppm
Carbon monoxide	9 ppm	9 ppm	50 ppm	25 ppm
Hydrogen sulfide	0.01 ppm	N/A	20 ppm	10 ppm
Ozone	0.08 ppm	N/A	0.1 ppm	0.05 ppm
Particulates	150 µg/m^3 per 24-hr, (PM 10) 65 µg/m^3 per 24-hr, (PM 2.5)	N/A	15 mg/m^3 (total)	10 mg/m^3 (total)
Formaldehyde	0.1 ppm (office)	N/A	0.75 ppm	0.3 ppm
	0.03 ppm (home)	N/A		
Nitrogen Dioxide	0.05 ppm	N/A	5 ppm	3 ppm
Radon	4.0 pCi/L	N/A	100 pCi/L	4 WLM/yr (working level months/year)

* Occupational Safety and Health Administration (OSHA) Permissible Exposure Limit — this level is a time-weighted average and is an enforceable standard that must not be exceeded during any eight-hour work shift of a 40-hour work week. ** American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Value — this level is a recommended time-weighted average upper limit exposure concentration for a normal eight to 10-hour workday and a 40-hour work week. N/A-Not Applicable or Not Established. (http://www.idph.state.il.us/envhealth/factsheets/indoorairqualityguide_fs.htm)

The values recommended by all four authorities (IDPH, ASHRE, OSHA, ACGIH) for temperature and humidity are quite comparable. The levels of gases cited in this document are recommended only for use as a guideline value to determine the amount of fresh outside air entering a building. The resource website also warns for cautious consideration of guidelines in a case specific manner. This is particularly true for industries where there are potential sources of carbon dioxide other than exhaled breath. The occurrence of higher amounts carbon dioxide as compared to IDPH recommendation will be obvious in industrial indoors due to exhaust gases

from instruments, internal combustion engines, dry ice, etc. Hence before applying guideline values for indoor evaluation care must to be taken to gather sufficient background information related to an indoor as, purpose, area, mode of ventilation, number of occupant and heating etc.

Indoor environments with significant deviation from the above stated values are regarded as non ideal for living. The causes for deviation from safety standards can be due to many reasons in modern constructions. The causes in modern buildings could be due to malfunctioning or failure of any of the systems as exhaust for indoor emissions, ventilation, heating, water supply and sewage disposal or also due to construction characteristics (*3*). This can also be a situation in the aftermath of natural calamities as hurricane, tsunami and floods (*4*).

As the spectrum of pollutants is determined by the purpose of indoors, examination of different indoors is essential for generating an archive of pollutants. As part of projects IFUMEP and HITEA (respectively Austrian and European FP7 initiatives) we investigated various indoor samples with exposure relevance collected from schools and inhabited buildings. To the best of our knowledge the outcome of the projects can be instrumental for governmental authorities in setting up guidelines for indoor safety and also for medical professional in clinical diagnosis in ambiguous situations as sick building syndrome (SBS) and building related illness (BRI). The issues of indoor safety in addition to impacting health care system also can influence economy by impacting directly or indirectly various structures of the social system eg. Insurance organizations, construction companies and building material manufacturing units etc.

The diseases sick building syndrome (SBS) and building related illness (BRI) are two classical examples relating poor indoor standards to health status of occupants. Both diseased conditions are attributed with many similarities but still differ in a subtle manner.

Indoor related health problems

Sick building syndrome (SBS)

Sick building syndrome is attributed with non objective physiological abnormalities. However it carries a greater importance as it could affect the whole or major part of the work force rendering them non-functional. A situation is regarded as SBS when a considerable percentage of (≥20%) building‘s occupants experience set of non specific symptoms such as headache, fatigue irritation

of mucous membrane (eye, nose and throat), eye symptoms (itching, redness and irritation), respiratory symptoms: nasal congestion, itching, coughing and running nose, throat symptoms: dryness and irritation (*5*). Since symptoms above mentioned are non quantifiable, inspection of buildings is a frequently resorted option for finding the actual cause for the disease. SBS is regarded as a multi factorial syndrome with the ventilation insufficiency regarded as prime responsible factor (*1*). While drawing conclusion with respect to the causes responsible for observed disease symptoms, multiple hypotheses must be considered. The causes can be ventilation rates, type of ventilation system, maintenance, chemicals or irritants from occupant activities, microbial contamination and off-gassing from building furnishings. A classical example for accumulating volatiles influencing individual health is documented for exposure to volatile organic compounds (VOCs) toluene, o-xylene and N-butyl acetate (*6*). Correlation of long term exposure to these substances is known for the manifestation of clinical symptoms such as concentration deficit, poor memory and increase in the reaction time (*6-7*). These are some of the evidences for ill effects of VOCs observed in adults working in automobile painting units. The given example may be an extreme situation of occupational hazard. However low levels exposure to above mentioned solvent vapors in houses or any non industrial environment cannot be completely denied (common solvents used in paints, varnishes, lubricants).

Building related illness (BRI)

The BRI are less common compared to SBS but often associated with more serious symptoms affecting small number of building occupants. The BRIs are classified as specific and non-specific based on observed symptoms. Specific BRI applies to a group of illnesses with a fairly homogeneous clinical manifestation and with known etiology (infections, allergic responses). These include legionellosis, hypersensitivity pneumonitis, humidifier fever, asthma, allergic rhinitis and toxic syndromes associated with exposure to chemical or physical agents. The non-specific BRI refers to a group of heterogeneous and non-specific, work-related symptoms such as irritation of skin, mucous membranes of the eyes nose and throat, headache, fatigue and concentration difficulties. The causes for non specific BRI are mostly related to inadequate ventilation, humidity and temperature fluctuation, chemical and biological contaminants from indoor and outdoor sources (*8*). The probable biological agents responsible for the above mentioned clinical manifestations are detailed in Chapter 1.

Review of analytical methods for indoor quality evaluation

The issues related to indoor evaluation are demanding and are often beyond the control of any of the traditional regulatory tools. Due to these obvious reasons indoor prevalence of various mycotoxins, individual‘s exposure to pollutants and toxicological consequences are less known. Considering the range of pollutants prevailing in indoors, microbes are only a part of the whole burden. However with their ability of rapid amplification (on availability of optimal humidity and temperature) they are still considered critical in determining the indoor environmental quality. Moreover mycotoxins are reported for causing acute inhalation toxicity (T2 toxin) in experimental animals (*9*). This property of mycotoxins makes the situation worse in both water damaged and healthy buildings (*10*). Hence it is desirable to have methods for onsite determination of both viable and dead microbes and/or their bioactive metabolites in indoor environments.

Nevertheless the present available methods for on-site determination of mycotoxins are highly focused for a particular species or class of analytes and may not be appropriate for comprehensive indoor evaluation. The reports of McKean et al. 2006 for synergistic effects of mycotoxins (*11*) once again stress the importance of multi mycotoxin investigation. The presently available methods are based on direct microscopy and taxonomy (*12-13*), microbial viability (*14-15*), genetic method (*16*), antibody based detection (*17*), analysis of microbial volatiles (*18-21*) and on non volatiles (*22-25*). Each of the sighted technique is with inherent merits and drawbacks. The methods based on direct microscopy are quicker but are questionable and challenging as concerning to individual‘s competency and matrix interference respectively (for quantitative analyses) (*26*). The culture based methods are well established for several decades. But as a whole only 1-10% of the airborne microbes are amenable for culturing on commonly used media. Due to this inherent drawback the culture based methods are considered cost, time and labor intensive. Unlike fungi, bacterial speciation are not straightforward, instead requires a series of technical procedures such as staining, biochemical tests and DNA sequencing (*27*). The major challenges for PCR screening of environmental samples (e.g. dust, infested building materials or furniture) are PCR inhibiting substances and non specific amplification of DNA. Besides this biological characteristics of spores (difficult structure to rupture) and

sensitiveness of PCR to DNA extraction procedures are two other factors that complicate the investigation (*28*). In spite of their higher sensitivity, genetic methods are not widely accepted in environmental analysis. This is mainly due to their inability to differentiate viable and non viable microbes. Hence PCR based methods may not be apt for monitoring indoor status, building renovation or quality of building materials. The methods based on antigen-antibody reaction are more suitable for the detection of larger molecules such as proteins and peptide (immunogens). Hence these are methods of choice for detection and quantification of allergens and other macro molecules (*29-31*). Similar to mycotoxins VOCs are also known to have synergistic sensory-irritation effects (*32*). The state-of-the-art technology GC-MS is a powerful tool for detecting hidden molds (*33*) and also for chemotaxonomy of fungal strains (*34*). Occasionally, extremely low MVOC concentrations and many disturbing concomitants indoors are reported to complicate analysis of microbial VOC in moldy buildings (*35*). However GC-MS is still one of the widely accepted techniques in indoor air quality evaluation. Secondary metabolites being inconsistent in their distribution throughout the fungal kingdom are widely accepted as unique markers for speciation and chemotaxonomy purposes (*36*). The same was also reported for fungal species found in indoor environments as well (*37-38*). Most of the LC-MS/MS methods focused on mycotoxins are dedicated for the detection of mycotoxins in food and feed commodities (*39-42*). There are few methods so far reported for the detection of handful number mycotoxins in indoor environments (*22, 43*).

Based on the literature survey we decided to investigate bioactives (such as microbial toxins) and other anthropogenic chemicals that are bound to indoor matrices. In this regard we developed mass spectrometry based methods GC-MS and LC-MS/MS capable of screening volatile and non volatile metabolites respectively. The methods were applied for the evaluation of indoor relevant matrices and SFD. The specific advantages of chosen analytical methods are described in chapters 2 & 3.

Chapter 1

Micro flora of indoor environments

“I have not failed. I've just found 10,000 ways that won't work.”
-Thomas A. Edison

1. Biological factors in indoor environment

The spectrum of pollutants found in indoor environments is determined by combination of animate, inanimate, climatic and geographical factors. Biological factors in indoors are a heterogeneous group of particles belonging to plants, animals and microbes. Though the list of indoor pollutants is endless, considering aims of the research projects HITEA and IFUMEP, our study objectives were limited to evaluation of relevant microbial secondary metabolites and mycotoxins. Unlike their counterparts biological entities (microbes and insects) multiply their number when the environmental conditions are conducive. Due to this reason the microorganisms predominantly found in outdoor environments are also seen prospering in indoors as well.

1.0 Mycotoxins and microbial secondary metabolites

Mycotoxins are secondary metabolites with no known biochemical significance in fungal growth and development. However the exact causes for the production of mycotoxins are yet to be determined. These secondary metabolites are inconsistent in their distribution throughout the fungal kingdom (*36*). A particular mold species may produce many different mycotoxins and/or may produce same mycotoxin as another species (*44*). Since mycotoxins can weaken the receiving host, fungi are assumed to use them as a strategy to improve the environment for their proliferation (*45*). Mycotoxins are produced mainly by the mycelial structure of filamentous fungi (*46*). The mycotoxin production varies according to the surrounding intrinsic and extrinsic environments (*46*). The classes of fungal secondary metabolites include polyketides, non-ribosomal peptides, terpenes and indole terpenes (*45*). It is well established that not all molds are toxigenic and similarly not all secondary metabolites from molds are toxic (*46*). In case of toxic secondary metabolites the modes of action of mycotoxins are by induction of carcinogenicity and cytotoxicity, disruption of macromolecule synthesis (DNA and RNA), competitive inhibition, impairment of membrane transport and immune suppression (*46*). Similar to fungi, bacteria are also known for producing secondary metabolites. Compared to fungi the mechanisms governing the bacterial secondary metabolite production are fairly better understood. The synthesis and secretion regulation is influenced by unique low molecular weight compounds, transfer RNA,

sigma factors and gene products formed during post exponential development (*47*). Under laboratory conditions secondary metabolites such as antibiotics are accumulated by the bacterial cultures at their stationary phase of growth. The exact purposes of secondary metabolites in bacteria are also not well known. Most of the known bacterial metabolites are viewed as bacterial pheromones (*48*). Bacterial secondary metabolites are regarded as both beneficial and deleterious (antibiotics and food contaminants). The properties and clinical relevance of various indoor relevant secondary microbial metabolites are described along with the species in this chapter. Moreover sum formulae and chemical structures of mycotoxins found during investigation of various indoor environments are presented in Appendix.

1.1 Bacterial species in indoors

Bacteria are important to humanity as natural scavengers of organic debris to producers of common food products (vinegar). Besides these they are also known for causing infections, allergic reactions and food contamination with extremely potent toxins. Many of the bacterial floras naturally occurring on human and animal skin are continuously emitted into the immediate surroundings (*49*). Apart from the microbes living symbiotically with humans and pets, antibiotic resistant strains of bacteria are gaining attention (*50*). The indiscriminate use of antimicrobial has lead to resistance among bacterial species responsible for infection among pet animals. Assessing antimicrobial resistance among bacteria as well as the risk of transmission of antimicrobial resistance to humans is other major challenge related to bacteria.

The bacterial species freely living in nature are rarely found amplifying in indoor environments. However exceptions are in the event of severe moisture damage due to natural calamities (floods, tsunami and hurricane) or inadequate maintenance of pluming and ventilation systems. Nevertheless the bacterial diversity in indoor environment too is sensitive for seasonal fluctuations (*51*). The bacterial species that are relevant to indoor environments are species of *Micrococcus, Bacillus, Nocordia, Coryneforms*, *Streptomyces*, *Staphylococcus, Enterococci*, *Salmonella* and *E.coli.* However most of the bacterial species are opportunistic pathogens. Species of *Streptomyces* are both opportunistic pathogens and indicators of water damage. The above mentioned species are briefly described to show their indoor and clinical relevance. For reader‘s information: With the exception of *Streptomyces* other species so for are not known for

amplification in indoor environments. Moreover due to limitations such as ionization properties of the analyte (see chapter 2) and commercial availability of the substance for the use as analytical standard all species listed could not be MS screened for their occurrence in indoor environments.

1.1.1 *Micrococcus*

Micrococci are common colonizers of human body and are rarely regarded as pathogenic to human beings. The occurrences of high levels of *Micrococcus* are frequently associated with poor ventilation in houses. Common *Micrococcus* species are *M. luteus, M. roseus, M. varians, M. denitrificans, M. freudenreichii* and *M. colpogenes*. The salt tolerant gram positive bacteria also survive minimal water availability and can grow on a wide range of unusual substrates- such as pyridine, herbicides, chlorinated biphenyls, and mineral oil (*52*). Rarely *Micrococci* are also associated with blood infections (sepsis), pneumonia and endocarditis (*53*).

1.1.2 *Streptomyces*

The genera are Gram positive spore forming bacteria with G+C rich genome. *Streptomyces* are oldest allies of mankind as producers of antibiotics and also rare causes for invasive infection in humans. They are accountable for the production two third of clinically important antibiotics. The diseases related to *Streptomyces* are catheter bacterimia, and *Streptomyces* pneumonia in patient with human immunodeficiency virus (HIV) infection (*54*). The *Streptomyces* metabolite valinomycin is known for causing mitochondrial toxicity (*55*) and inflammatory responses in human alveolar epithelial cell line (*56*). Species of *Streptomyces* are frequently reported for their co-occurrence with fungi in water damaged buildings (*55, 57*). The clinical relevance of all known *Streptomyces* metabolites is yet to be ascertained. Some of the widely used *Streptomyces* metabolites as antibiotics are oligomycins, valinomycin, puromycin, geldanamycin, and bafilomycin. Nevertheless metabolites such as antibiotics can also be beneficial for purposes other than their intended therapeutic application. In our study we used them as chemical bio markers for detection of *Streptomyces* in indoor environments. (included in our method, publication 1 [table 1])

1.1.3 *Bacillus*

Bacilli are the ubiquitous bacterial genera living both as free living in nature and as pathogen associated with animals. These bacteria evade adverse environmental conditions by producing endospores. Species of bacillus are known for causing food borne infection and veterinary diseases. The insect pathogens *B. thringenesis, B. sutilis, B.anthracis and B.cereus* are extensively studied species because of their economical and clinical significances (*58*). Some of the macromolecular toxins of *Bacillus* are emetic toxin (ETE) and enterotoxins: HBL, Nhe, and EntK (*B.cereus)*, PA+LF and PA+EF (*B anthracis*) and delta-endotoxin *(B. thringenesis).*

1.1.4 *Coryneforms*

Corynebacteria occur commonly in nature as inhabitants of soil, water, plants, and food products. These are Gram positive, rod shaped bacteria with aerobic and facultative anaerobic modes of respiration. Similar to *Actinobacteria*, *Corynebacterial* genome is composed of high G+C content. These organisms are known for infecting both domesticated animals and humans. Some of the *Corynebacterial* infections are diphtheria, urinary infections and catheter bacterimia (*59*). The virulence factors of *Corynebacterium diphtheriae* responsible for diphtheria are extensively studied bacterial toxins.

1.1.5 *Staphylococcus*

Species of *Staphylococcus* are also the normal flora of healthy individuals (*49*). The dispersal of *Staphylococcus* into air mainly occurs with desquamation of cells. *S. aureus* are responsible for several skin infections, pneumonia, meningitis and sepsis. The methicillin-resistant *Staphylococcus aureus* (MRSA) strains are often found associated with hospital and clinical practices. Moreover several studies in veterinary hospitals have also indicated the presence of MRSA strains on skin surfaces of pet animals. These species are regarded as threats causing community-acquired infections. *Staphylococcus aureus* produce species specific endotoxins such as superantigens, Exfoliative toxins (EF) and toxin capable of acting on cell membrane (*60*).

1.1.6 *Enterococci*

Enterococci are gram positive bacteria tolerant to a wide range of environmental conditions. These species are known for co-occurrence with other species of *Staphylococci*, *Pseudomonas aeruginosa* and heterotrophic bacteria in water parks. Hence *Enterococci* are often investigated along with other bacterial species for ensuring the safety of public health. The species *E. faecalis* and *E. faecium* are frequent commensals of humans. *Enterococci* are known for causing bacteremia, bacterial endocarditis, diverticulitis and meningitis (*61*). Literature availability regarding their toxins and other metabolites are limited.

1.1.7 *Salmonella*

These are gram negative, rod shaped non-spore forming bacteria. Species of *Salmonella* are zoonotic and can be transferred between humans and other animals. *Salmonella* are tolerant to freezing temperatures for many weeks. This long term survival ability of the species makes them critical in indoor environments. *Salmonella* are known for causing gastroenteritis, salmonellosis and for the inhibition of innate immune system by the secretion of AvrA toxin (*62*). Critical indoor sources are poorly maintained kitchens, stagnant polluted surface water (eg. improper plumbing, hidden water lodging) and inadequately preserved meat.

1.1.8 *E.coli*

Escherichia are gram negative rod shaped bacteria living in lower intestine of endothermic animals. Generally these are harmless microbes with exception of serotypes responsible for food poisoning. The major modes of transmission of pathogenic strains are by fecal-oral transmission. The indoor relevance of *E.coli* are reported by of Rosas I et al. 1997 (*14*) for the detection of antibiotic multi resistance *E coli* serotypes in settled floor dust samples derived from the houses of Mexico City.

1.2 Fungal species in indoor environments

The fungal growth in the indoors are undesirable as they can cause diseases to occupants and damage to buildings. The problems directly associated with fungi and building are due to rot fungi *Serpula* and *Proria* that can deteriorate mechanical firmness of wooden structures. In addition to rot fungi, the other fungal species associated with damp buildings are gaining attention because of their clinical relevance to Sick Building Syndrome (SBS) and Building Related Illness (BRI). There are many reports concerning prevalence of new fungal species in indoors. However all reported species are not proven for their relevance to indoor safety and occupational hazards by exclusive studies. In the next parts of the chapter clinical and toxicological aspects of various indoor fungi and their role in manifestation of various symptoms observed in SBS and BRI are discussed.

The fungal secondary metabolites production in indoors and microbial succession are determined by two major factors humidity and temperature. Based on microbial succession indoor fungi are classified into three different classes. Primary colonizers or storage molds that can grow at $a_w < 0.8$, secondary colonizers or phylloplane fungi - requiring a minimal a_w between 0.8 and 0.9 and tertiary colonizers or water damage molds that grows at $a_w > 0.9$ (*63-64*).

“Restlessness is discontent and discontent is the first necessity of progress.”
-Thomas A. Edison

1.3 Fungi and buildings

1.3.1 *Meruliporia incrassate (Poria Incrassata)*

The *Poria* invasion into a building is always considered catastrophic. *Meruliporia incrassate* is also known as *Poria incrassata,* which is a common dry rot fungus found in the United States. The fungus is capable of infiltrating foundation, wood, concrete and soon the entire house. In comparison to other decaying fungus this species is highly temperature sensitive and hence only grows in the more shaded parts of the structures and not on wood exposed to bright sunlight (*65*). The control and remediation strategies are relatively simple - as adequately heating and eliminating moisture sources in these buildings. However due to its secretive mode of penetration always makes controlling becomes a challenging task. Once well established it can destroy large areas of floors and walls. Literature availability regarding the toxins and other metabolites of *Poria* is limited.

1.3.2 *Serpula lacrymans*

The temperature sensitive Basidiomycete *Serpula lacrymans* is rarely found outside the built environment. This species is particularly common in poorly maintained old buildings of northern Europe. However distribution of *Serpula* and reports for its occurrence in other parts of the world are scanty. The reports for its occurrence in forest or other natural habitat in Europe are confined to the only reports of Palfreyman et al. (*66*). The fungi require elevated moisture levels for survival (>20% moisture content). The predominant carbon sources are wood and other cellulose rich matrices. Fungi use a non-enzymatic mechanism for depolymerisation of cellulose. The species is versatile and can also grow on non woody materials as plaster, brick and stones. From such alternate sources the organism extracts calcium and iron essential for facilitation of degradation processes (*67-68*). It uses calcium and iron extracted from building materials to aid the breakdown of wood, which results in brown rot (*69*). So far no allergenic compounds are known for this fungus. However asthma and extrinsic allergic alveolitis are associated with *Serpula lacrymans* (*70-71*).

1.4 Fungi and health

1.4.1 *Stachybotrys*

In the indoor context the *Stachybotrys* species are regarded as critical indicators of water damage. They are commonly referred as black mold due to their dark pigmentation. The genus *Stachybotrys* is worldwide in distribution, (*72-73*). Corda in 1837 was the first to isolate *Stachybotrys* growing on domestic wallpaper. The first experimental reporting for equine illness is credited to Drobotko et al. (1940). He also coined the term ―stachybotryotoxicosis" and described symptoms of the disease. The stachybotryotoxicosis in human were mainly observed among the fodder handlers and others in contact with musty straw. They suffered due to dermatologic and respiratory disorders. Primarily disease manifestations were seen on the skin, dermatitis on scrotum, medial thighs, axilla and less frequently on hands and other areas. Considering the lesion location the transmission was thought rather due to aerosolisation of offending substance (*74*).

The dematiaceous *Stachybotrys* species are relatively stable over a wider temperature range (<60°C). However they require a high a_w greater than 0.9 for their proliferation. Hence *Stachybotrys* are regarded as the tertiary colonizers of indoor. *Stachybotrys* species produce wide range of metabolites with complement activation, receptor antagonist, phytotoxic, cytotoxic, and cytostatic properties. The toxins are generally with empiric formula of $C_{25}H_{34}O_6$ or $C_{26}H_{38}O_6$ which are structurally comparable to trichothecene class of metabolites produced by other fungal species. Some of the biomarker metabolites of *Stachybotrys* are spirolactams, spirolactones, phenylspirodrimanes, stachybotrylactam, satra toxins and cyclosporins (FR901459) (included in our method, publication 1 [table 1]). The Trichothecenes are assumed to act as protective shield for fungus against adverse conditions such as sun-light, UV light, X-rays, heat (up to 120°C), and acids. The reports for satratoxin G–albumin adducts (in vivo) upon human and animal exposures to *S. chartarum* or spores are some evidences for probable accumulation of *Stachybotrys* mycotoxins in human body (*75*). This further emphasizes the criticality of *Stachybotrys* in relation to inhabitant's safety living in water damaged indoor environments.

1.4.2 *Aspergillus*

Aspergilli are one of the primary colonizers of the indoor environments. They grow around a_w below 0.8 and temperatures range of 30-37^oC. On the availability of moderate to high relative humidity *Aspergilli* undergo species succession. During the favorable condition species grow and aerosolize innumerable number of spores making them major indoor contaminant. Moreover affecting the indoor air quality *Aspergilli* are also known for causing various respiratory disorders and allergic reactions. Some species of *Aspergillus* can induce type I or atopic allergy, whereas *A. fumigatus* is one of the major causes of type III hypersensitivity pneumonitis and allergic sinusitis (*76*). The species are known for producing carcinogenic and tremerogenic toxins. The mycotoxins of *Aspergilli* are aflatoxins (*A.flavus*), sterigmatocystin (*A. versicolor*), ochratoxins (*A. ochraceus*), austins (*A. ustus*), patulin (*A. terreus*) and fumitremorgins (*A. fumigatus*) (*77*) (included in our method, publication 1 [table 1]).

1.4.3 *Trichoderma*

Trichoderma species are ubiquitous in their distribution. They are common isolates of soil, wood and wood based products. They are tertiary colonizers of water damaged indoor environment. Thrane U et al. (2001) reported for the occurrence of 44 different strains of Trichoderma in water damaged buildings (*78*). Two species *T. longibrachiatum* and *T. citrinoviride* are also known to be opportunistic pathogens in immune suppressed patients (*79*). Isolates of *Trichoderma* are known to produce mycotoxins such as the trichothecenes trichodermol, trichodermin (acetyl ester of trichodermol) and other esters of trichodermol, gliotoxin and viridian (included in our method, publication 1 [table 1]). Besides mycotoxins numerous low molecular weight compounds such as volatile pyrones and lactones, ribotoxic peptaibols, cytotoxic proteins and iso-nitriles are known to be produced by *Trichoderma* species (*80*). Still some controversy exists regarding the production of diacetoxyscirpenol by indoor isolates (*81*). As it would require trichothecene synthesis over iso-trichodermol (*82*). The only report for indoor isolate for trichothecene bio synthesis is by Corley et al. 1994 (*83*).

1.4.4 *Exophiala (Black Yeasts)*

The Black Yeasts or *Exophiala* are absent in outdoor environments. The fungi are oligotrophes and grow at temperatures above 37°C (*84*). They have an extracellular polysaccharide capsules which is regarded to be the virulence factors. The versatile adaptability of Black yeast to human habitat is by virtue of stress protection provided by melanin pigmentation. High colony counts of Black yeasts are common occurrence in steam rooms and public bathing facilities along with other tertiary colonizing mycota (*85*). It is hypothesized that *Exophiala* black yeasts live on trace amounts tannins precursors and other polyaromates (*86*). In indoor environments they are suspected to survive by the assimilation of trace amounts of VOCs while growing on unusual substrates. *Exophiala dermatitidis* is reported to be a rare etiologic agent of central nervous system among healthy adolescent patients (*87*). The two other species known for human dermal infections are *Exophiala xenobiotic* (cutaneous) and *Exophiala jeanselmei* (sub cutaneous) (*88*).The soil derived *Exophiala pisciphila* produce two indole alkaloids and a chromone derivative with cytotoxic properties (*89*).

1.4.5 *Phialophora*

Phialophora require high relative humidity, similar to other tertiary colonizers of indoors for their survival. They are characteristic of producing flask shaped phialides with round or ovoid conidia. *Phialophora* are known for infecting plants and animals (*90*). They are common saprobes of soil, wood, pulp or any other plant materials. The reports of *Phialophora verrucosa* in potted plants in hospitals along with other potentially pathogenic fungi (*91*) suggests existence of other sources harboring pathogenic strains in indoors. The clinically important strain *Phialophora richardsiae* is associated with chromoblastomycosis and phaeohyphomycosis. To the best of our knowledge there are limited reports describing mycotoxins or *Phialophora* metabolites. Species *Phialophora asterris* is known for the production of (–)-cryptosporiopsin with antibiotic properties and antimicrobial properties against an array of plant pathogenic fungi and other microorganisms (*92*).

1.4.6 *Ulocladium*

The presence of *Ulocladium* is considered as an another potential indicator for building moisture damage. They form the tertiary colonizing mycota of indoor environment and require higher relative humidity for spore germination and proliferation. They are phylogenetically similar to *Alternaria* but are differentiable from later in terms of chemical markers. *Ulocladium* can degrade cellulose and are frequently found growing on drywall paper, ceiling tiles and other cellulose rich materials. The species *U. atrum, U. botrytis* and *U. chartarum* are common indoor isolates. Species specific mycotoxins of *Ulocladium* are curvularin and interfectopyrones and derivatives of altertoxin I (*93*) (included in our method, publication 1 [table 1]). Known health implications of *Ulocladium* are keratitis (*U. atrum)*, oncomycosis (*U. botrytis)* and other cutaneous infections (*U. chartarum).*

1.4.7 *Fusarium*

Fusarium is a large genus of filamentous fungi found in soil. They are investigated worldwide for their role impacting food and feed chains. Predominantly, outdoor species of *Fusarium* are found associated with indoor occurrence. They gain relevance with their access into indoors due to ventilation, wind currents and also due to contaminated grains or other food commodities. Some of the species are opportunistic pathogens of human and animals. Among immune individuals *Fusarium* species are reported for causing body and blood infections (*Fusarium solani*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Fusarium proliferatum* and, rarely, other *Fusarium* species) (*94-96*). These species are reported for onychomycosis (*97*) and keratomycosis or mycotic keratitis (*98*) among individuals with compromised immune system. The major toxicological threats due to *Fusarium* are mainly due to mycotoxin entry through food and feed chains. More than 50 species are known for producing mycotoxins on cereals or cereal plants such as barely, wheat and maize. The main toxins produced by these *Fusarium* species are zearalenon and its derivatives, fumonisins (neurotoxin and carcinogen) and trichothecenes (neurotoxin), beauvercin and enniatins, butenolide, equisetin, and fusarins (*99*) (included in our method, publication 1 [table 1]).

1.4.8 *Chaetomium*

The *Chaetomium* species require relatively high humidity for survival and proliferation and (*100*). Spores are produced within flask-shaped bodies (perithecia) and not readily exposed to air. The toxic ascomycete genus *Chaetomium* is other most frequent indoor fungal contaminant (*100*). In indoor environments *Chaetomium* isolates were reported from kitchens, bathrooms, wall paper, mattresses, carpets and window frames (*101*). The presence of spores in air samples is considered as an indication of a mould problem in the buildings. Most of the species are prolific producers of cellulases. Due to their strong destructive nature, *Chaetomia* species are often used for testing cellulose based material resistance to mould growth. The widespread and common species is *Chaetomium globosum*. *Chaetomium* are known for causing economic damages to mushroom cultivation. The species are associated with oncomycosis and invasive mycotic Infections (*Chaetomium perlucidum).* Toxic metabolites produced by *Chaetomium* are chaetoglobosins, sterigmatocystin and o-methylsterigmatocystin (*Ch. globosum, Ch. gracile, Ch. homopilatum and Ch. Virescens*). Moreover *Ch. Globosum* are attributed for production of antibacterials, chaetocins and cochliodinol (*44*) *(102)* (included in our method, publication 1 [table 1]).

1.4.9 *Phoma*

The *Phoma* species can be found growing on plant debris and also dispersed in soil. In indoors they are one of the tertiary colonizing mycota requiring relatively high a_w for their proliferation. These species are often reported for their growth in bath rooms (wooden constructions, wall paper, in settled dust), swimming pools (sporadic) and other parts of buildings accommodating high humidity (*103-104*). The common species in indoor environments are *Phoma glomerata* and *Phoma macrostoma*. These species are mainly regarded as contaminants. However rarely they cause infections among individuals with a weakened immune system.

1.5.0 *Cladosporium*

Cladosporium are predominantly found in outdoor air. Due to their ubiquitous presence they are not regarded as important indoor species, unless and otherwise present in extremely high concentrations (*105*). When present in indoors they are regarded as secondary colonizers (*106*). They are characterized by darkly pigmented mycelia and brown spores produced on branching

chains. They grow on window sills, painted walls, and other cellulosic materials. Common indoor species are *Cladosporium herbarum, Cladosporium sphaerospermum, Cladosporium cladosporioides and Cladosporium macrocarpum.* The hyper parasite of rust fungus *C. tenussimum* is reported for producing cladosporals (*107*). A few species are known for producing toxins and it is one of the common fungal agents in indoors responsible for inducing allergic reactions.

1.5.1 *Penicillium*

Pencillium species are primary colonizers of indoor environment and grow at a_w below 0.8. In the absence of adequate R^H they imbibe water from wet surfaces and continue to proliferate. Spores are dry, 3-5μm in diameter and are amenable for easy dispersal by wind currents. A wide variety of *Pencillium* species are known to grow on indoor surfaces as drywall, wood, carpet, painted surfaces, wallpaper, and various types of household articles. Many species are producers of mycotoxins such as penicillic acid (*P. aurantiogriseum*), viridicatin (*P. viridicatum*), ochratoxins (*P. verrucosum*), luteoskyrin (*P. islandicum),* rugulosin (*P. variabile)*, penitrem A *(P.crustosum*), patulin and griseofulvin (*P. griseofulvum*), citrinin *(P. citrinum)* and rubratoxin (*P. crateriforme)* and many others (*105, 108-109*) (included in our method, publication 1 [table 1]). Species of *Penicillium* are non pathogenic to human, however they are known for causing food spoilage. Inhalation of *Penicillium* spores containing mycotoxins is implicated as one of the contributing factor for Organic Dust Toxic Syndrome (ODTS) and Non-infectious Fungal Indoor environmental Syndrome (NIFIES).

1.5.2 *Eurotium*

Eurotium is a xerophylic fungus and a primary colonizer of indoor environments. These species can be found growing on a variety of substrates with limited water availability. Species *E. herbariorum, E. amstelodami, and E. rubrum* are common building isolates reported from Canada and rest of the world. Toxicological reports concerning *Eurotium metabolites* are limited. However reports of various species of *Eurotium* and their metabolite spectrum are available. Some of the *Eurotium* metabolites are neoechinulin A, neoechinulin B and epiheveadride (*E. amstelodami* and *E. rubrum),* neoechinulin A&B, cladosporin (included in our

method, publication 1 [table 1]) and epiheveadride (*E. herbariorum).* Minor aggregates of flavoglaucin, auroglaucin and isotetrahydroauroglaucin. Echinulin, preechinulin, and neoechinulin E are production by all aforementioned species of *Eurotium* (*110*). *Eurotium* are related with inhalation health risks among individuals with weak immune system.

1.5.3 *Wallemia*

The *Wallemia* are generally xerophytic fungi (primary colonizer), capable of growing over a wide range of water activity from 0.69 to 0.997 (*111*). *Wallemia sebi* is commonly found in indoors closer to agricultural fields. The xerophilic fungus can grow on range of matrices spanning between dry wall, salted fish or jam. In some parts of the world *Wallemia* are suspected to be the causative agent for farmer‘s lung disease. Similarly the airborne *W. sebi* is associated with human allergies, particularly bronchial asthma (*112*). Elevated levels of immunoglobulin G (IgG) antibodies are reported among Finnish farmers exposed to *W. sebi (17).*The fungus produces walleminol A, a toxic metabolite with an inhibitory dose effects similar to penicillic acid (*113*)

1.5.4 *Engyodontium*

Engyodontium are ubiquitous in their distribution. The adaptability of species makes them versatile survivors. The most common species, *E. album*, is a cottony, white mold producing numerous, tiny dry conidia. The number of reports for its incidence or characterization is limited. These species are regarded as opportunistic and infectious pathogens among immune compromised individuals (*114*). The first report of *Engyodontium* occurrence (to best of our knowledge) and also for the incidences of keratinolysis among children living in water damaged homes are from Slovakia (*115*). None of the species are described for production of any of the mycotoxins.

1.5.5 *Petriella*

Pteriella are common indoor fungi observed in bathrooms, underneath kitchen sinks and also on other wet wooden structures. The fungal proliferation is relatively slow, produce cirrus reddish brown ascospores. They are reported from tape samples, surface swabs, carpet dust and also

observed in spore-trap samples from houses undergoing renovation. Species *P. sordida* and *P. setifera* are reported from indoors but till date none of the species are associated with production of mycotoxins or any other harmful health implications (*116*).

1.5.6 *Alternaria*

The species *Alternaria* are predominantly found in outdoors. Their occurrence indoors could be attributed to exchange of air between indoor and outdoor environment. They produce large crosswise and lengthwise septate condia. The fungal spores can germinate at 25^{o}C and a_w of 0.85. These species are capable of degrading cellulose and can be found growing on drywall paper, ceiling tiles and wood materials. The allergic reactions (penumonitis, bronchitis) are frequent observations among individuals frequently exposed to saw dust containing *Alternaria* allergens (*117*). *A.alternata* is the most common cause of allergy and asthma among children. Some of the known mycotoxins of *Alternaria* are alternariol, alternariol monmethylether (AME), altenuene, altertoxin and tenuazonic acid (included in our method, publication 1 [table 1]).

1.5.7 *Acremonium*

The genus *Acremonium* are widespread in soil and plant debris. In indoor they are frequently found growing in HVAC systems (heating, ventilation, and air conditioning) and humidifiers. Common indoor species are *A. strictum, A. terricola, A. bacillisporum*. Species *Acremonium* include many pathogenic strains which are known for causing opportunistic infections in human (*116*). A few species are known to produce the mycotoxin citrinin with nephrotoxicity in experimental animals (*118*) (included in our method, publication 1 [table 1]).

1.5.8 *Absidia*

Absidia are the zygomycetes that are frequently found in soil and plant debris (*119*). The species are known for their prevalence in indoor environments (surface and air samples). None of the species are so far are reported for producing any mycotoxins on building materials. However the species *Absidia corymbifera* is associated with zygomycosis in hospitals. The primary sources of infection are not clearly known. Nevertheless the secondary sources of infection are due to

infected elastoplast adhesive bandage rolls, contaminated ventilation systems, and inadequately maintained patient shower cabins (*120*).

1.5.9 *Mucor*

Mucor is another zygomycetes frequently found in soil, plant debris and animal dung. Majority of *Mucor* species are not known for producing any mycotoxins in indoors or in natural habitat. However 3-nitropropionic acid was reported in the culture extract of *Mucor circinelloides* isolated from grass silage (*121*) (included in our method, publication 1 [table 1]). Some strains of *Mucor* are reported for causing opportunistic human infections and Mucormycosis (*119, 121-122*). In a rare case, species *Mucor circinelloides* is also known for causing zygomycosis.

1.6.0 *Myxotrichum*

This ascomycete (Myxotrichaceae) is frequently found in soil and in indoor environments growing on paper substrates, damp drywall, decomposing carpets and cardboard (*123*). Species as *M. deflexum*, produce a pinkish red pigment and may produce visible stains on the paper surface. No reports of mycotoxins, pathogenicity or allergy are known.

1.6.1 *Ophiostoma*

Ophiostoma belongs to species ascomycete and genus *Ophiostoma* and known as plant parasites. The spores produced are sticky and are not readily airborne unless disturbed (*124-125*). The *Ophiostoma* presence in indoors are not related to any sort of building moisture problems, but as a direct consequence of their association with timber used for construction. Hence they are commonly referred as ‗lumber molds'. There are no reports for production of mycotoxins or disease associations for this genus of fungi.

1.6.2 *Scopulariopsis*

Scopulariopsis, a soil saprophyte and a common isolate from air and cellulose rich building materials. Several species are known for causing opportunistic infections. *S. brevicaulis* is a strain resistant to wide range of antifungal agents and associated with onychomycosis (*114, 122*). The other species often reported from indoor environments are *S. brevicaulis, S. brumptii,*

S.candida, and *S. asperula.* Reports for production and release of mycotoxins or other toxic metabolites do not exist.

1.6.3 *Paecilomyces*

Species *Paecilomyces* are isolated from air, damp walls, wet plaster, carpet dust, HVAC system, soil, indoor air, and wood (*126-129*). These species are generally non pathogenic but are emerging as opportunistic pathogens in immune compromised hosts causing mycoses. Infections due to *Paecilomyces* are referred to as paecilomycosis. Paecilomyces are also known for causing allergic alveolitis (*130*). Separate isolates of *Paecilomyces lilacinus* from soil and from biopsy samples of patients with oculomycosis are known to produce peptide mycotoxin "leucinostatins". Toxicological studies showed that leucinostatins have some potent effects on liver cells on oral administration. Similarly leucinostatins are also reported for causing uncoupling in rat liver mitochondrial system (*131*). None of the other species are reported for production of any myctoxins or toxic metabolites till date.

1.6.4 *Sphaeropsidales*

The *Sphaeropsidales* are frequent co-isolates with other common indoor fungi as *Cladosporium* and *Penicillium* from indoor settled dust (*132*). So far no adverse health effects of fungi or their metabolites are known. The *Sphaeropsidales* sp. (strain F-24'707) is reported for producing cladospirone bisepoxide, a clinically important compound with selective antibiotic, antifungal and antitumor properties (*133*).

1.6.5 *Tritirachium*

Tritirachium is a mitosporic fungus commonly isolated from paper, jute, textiles, adhesives, and gypsum used in plasterboards. Its natural habitat is soil and decaying plant material. It is an insect pathogen. In humans *Tritirachium* are known for causing superficial mycoses, as corneal ulcer, otomycosis and seborrheic scalp dermatitis (*134-135*). None of the species of *Tritirachium* are reported for producing metabolites with toxic properties.

In the forth coming chapters application of microbial metabolites as markers for indoor evaluation are discussed. The intricacies involved in method development, method validation pertaining to HPLC-MS/MS method are described in the chapter 2.

“Just because something doesn't do what you planned it to do doesn't mean it's useless.”
-Thomas A. Edison

Chapter 2

Identification and quantification of indoor mycotoxins and bacterial secondary metabolites by high performance liquid chromatography and tandem mass spectrometry.

“The value of an idea lies in the using of it.”
-Thomas A. Edison

2 HPLC-ESI-MS/MS

High performance liquid chromatography (LC) coupled with mass spectrometry (MS) is one of the most powerful analytical tools used in modern organic chemical analysis. In the past coupling of LC and MS was not feasible due to lack of adequate interfaces which could maintain required vacuum of 10^{-6} to 10^{-7} Torr (1 Torr\133.3 Pa) under higher liquid flow rates (*136*). The issues pertaining to magnitude of vacuum are better explained by comparison of quantities of introduced mobile phase along with analytes into MS, in cases of GC-MS and LC-MS systems respectively. Conventionally in GC-MS the volume of gas entering MS, is 0.5-2 ml/min. Whereas in the LC-MS it is of the order of 350 ml/min for methanol and greater than 1000 ml/min of water with a liquid equivalence of 1 ml/min methanol or water (*136*). Subsequently hindrances related to vacuum due to various liquid flow rates were made feasible by designing adequate interfaces. An account of different interfaces that evolved over a period of time with technological advancement is listed in interface section.

As a primary requisite for ESI-MS analysis, a molecule of interest must exist either as a preformed ion in a solution or must be amenable for charging through processes as protonation/deprotonation, adduct formation, or electrochemical oxidation/reduction. The assumption that analytes with significant hydrophobic portions generally have a higher ESI response compared to polar analytes may not be true in all cases. But as a rule analytes having poor ionization properties due to lack of ionizable groups are unfavorable for ESI. The wider acceptance of LC-MS over chromatography alone is due to features as selectivity, chromatographic integrity with mass detection (unaffected by eluent composition or flow rates), peak assignment, feasibility for structural elucidation and quicker method development. The separation power of newer chromatographic systems coupled with the advanced mass spectrometers has enabled analysis of complex samples with a high degree of confidence. Thus LC-MS has become indispensible technology in pharmaceutical industries and other laboratories dealing with trace amounts of analytes such as forensic science, doping control and environmental analysis (*137*).

Now a day the instrument configuration of LC or MS alone or combination of both can be customized according to the purpose and individuals preference. Commercially combinations of HPLC/UPLC along with assorted combination of detectors as single quadrapole, triple quadrapole (where Q2 acts as collision cells) or combination of quadrapole and TOF (Time of flight) are available for laboratories. Each of the available combination is with inherent advantages and limitations. As mentioned earlier a HPLC-single quadrupole combination which is cost effective may not be appropriate for the purpose of structural elucidation but apt for routine quality control evaluation. Similarly a triple quadrupole analyzer in comparison to combination of quadrupole-TOF is considered less suitable for accurate mass measurement. The construction, principal, advantages and drawbacks of different mass analyzers are detailed later in this thesis.

The methods based on LC-MS/MS are regarded more reliable in comparison other analytical methods such as chromatography or chromatography coupled to any of the optical detectors. This is due to the fact that the generated mass spectra are ~~–m~~ostly cleaner and supposedly less variable" (also depends on sample complexity) and which are also ideal for use in transferable libraries for comparison of results by different users (*138*). The LC-MS/MS data are due to fragmentation of a single ion in a controlled environment (collision cell). The features mass selection and selective fragmentations are of utmost advantages in avoidance of interferences and elimination of ambiguity in cases of low abundant analytes. The soft ionization interfaces such as electrospray or atmospheric pressure chemical ionization (ESI or APCI, respectively) are popular among the commercially available interfaces (*138*). Moreover the ESI's ionization feasibility over a range of molecular class (very polar to semi/mid polar) and molecular weights made it a single most used interface for ionization. It is also worth mentioning that the lower limits of detection of LC-MS/MS over GC-MS procedures (anabolic steroids as well as glucocorticosteroids) is an another reason making it method of choice in doping control and forensic science (*137*).

"A man may die, nations may rise and fall, but an idea lives on."
-John F. Kennedy

2.0 Interface technologies

As described earlier interfaces play a crucial role in linking complimentary technologies/techniques and thereby widening their scope (structure analysis, qualitative and quantitative). Some of the earlier interface technologies are moving wire and moving belt interfaces, molecular separators, direct liquid introduction (DLI), flow-FAB interface and the Thermospray (TSP) (*136*). The advent of TSP has given way to the soft ionization methods such as APCI (Atmospheric pressure chemical ionization) and ESI (Electrospray ionization) commonly used in LC-MS/MS (*136*).

2.1 Atmospheric pressure chemical ionization (APCI)

Earlier APCI interfaces could be visualized as an evolved form of TSP supplemented with hot Nitrogen gas. Nevertheless the use of the hot gas was to bring about quicker vaporization of the solvent and also for facilitating analyte transfer to the ionization chamber (radioactive beta source, specific to earlier interfaces). The ionization produces an atmospheric pressure reagent which later releases analyte ions by ion-molecule reactions. Ions generated in this way are transferred to the mass analyzer through an aperture and are later subjected for the analysis. Modern APCI interfaces are equipped with a heated pneumatic nebulizer probe and a high-voltage needle to produce a corona discharge responsible for solvent ionization. APCI is very effective for the analysis of low and medium polar chemical entities or when relatively non-polar solvents are unavoidable. Hence the APCI interface can be frequently found in environmental and pharmaceutical laboratories.

2.2 Electrospray ionization (ESI)

The idea underlying ESI was put forth by Beuhler et al. which hypothesizes ~~in~~troduction of sufficiently larger energy may bring about vaporization prior to decomposition"(*139*). ESI is a soft ionization interface suitable for generating intact ions of analytes with high molecular masses and wide range of polarities. The essential features of ESI interface are illustrated in Fig 1. However it is worth mentioning the spray position in ESI can be either on axis (as in Fig 1) or off axis. The earlier electrospray sources were made of a capillary stainless-steel needle with grounded side connected to the of a high-voltage source and placed in a chamber (a metal-coated

glass cup) that could act as counter-electrode. Since the objective of our research project was to scan for the natural occurrence of microbial metabolites such as trichothecenes, peptaibols and antibiotics we resorted for the use of ESI interface for the intended multi target detection method (detailed in publication 1).

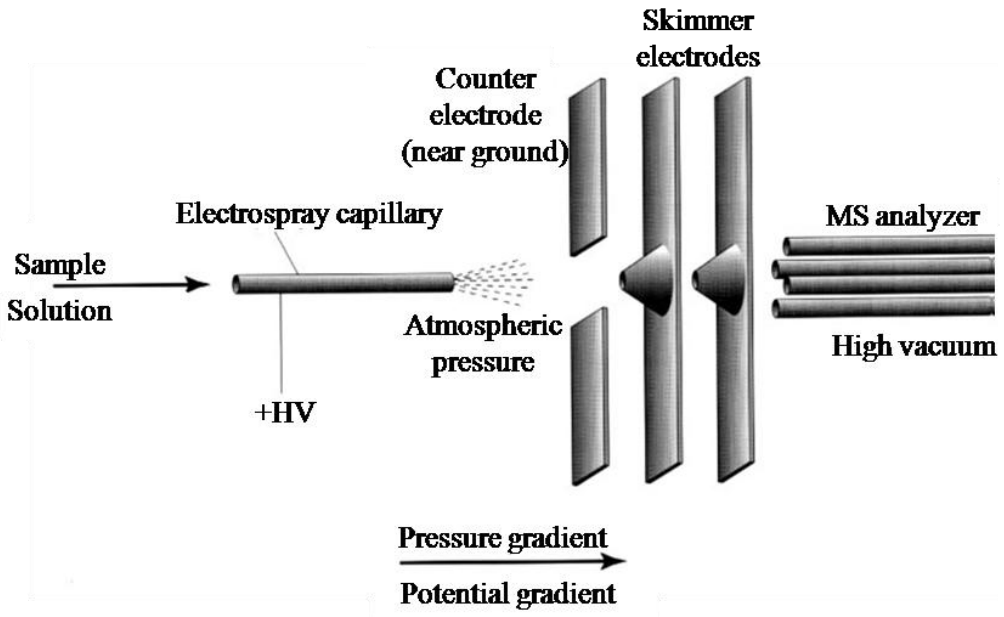


Fig 1 Essential features of on axis ESI interface Gaskel et al. 1997

2.2.1 Requirements of ESI

a) Solution characteristics

The solvent characteristics play a pivotal role in determining ESI performance. Nevertheless the solvent composition used for ESI depends on the analyte, application and mode of MS operation. Generally it is convenient to create a stable spray in a positive ion mode, with conductive solutions with at least 50% of a moderately polar organic solvent such as methanol or acetonitrile. Generally aqueous solutions with high surface tension are not desirable for adjusting ESI parameters. On the other hand non polar liquids (as hexane, trichloromethane etc.) with very low surface tension, high volatility and low dielectric constant are equally challenging for producing a stable Taylor cone. In addition to producing a stable Taylor cone an ideal solvent is expected not to cluster with the analyte molecule or contribute alone or with added electrolyte to background/noise in mass spectra. Besides all the above mentioned criteria solvent/mobile phase

characteristics as flow rate and applied voltage can also influence formation of a stable spray and thereby ESI efficacy.

b) Solvent and ionization

Conductivity of a solution is an important aspect determining the efficiency of the ionization process. The moderate uses of ionic species (volatile buffers, weak acids and bases) for charge separation are usual practice in LC-MS/MS. The solvent mixtures compatible with positive mode of MS are acidified mixture of acetonitrile/water and methanol/water supplemented with neutral salts. The salts (neutral) facilitate the analysis of polar and neutral analytes by the forming adducts with them. Acidic solutions facilitate protonation of the analyte, thus ideal for the analysis of analytes such as proteins and organic molecules that have basic functional groups. In order to accomplish successful analysis in the negative ion mode, it is necessary to use a solvent that creates stable anions. Since the objective of our study was to analyze both acidic and basic metabolites within a single run time (with minimal number of sample injections), we optimized a balanced condition of chromatographic solvents (methanol water mixture) by the addition to acetic acid (1%) and ammonium acetate (5 mM) (publication 1, materials and methods).Though fluorinated solvents create a stable deprotonated anions, they are not widely accepted due to environmental concerns. However an exception to fluorinated compounds is Trifluoroacetic acid (TFA), the addition can cause severe background noise in negative ESI mode hence cannot be generalized.

2.2.2 ESI mechanism

The mechanism involved in electrospray can be sub divided into three sequential events; a) droplet formation, b) droplet shrinkage and c) gaseous ion formation (Fig 2) (*140*).

a) Droplet formation

The solution delivered to the tip of electrospray experiences an electric field due to high potential of the capillary. Assuming a positive potential, cations undergo accumulation at the surface, which are eventually drawn out in the form of a "Taylor cone". At a sufficiently high imposed field the Taylor cone is further drawn to a filament which produces positively charged droplets. The formation of positively charged droplets takes

place at a point when the surface tension of the droplet exceeds the applied electrostatic force by a process known as budding. However the diameter of the droplets so formed are influenced by a various other parameters as well, such as applied potential, solution flow rate and properties of solvents.

b) Droplet shrinkage

The droplets traversing a pressure gradient towards the analyser tend to reduce in diameter due to solvent evaporation and collision warming. The process of shrinkage continues till the ―Rayleigh's limit" is reached, at which the magnitude of the charge is sufficient to overcome the surface tension holding the droplet together, leading to a ―Coulomb explosion". It is envisaged that continuous depletion of the droplet size is due to solvent evaporation and fission, respectively. The process continues until droplets containing a single ion are formed.

c) Gaseous ion formation

Gaseous ion formation alternatively known as ion evaporation and ion emission is envisaged to occur due to repulsion between the charged ions and the other charges of the droplet. The event of repulsion continues until the radius of daughter droplet depletes to an extent where surface charge density is sufficient enough to desorb ions into ambient gas (*141*). This model accommodates the formation of multiply charged gas phase ions. The desorbed ions occasionally carry solvent or solute species along with them which are also amenable for mass analysis. These adduct are referred as ―quasi-molecular ions". The sequential events mentioned above are accepted amidst challenges, as working hypothesis (*142*). The dominant reaction in positive ESI is oxidation and reduction in negative ESI (*143*).

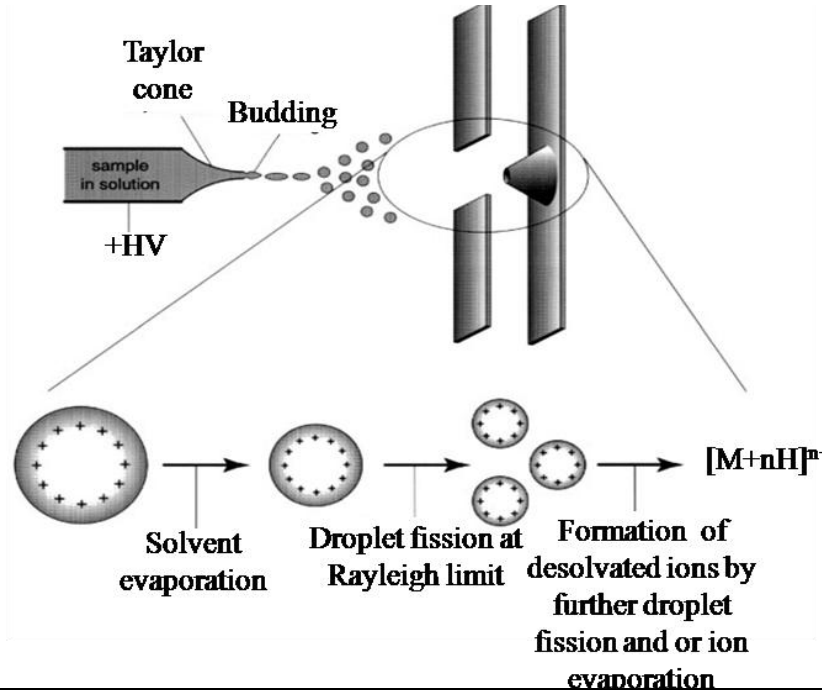


Fig 2 Droplet formation in ESI interface Gaskel et al. 1997

Moreover factors such as choice of bath gas (inert gas), gas flow rates and temperature also play a crucial role in determining success of a particular experiment. Hence these parameters should be optimized according to the specific needs of an experiment and also apparatus set up. For example, higher gas flow rates prevent less mobile ions from reaching the capillary. On the contrary extremely low gas flow rates adversely affect sensitivity due to excessive ion salvation. Similarly optimum temperature can influence the mass spectral quality (higher S/N) by minimizing the response of solvent clusters which are more volatile than the analyte molecules.

2.3 Advantages of ESI

The features of ESI which popularized the technique are

a. It is a mild ionization technique that can produce both protonated and deprotonated molecular ions of polar, non-volatile, high molecular weight (130 kDa) and themolabile compounds with ease.
b. Formation of multiply charged ions of the type $[M+nH]n^+$ (the equivalent is also true in the negative ion mode) (*136*). This can be attributed for the presence of various ionizible groups on macromolecules such as proteins or nucleotides.

c. Conventionally ions in ESI are formed because of protonation or deprotonation or due to adduct formation. This directly reflects the acid-base equilibrium in solution. In this context, ESI can also be relied for additional information about the analyte properties in the liquid phase.

2.4 Challenges working with ESI

The phenomenon of coronal discharges observed in the negative mode makes ESI more challenging compared to MS operation in positive mode. Due to coronal discharges, gas phase analyte and solvent molecules undergo ionization generating significant noise, loss of signals and, arching. Under extreme cases coronal discharge may also damage electronic components. In order to minimize coronal discharges electron scavenging gases or halogenated solvents or combination of both are recommended as a preventive measure (*143*).

2.5 Intricacies associated with ESI-MS/MS

2.5.1 Instrumental sensitivity

Fundamentally ESI sensitivity is governed by two factors; a) the efficiency of formation of gas-phase ions and b) efficiency of ion transfer through the various stages of the mass spectrometer to detector. The accurate measurement depends on the measured fraction of ions, type of mass analyzer and also on the mode in which MS is used. For example, an ion trap in batch processing mode is more suited for full-spectrum analysis with higher sensitivity compared to a quadrupole instrument (*144*). On the other hand a quadrupole instrument operated in selected ion monitoring (SIM) mode can accomplish highest sensitivity for a single analyte compared to an ion trap mass analyser. Among the two criteria mentioned above, it is accepted that the predominant factor limiting the sensitivity is efficiency of ion transfer through the mass spectrometer (*143*). Since ions are not amenable for immediate measurement after their expulsion from the droplets, none of the assumptions are proven substantially. Assuming ―ion transfer loss" is true, modification to triple quadrupole with ‗‗ion funnel'' and ―multi-capillary inlet" succeeded in gaining approximately 23 fold higher ion transmission than conventional ESI ion optics (*145*). Another approach to rectify transfer losses was attempted by Tang et al. by means of improving the efficiency of analyte charging. Considering this as basis, Tang et al. developed a microarray of

electrospray emitters that would enable multiple stable Taylor cones simultaneously. The use of this microspray emitter facilitated operation at higher flow rates and accomplished higher sensitivity of the order 2–3 fold compared to single Taylor cones ESI (*146*). Some of the modern mass spectrometers (e.g., QTrap 5500 AB SCIEX) have adopted another simple approach of installing a larger orifice permitting transmission of relatively higher ion current.

2.5.2 Detection limits and linear dynamic range

An extended linear dynamic range on both extreme ends is desirable for improved quantitative analysis and also for better limits of detection (LOD). LOD is defined as the lowest amount of analyte in a sample that can be detected, but not necessarily feasible for the quantification. With regard to higher concentrations, the factors limiting ESI are not completely understood. However the probability of electrical charge deficiency to bring about ionization of all molecules cannot be completely ruled out. Hence combination of parameters promoting effective droplet evaporation, charging and transmission of gas-phase analytes are adopted as a convention. At lower concentrations (bio-analytics with critical sample volume) detection limits are constrained by background/noise and interference originating from multiple sources. As a result bio-analytics using ESI-MS requiring very low detection limits rely upon tandem MS in the selected reaction monitoring (SRM) mode. The SRM differentiates target molecules from the interferences due to the unlikeliness of interferences leading to formation of fragments that are identical with the fragments formed due to disintegration of molecules of interest. Because of this SRM is regarded advantageous in overcoming ambiguity due to interference or chemical noise.

2.5.3 Random noise

The ubiquitous signal (noise) which has a probability of occurrence at every m/z value is referred as ―random noise". The sources of random noise in ESI-MS are not completely known. According to one speculation random noise is that fraction of ions which reaches the detector without going through the mass analyser. Another potential source of random noise could be due to the passage of large, highly charged clusters of analyte and solvent molecules through the mass analyzer and arriving at the detector. Under conventional conditions, some ESI droplets may not desolvate completely, and thus highly charged solvent/analyte/counter ion clusters could

be formed (*143*). Additionally an involvement of cosmic rays in random noise formation is also speculated.

2.6 Mass analyzers

Mass separation and measurement

Depending on the required mass resolution various mass separation technologies can be combined and hyphenated with chromatography. The mass resolution (or resolving power) of the mass analyzer is the ability to distinguish between ions differing in the mass/charge (m/z) ratio by a small increment. It may be characterized by giving the MS peak width, measured in mass units, expressed as a function of mass, for at least two points on the peak, specifically at fifty percent and at five percent of the maximum peak height (*147*). Three popular mass separation technologies associated MS are qudrupoles, ion traps and Time of flight (TOF) mass analyzers.

2.6.1 Iontrap mass analyzer

The ion-trap mass analyzers can be either 3D ion trap (Paul traps) or the 2D ion trap (quadrupole or linear ion traps). The 3D ion trap consists of circular electrodes and two ellipsoid caps on top and bottom. Contrary 2D ion traps are made of four rod quadrupoles ending in lenses that reflect ions forward and backward in that quadrupole. The mass separation is brought about by changing the electrode voltages to eject an ion from the trap, producing mass spectra. The advantages of the ion-trap mass analyzers are the condensed size and the ability to trap and accumulate ions. The trap feature is exclusively beneficial for the analysis of low abundant molecules and also for generating a cleaner mass spectrum with higher signal-to-noise ratio (S/N). The major drawback concerning ion-trap mass analyzer is their low mass accuracy compared to other mass analyzers (exception quadrupole ion traps). This is observed even for the most abundant masses with relative errors around 1 % (*148*). Thus compared to other available mass analyzers 3D ion traps are less frequently opted choice by mass spectrometry community.

2.6.2 Quadrupole mass analyzer

As the name indicates quadrupolar electric fields rather than magnetic fields bring about the mass separation. It consists of four cylindrical rods arranged in parallel with opposite rods connected by electrical circuits (Fig 3). A radio frequency (RF) voltage is applied between one pair of rods and the other. Moreover a direct current voltage is then superimposed on the RF voltage (Fig 4&5). Ions travel down the quadrupole between the rods. The mass separation is based on the trajectory of ions in oscillating electric field. Ions with a distinct m/z value pass through and reach detector, leaving behind all other ions. The ions with unstable trajectories collide with the rods and or are eliminated by vacuum pumps. This particular feature is of utmost advantage for developing SRM methods capable of screening large number of target analytes (publication 1).The modern qudrupole mass analyzers can scan a whole mass range of eg., 50-850 m/z within a short time span. These qudrupole mass analyzers when coupled to modern GC or LC can enormously reduce the scan time. Similarly qudrupole mass analyzers operated in selected ion monitoring (SIM) mode can achieve higher sensitivity compared to other modes (*149*). In addition to the above mentioned advantages quadrupole mass analyzer can also be used as linear ion traps (2D traps) by applying specific RF voltage.

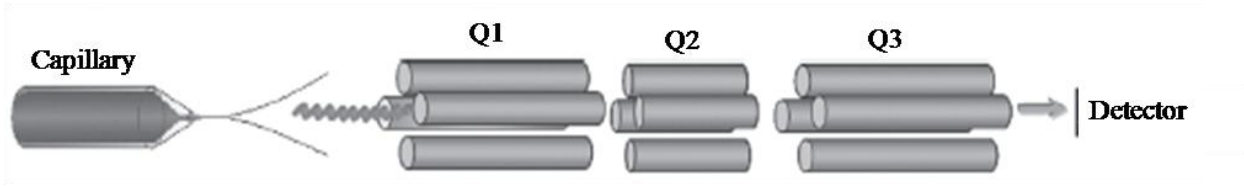


Fig 3 Hybrid instrumentation- with triple quadrupole mass analyzer Ref:NBK56011

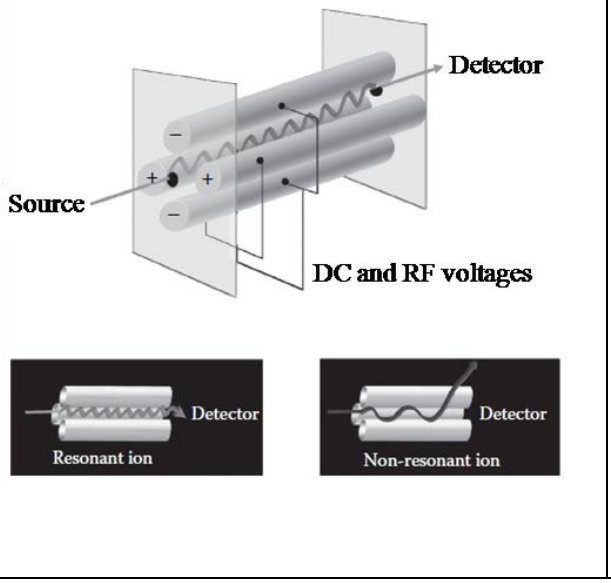


Fig 4 Quadrupole mass analyzer.Ref:NBK56011

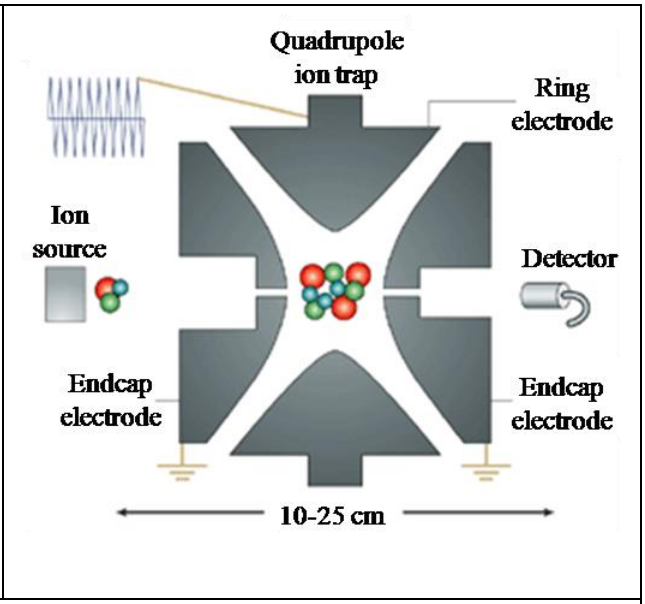


Fig 5 Quadruple ion trap – section view Glish et al. 2003

2.7 Architecture of the triple quadrupole mass spectrometer

The application of quadrupole mass analyzers can be seen in several commercially available instruments eg. QTrap 2000, 4000, 5500 (AB SCIEX) (Fig 6). Here is only a brief portrayal of construction of triple quadrupole mass spectrometer and different operational modes associated with it are given. As described above the quadurpole mass analyser consists of 4 cylindrical rods arranged in parallel with opposite rods connected by electrical circuits.

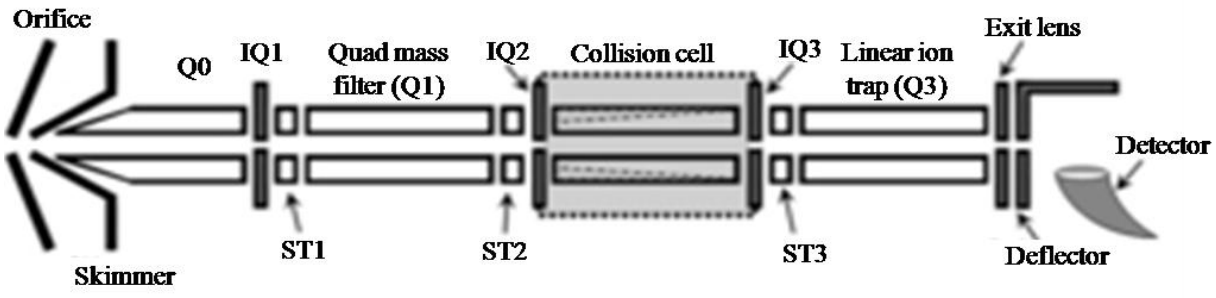


Fig 6 QTRAP 4000 mass spectrometer schematic: ST, stubbies; Q, quadrupole rod set; IQ, interquad lens (Ref: springerimages)

These instruments are equipped with three quadurpoles designated as Q1, Q2 and Q3. A triple quadrupole mass spectrometer can be operated in different modes. Each of the operational modes is with inherent advantages and could be attributed to operations of MS in different combinations of quadrupoles. In all the modes (SIM/MRM/SRM, detailed later) the precursor ions are primarily scanned in the first quadrupole (Q1), selected ions are transferred to second quadrupole (Q2) which functions as collision cell, fragmenting the precursor ions. The resultant products are accelerated out from Q2 and are monitored in the third quadrupole (Q3). It is worth mentioning in the above mentioned commercially available instruments the third quadrupole (Q3) can also be operated as linear ion trap by applying only with RF voltage to Q3.

2.7.1 Experiments with tandem quadrupole mass spectrometers

In literature the terms tandem MS experiments and tandem MS modes are frequently used in an interchangeable manner referring one and the same aspect. Many different experiments can be conducted using tandem mass spectrometers, namely product ion scans, precursor ion scans, neutral loss scans, multiple reactions monitoring (MRM), selected reaction monitoring (SRM) and scheduled reaction monitoring (sSRM). Various triple quadrupole and trap operation modes are summarized in below shown table. Hopfgartner et al. 2004. In our study we predominantly used schedule reaction monitoring for screening various indoor matrices. In the event of ambiguity due to co-eluting matrix molecules, molecular identity was confirmed using EPI scans in an another complimentary experiment. These two experimental modes are detailed at a later part with examples. However its note worthy, the information dependant acquisition (IDA) can combine both the scan and confirmatory analysis into a single run.

If only the product ions of a particular parent ion mass are monitored in an experiment it is called a product ion scan. This is particularly beneficial to determine the structurally significant fragments ions for a selected precursor ion. Similarly experiments exploring identities of all possible precursor ions in a sample that decomposes to produce a specific product ion is designated as precursor ion scan. This is used for the identification of a chemical class of compounds (*150*). Unlike the above two, neutral loss scans monitor all pairs of precursor ions and product ions that differ by a constant neutral loss (*151*). SRM is a selective experiment. In this both mass analyzers are set to monitor a selected m/z value. This is analogous to selected ion

monitoring (SIM) of single quadrupole mass spectrometers. SRM has an increased sensitivity and selectivity for the target compound identification and quantification. In SRM only specific product and precursor masses of analytes are measured instead of scanning over a range of m/z values. Moreover when SRM is adopted in a schedule manner (ie. anlyte monitored only around an expected LC retention time) it is designated as scheduled reaction monitoring (sSRM).

Mode of operation	Q1	Q2	Q3
Q1 Scan	Resolving (scan)	RF only	RF only
Q3 Scan	RF only	RF only	Resolving (scan)
Product Ion Scan (PI)	Resolving (fixed)	Fragment	Resolving (scan)
Precursor Ion Scan	Resolving (scan)	Fragment	Resolving (fixed)
Neutral loss scan (NL)	Resolving (scan)	Fragment	Resolving (scan offset)
Selected reaction monitoring (SRM)	Resolving (fixed)	Fragment	Resolving (fixed)
Enhanced Q3 single MS (EMS)	RF only	fragment	Trap/scan
Enhanced product ion (EPI)	Resolving (fixed)	fragment	Trap/scan
MS^3	Resolving (fixed)	fragment	Isolation/fragment trap/scan
Time delayed fragmentation (TDF)	Resolving (fixed)	Trap/ fragment	Fragment/ Trap/scan
Enhanced resolution Q3 single MS (ER)	RF only	No fragment	Trap/scan
Enhanced multiply charged (EMC)	RF only	No fragment	Trap/scan

Summary of various triple quadrupole and trap operation modes. Hopfgartner et al. 2004

a) Scheduled reaction monitoring (sSRM) for multi target detection

The application of selected reaction monitoring to multiple product ions from one or more precursor ions is referred as multiple reaction monitoring (MRM) (*152*). Besides when analyzers are scheduled dynamically for monitoring m/z are accredited as sSRM. This feature of sSRM was adopted for developing our multi method consisting of 187 target analytes. The Applied Biosystems 4000 Q TRAP™ MS instrumentation operated with software Analyst® 1.5 or higher versions could accommodate more than 300 analytes without any compromises in duty cycle or dwell time (our current multi method status). This feature is particularly beneficial for the analysis of closely eluting substances as well. Aforementioned trait has been effectively adopted by us (Fig 7) and other researchers for building multi analyte detection methods (*23, 153-155*). The sSRM features as dynamic scan windows and dwell time optimization can be better appreciated when compared to features of previous version of instrument

software (Analyst® 1.4). The Analyst® version 1.4 with the static scans windows (periods for convenience this is designated as classical SRM (cSRM)) and period switching follow a strict retention time (RT) requirement for monitoring of analytes. However slight RT shifts are frequently experienced problem in chromatography. These RT shifts can severely affect the analysis of target compounds, particularly which are eluting close to time periods. Similarly the time lapse during the period switching may additionally prevent monitoring of analytes eluting at that particular point of time. These problems can affect the efficiency of multi target detection methods and also can be hindrances for method expansion goals.

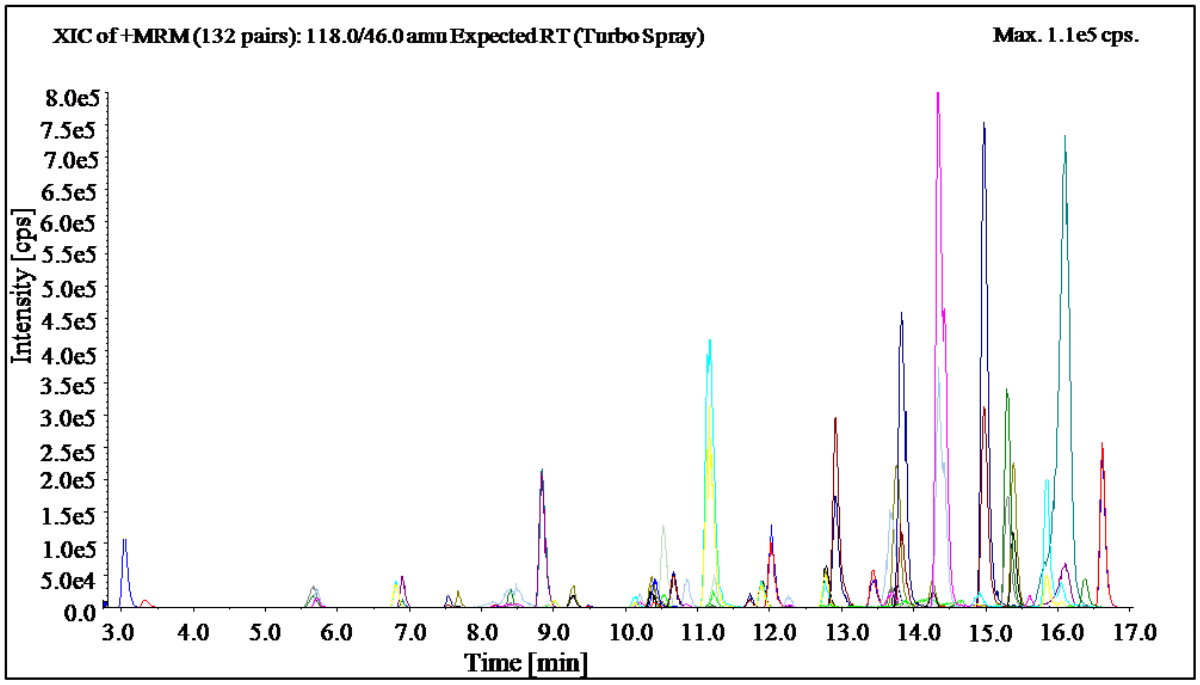


Fig 7 Extracted ion chromatogram with scheduled reaction monitoring (SRM)

Advantages employing sSRM over classical SRM (cSRM)

Distinct to the cSRM (static scan windows) the sSRM automatically associates retention times with transitions. It creates an optimized acquisition method where each transition is monitored only across an adept time window designated as SRM detection window. The principle is to monitor the transitions only when necessary. Based on the analytes‘ retention times, the sSRM algorithm decreases the number of concurrent SRM transitions monitored at any point of time, allowing both cycle time and dwell time to remain as low as possible at higher levels of SRM multiplexing (*154*). The scheduled SRM is advantageous due to more data points per peak, better

reproducibility (coefficients of variation (CVs) <5%) and superior signal-to-noise ratio (S/N), even when the number of SRM transitions is doubled. As a consequence sSRM can acquire three times more data points on an average compared to cSRM and enhance of limits of quantification by a factor two or even higher (up to six), depending on the analyte transitions (*154*).

b) Enhanced product ion Scan (EPI)

The term ~~–e~~nhanced" is used when third quadrupole (Q3) is operated as LIT (linear ion trap) (*156*). As compared to earlier modes of MS operation in EPI produce MS/MS spectrum with higher S/N ratio (Fig 8). The reasoning for this could be the availability of higher cycle time. Hence EPI can be regarded as SIM of an exclusive analyte or an ion over an entire chromatographic run. Meanwhile user can define series of collision energies for fragmentation of precursor ion, generating series of mass fragmentation spectrum.

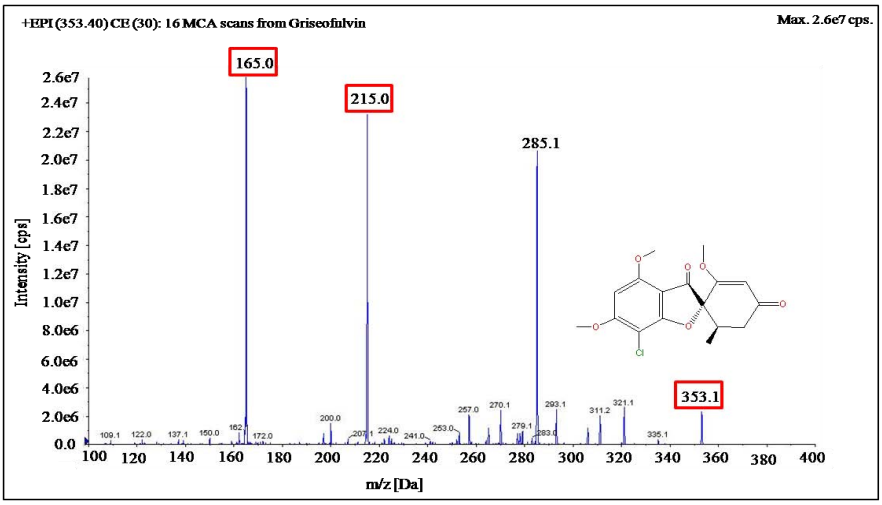


Fig 8 Enhanced product ion (EPI) scan of Griseofulvin (*Penicillum griseofulvum*)

Once again this feature is common in triple quadrupole mass spectrometers. The fragmentation is by collision activated dissociation in (collision cell [Q2]). In this mode the precursor ion to be fragmented is first selected in Q1 with mass window of 1-4 amu wide, filtering out all other ions. The fragmented ions are trapped in Q3 and scanned at a specific scan speed appropriate for fragment ion resolution. This is mostly useful confirming low abundant analytes. In our study the trap function was useful in differentiating the true analyte signals from signals due to ionization of co eluting matrix molecules (publication 1, matrix interferences for: griseofulvin gibberllic acid and fumitremorgin C).

2.8 Criteria for LC-MS/MS method development

Before getting into method development it is essential to formulate clear objectives and outlines for the analytical method. If the method is intended for quantitative or semi quantitative applications, method validation with appropriate certified matrix/matrices reference material/s are obligatory. Considering purpose of the method attention should also be paid for standardizing specific extraction procedures for dissimilar matrices. Ideally chromatography should produce narrow peaks (minimum 12-15 data points). A similar criterion can also be found in decision 2002/657/EC for achieving best method sensitivity (*157*). Irrespective of purpose of the method, all methods should achieve optimal separation of compounds accompanied by high signal-to-noise ratio (S/N) in MS. If methods are intended for detection and quantification of larger number of target molecules, emphasis should be also on achieving optimal cycle time for each target.

2.8.1 Systematic multi target detection method development and validation

The constants related to an LC-MS method can be broadly divided into three sets of parameters. These include parameters related to pre chromatography (sampling, extraction, analyte stability, cleanup, and pre concentration), chromatography (mobile and stationary phases, gradient, temperature, and flow rates) and mass spectrometry (interface, ionization, temperature, scan mode).

Since standardization of pre chromatographic parameters (sample size, injection volume, extraction time, extraction solvent, extraction assistance (shakers, sonication, and microwave), derivatization and clean up) are dependent on complementary assays, optimization of these parameters are generally performed once the LC-MS/MS method development is completed. The earliest step in method development is gathering relevant particulars about targets analytes. These include commercial availability, physicochemical properties, pH tolerance, stability and solubility in relevant polar and apolar solvents, photo stability, p*K*a, log*D* and log*P* *(158)*. Subsequently positive and negative ESI full-scan spectra are acquired by syringe infusion or with flow injection analysis using suitably dissolved pure analyte standards. This is mainly performed for confirming the integrity of analytes and to identify characteristic MRM transitions. In the event chromatographic solvent/s entering the mass spectrometer are different than used for

dissolution of the analytes, stability and ionization are re evaluated by diluting in relevant mobile phase (isocratic) or 1:1 mixture of mobile phases (gradient). Situations where the extract is highly incompatible with the early of eluent composition, solvent removal and subsequent reconstitution in a suitable solvent can be another alternative option. Any of the additional steps followed by sample extraction as drying and reconstitution is invariably attributed with loss of analytes. Hence emphasis should be on minimizing the number of procedures post sample extraction. Using protonated $[M+H]^+$ or deprotonated $[M-H]^-$ or $[M+NH_4]^+$ molecular ions as the precursor ions ($[M+nH]n^+$ and $[M-nH]n^-$ for macromolecules as proteins and carbohydrates), product ion spectra are obtained. Moreover the SRM transitions are selected by manual or automatic optimization of all MS/MS related voltages. At this stage a minimum of two SRM transitions are selected in order to minimize false positive results. It is highly unlikely (but not impossible) that transitions due to a co-eluting metabolite or any endogenous component match with both transitions of a target analyte. The MS parameters, such as declustering potential, sprayer voltage, capillary temperature, tube lens potential, skimmer voltage, collision energy, sheath gas and auxiliary gas pressure are optimized by monitoring the pre-selected SRM (publication 1 [materials and methods & table 1]) transitions or set to default values. Nevertheless in situations where the number of target analytes are considerably large, MS parameters that are suitable for majority or analytes is regarded as optimal. The same principal is applicable even for optimization of extraction procedure. To summarize, methods focusing on multi-analytes detection may be regarded as methods with certain compromises in employed parameters which are inevitable.

The logical next step in a multi method development is screening for an optimal LC gradient of mobile phases for separation of the analyte mixture. Concurrently care must be taken while adjusting gradient in order to prevent co-elution and aggregation of too many analytes to any particular region of chromatogram. It is important to have optimal dwell times and cycle times for sensitivity and precision of the method. In the events where dwell time and cycle time are severely compromised, the method should be split into another parallel method by transferring a certain number of analytes. For the analysis of co-eluting analytes with structural similarity,

polarity switching can be an option (in rare cases as 15-Acetyldeoxynivalenol, 3-Acetyldeoxynivalenol) for reducing dilemma related to origin of transitions.

2.8.2 Method performance evaluation

In our study the method qualification was conducted to assess accuracy, precision and ruggedness of the method. The LC retention time and the intensity ratio of the two sMRM transitions were verified with the values of authentic certified standards. The permitted deviation of ±0.1 min of expected retention time and 30% rel., respectively were accepted. The confirmation of positive analyte identification was obtained by the acquisition of two sMRMs per analyte yielding 4.0 identification points according to commission decision 2002/657/EC (exceptions moniliformin and 3-nitropropionic acid, only one fragment ion) (*157*). The coefficients of variation (CVs) of the whole method were calculated using Validata®, a Microsoft Excel macro (*159*) from linear, 1/x weighted calibration curves obtained after the analysis of the spiked samples. This software tool was also used for the calculation of the repeatability of the method at the lowest and the highest concentration level and of the 95% confidence interval of the slopes of the calibration functions of the three sample types. From the latter, the 95% confidence interval of apparent recovery (R_A), signal suppression enhancement (SSE), and extraction recovery (R_E) were calculated according to the law of error propagation. Limits of detection (LODs) were calculated at the lowest evaluable concentration levels of both spiked samples as well as of liquid standards using "S To N" script of Analyst® 1.5. The complete data pertaining method development, validation and application can be found in attached publication Vishwanath et al. 2009 (*23*).

To estimate the matrix effect in cases of other indoor relevant matrices (building materials) the diluted extracts were fortified with a multi-analyte standard on one concentration level and the resulting peak areas were compared to corresponding peak areas of the liquid standards (zero matrix). The efficiencies of extraction solvents (methanol, ethyl acetate, acidified acetonitrile water mixture) were investigated by comparing the peak areas of indoor relevant analytes (publication 1). The extraction solvent optimization was done by spiking settled floor dust samples collected in our offices.

2.8.3 Method validation requirements

The validation is defined ~~as~~ a process of proving that an analytical method is acceptable for its intended use". The validation is ideally performed before employing a method for routine analysis. However, methods are also (re)validated whenever the conditions change for which the method has been validated (eg. an instrument with different characteristics or samples with a different matrix) or whenever the method is changed and the change is outside the original scope of the method. The method validation procedures are extensively described for marketed drugs in United States, Europe and Japan. Some examples of available guidelines or recommendations are United States pharmacopeia 25 (USP 25) (*160*), EU Legislation – Eudralex (European commission), and EMEA guidelines (European medicines agency).

The validation procedure can be useful as to determine uncertainties related to matrix effect, extraction losses and analyte stability (in a solvent or a solvent mixture). The method validation should include use of appropriate certified reference material (CRM) for determining the method accuracy. In cases when suitable CRMs are unavailable or does not exist for all analytes, any blank matrix close to intended purpose can be spiked at relevant concentration levels and used for validation purpose. In our study method validation was carried out by spiking CRM SRM 2583® from National Institute of Standards and Technology, Gaithersburg, Maryland. SRM 2583 is certified for five trace elements and is composed of dust collected from vacuum cleaner bags used for cleaning of interiors of dwellings. Moreover performance characteristics of a method such as efficiency of solvent extraction, analyte recovery, and matrix effects are carefully criticized in relation to certified analyte standards and blank matrix extracts (CRM without spiking). All validation results are compiled and published Vishwanath et al. 2009 (*23*). The stability of analytes over measurement time was assessed by comparing 24h bench-top room temperature incubated standard mixture to zero-hour incubated standards. The efficiency of extraction and recovery were differentiated. The extraction efficiency is defined as the recovered amount of analyte from spiked samples after extraction. According to IUPAC definition the apparent recovery (abbreviated as R_A) denotes the ratio of an observed value obtained from a calibration graph divided by a reference known or theoretical value. This is also often referred to

as overall or total recovery of a method (*161*). In principal the apparent recovery of a method is the amount analyte recovered combining extraction losses and losses due to matrix effect.

2.9 Indoor investigation and relevance of various indoor matrices

The material sampling plays a crucial role in indoor investigation and damage estimation. Hence collections of wide range of materials with different physical and chemical characteristics are advantageous. The indoors (particularly homes) with respect to the matrices can be defined as any environment which is ~~–a~~ccording to individual's preferences". The matrices that are found in indoor environment are generally diversified and are difficult to generalize. Hence in our study we focused on those matrices with highest probability of exposure to occupants (dust) and higher microbial infestation probability.

2.9.1 Building materials

Water activity (a_w) and organic content of indoor matrices determine the microbial spectra found in an indoor environment. The raw materials used in buildings can be broadly classified into inorganic and organic materials. Apart from the structural materials (metals) used in buildings all other materials such as mortar (MR), carton gypsum board (CG), chipboard and mineral wool can absorb or adsorb variable amounts of moisture, thereby can promote microbial growth to different extents. The matrices mortar and carton gypsum board could be regarded as extremely inorganic and organic rich sources with respect to microbial growth respectively. Hence in the event of moisture damage investigation of various indoor matrices are inevitable to get an adequate picture of indoor status.

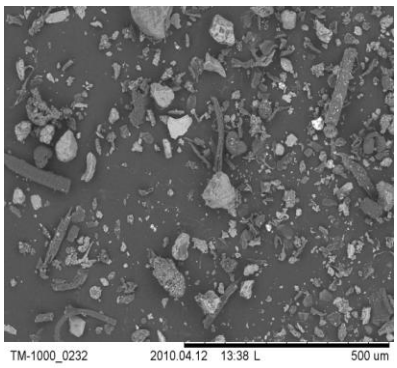
2.9.2 Settled floor dust (SFD)

Since the process of degradation in indoors is typically slow, settled floor dust can be as valuable as any well preserved fossil evidence for indoor environmental evaluation (*162*). Indoor Settled Dust (ISD) or Settled House Dust (SHD) or Settled Floor Dust (SFD) is a matrix formed due to the deposition of aerosols (outdoor and indoor) and outdoor particles brought due to foot trafficking (Fig 9). The particles that are brought by foot trafficking mainly contain sand, loam and are generally rich in silica. Formation of settled dust by aerosol sedimentation depends mainly on the particle size. The particles with aerodynamic diameter greater than 100 µm readily sediment to form SFD. Whereas very small particles (<0.1 µm) accumulate into preexisting SFD

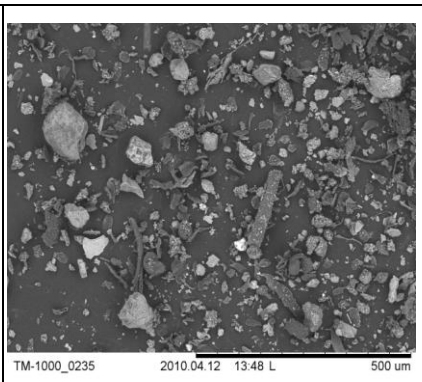
due to particle diffusion. The bio aerosol fraction are generally of the order of 0.3-100 μm consisting of spores (~3-13 μm), pollens (~10-100 μm), fragments of mycelia, plant fibers, viruses (<0.1 μm) and bacteria (0.3-11 μm) (*163-164*). On the other hand the aerosols of anthropogenic origin contain weathered fraction of furniture, textile fibers (~12-100 μm), keratin (~10-100 μm) and building construction materials (~3-100 μm) (*165-166*). Moreover auxiliary physical parameters such as humidity, temperature gradient, air flow, area, surface charge can have considerable influence on deposition of particles (*167-170*). Based on the particles size aerosols are classified into sedimentary and respirable suspended particles (RSP). The aerosol fraction with higher surface area in addition to contributing for the dust formation also acts as sink for adsorbing volatile and semi-volatile organic compounds such as polycyclic aromatic hydrocarbons (*171-173*). During our investigation we evaluated various dust samples such as dust collected as methanol swabs at different heights, dust samples collected using vacuum cleaners and fine dust particles adsorbed to vacuum cleaner dust bags.

“You have to dream before your dreams can come true.”
-Dr. A P J Abdul Kalam

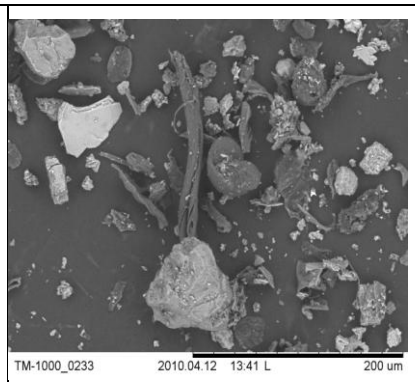
Transmission electron microscopic (TEM) image of indoor settled floor dust



SRM 2583®, Focus 1(500µm)



SRM 2583®, Focus 2(500µm)



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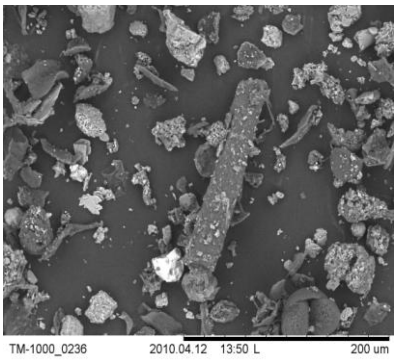

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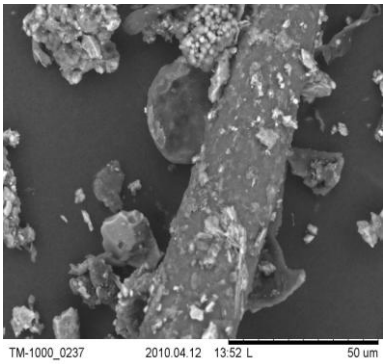
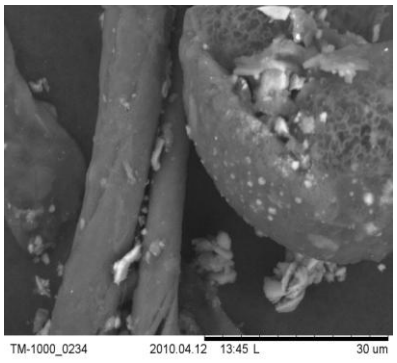
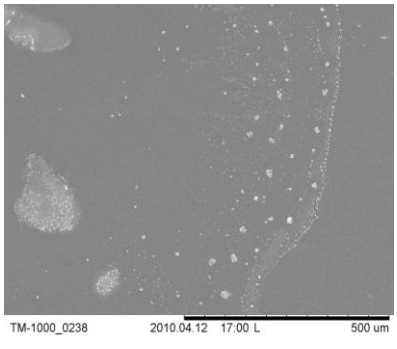


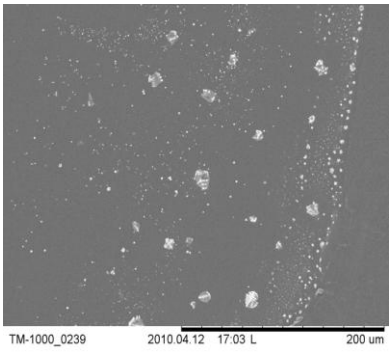
SRM 2583®, Focus 1(50µm)



SRM 2583®, Focus 1(30µm)



Acetonitrile extract SRM 2583® Resolution (500µm)



Acetonitrile extract SRM 2583® Resolution (200µm)

Dust certified reference material SRM 2583®

Fig 9 Courtesy TEM: Prof. Siegfried Reipert and Bhuma Wysodil, M.Sc, Vienna biocenter, MFPL laboratories, Dr. Bohr-Gasse 9, Vienna, Austria.

Unpublished data:

2.10 Materials and methods

a) Study sites and sampling

The methanol swabs of settled floor dust and building surfaces were collected from primary schools in Spain, Finland and The Netherlands. The buildings were inspected for moisture damage / dampness / visible mold by trained personnel following a common protocol and using standardized checklists and surface moisture recorders. Visual observation data were collected on building characteristics, moisture damage, and other potential contributors to poor IAQ including observations on maintenance, sources of dampness/mold, and particles. The samples were collected from different locations/rooms in the school buildings, categorized based on their use and potential exposure duration of pupils and teachers. An average of 15 sampling locations was targeted in each school.

For the methanol swabs sampling we used 7mL glass vials equipped with Teflon lined caps (Supelco Cat.Nr. 27150-U) which were pre-filled with 3 mL HPLC grade methanol (JT Baker Cat.Nr 8402); in a few cases where the dust amounts were large, 3 mL extra of methanol were added. Foam swabs (Foamtips Pro, Chemtronics Cat.Nr CFP-50) were wetted with methanol and moved across the sampling area; the adhered dust was thoroughly transferred from the swabs into the methanol filled vials. The methanol swabs samples were collected following a similar procedure from surfaces with visible damage. All vials were transported to the study centers on the day of sampling, sealed with parafilm, and stored at -20 ° C. Shipment of the samples was done at RT; the samples were subsequently stored until analyses at -20 °C.

The building materials gypsum board, chipboard, wood, sand, wall paper, mineral fiber (MMF) and vacuum collected settled floor dust were procured from water damaged buildings of Finland and Sweden. Moreover additional settled floor dust samples were also collected from control buildings (with no moisture damage) and renovated buildings (buildings earlier reported for water damage).

b) Sample preparation and analysis of fungal and bacterial metabolites:

The HPLC-MSMS analyses were performed as described in detail by Vishwanath et al. (2009), with additional 58 analytes being integrated in the protocol. The methanolic suspensions were shaken for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany). The particulate matter was allowed to settle for 10 min, and 100 µl of the clear upper methanolic layer were transferred into glass vials equipped with glass microinserts. Upon addition of 100 µl water, the diluted raw extract was directly injected into the HPLC-MS/MS instrument. For building material the metabolite extraction was done as described by Vishwanath et al. (2009).

The section describing detection and quantification in the scheduled Multiple Reaction Monitoring (sMRM) can be found in attached publications (Vishwanath et al. 2009) (*23*). However results were not corrected for incomplete extraction and/or signal suppression/enhancement due to co-eluting matrix constituents, as matrix-matched calibration was found to be insufficient for correction of these effects in the extremely heterogeneous matrix dust (Vishwanath et al. 2009) (*23*).

2.11 Statistical analysis

Basic statistical tests were conducted to identify significance of toxins adhering to a particular matrix. The statistical package R console was used for data normalization as well as for identification of metabolite significance. The data was normalized by the addition of a constant of factor (0.01) to every data point. This was followed by verification tests for normal distribution of data (–Kolmogorov-smirnov test and theoretical normal distribution test"). In the event these tests were negative data was transformed using the $\log_{10}$ normalization. The same transformation was applied even for constant factors. Therefore significance tests were performed against $\log_{10}$ (0.01). The normalized data was subjected to Welch t-test or unequal variance t test. The test assumes that groups of data are sampled from Gaussian populations, but does not assume those populations have the same standard deviation.

2.12 Summary of results

Matrix specific microbial infestation

Building material –Man made fiber (MMF) (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean (Log_{10})**	**No. positives**
Altenuene	33.12	33.12	n.a	-	1/93
Alternariol	18.77	13.18	8.80	-	3/93
Alternariol monomethyl ether	48.91	1.19	67.69	-	3/93
Altersolaniol A	608	608.00	n.a	-	1/93
Andrastin A	59.52	59.52	n.a	-	1/93
Aspyrone	8520	8520.00	n.a	-	1/93
Averantin	15.42	15.42	n.a	-	1/93
Averufin	24.74	24.74	n.a	-	1/93
Beauvericin	0.37	0.12	0.43	-1.87	9/93
Chaetoglobosin A	82.8	82.80	n.a	-	1/93
Chanoclavine	21.23	21.23	17.81	-	2/93
Chloramphenicol	54.44	18.78	58.79	-1.81	5/93
Emodin	51.40	20.21	98.74	-1.51	14/93
Enniatin A1	0.35	0.23	0.32	-	4/93
Enniatin B	0.36	0.29	0.19	-1.80	12/93
Enniatin B1	0.50	0.50	n.a	-	1/93
Enniatin B3	0.03	0.03	0.01	-1.97	5/93
Equisetin	8.64	6.00	7.48	-1.58	14/93
Festuclavine	14.05	14.05	n.a	-	1/93
Fumigaclavine	218.24	218.24	168.96	-	2/93
Kojic acid	878.08	693.60	684.21	1.79	4/93
Macrosporin	84.58	123.12	57.07	-	3/93
Nigericin	0.18	0.18	n.a	-	1/93
Nonactin	1.27	1.25	0.27	-1.88	5/93
Norsolonic acid	219.04	28.96	286.85	-	3/93
Ochratoxin A	20.15	20.15	11.85	-	2/93
Physcion	96.69	52.68	116.44	-1.59	10/93
Sterigmatocystin	57.76	57.76	55.36	-	2/93
Viridicatin	41.60	41.60	n.a	-	1/93

Table 1 Building material –Man made fiber (MMF)

Building material -Wood (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Alternariol	398.40	398.40	n.a	-	1/14
Alternariol monomethyl ether (AME)	55.20	55.20	n.a	-	1/14
Beauvericin	0.60	0.60	n.a	-	1/14
Chaetoglobosin A	200.23	160.00	174.66	-0.92	4/14
Chloramphenicol	13.82	13.82	n.a	-	1/14
Emodin	117.31	46.12	162.70	0.55	10/14
Enniatin A	0.28	0.28	n.a	-	1/14
Enniatin B	0.46	0.51	0.29	-	4/14
Equisetin	0.28	0.28	n.a	-	1/14
Festuclavine	3.26	3.26	n.a	-	1/14
Kojic acid	1968.00	1968.00	n.a	-	1/14
Macrosporin	123.12	123.12	n.a	-	1/14
Meleagrin	53.36	17.00	60.26	-0.91	5/14
Monactin	0.53	0.53	n.a	-	1/14
Mycophenolic acid	89.36	89.36	1.84	-	2/14
Ochratoxin A	5.02	5.02	n.a	-	1/14
Physcion	48.00	48.00	19.68	-	2/14
Stachybotrylactam	1.00	1.00	n.a	-	1/14
Sterigmatocystin	47.24	20.64	43.85	-0.36	7/14
Valinomycin	8.60	3.97	12.82	-1.00	6/14

Table 2 Building material -Wood

Building material -Chipboard (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Aspyrone	405.33	386.40	127.1	-	3/4
Macrosporin	10.50	6.91	8.2	0.90	4/4

Table 3 Building material -Chipboard

Building material-Sand (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Enniatin B3	0.01	0.01	n.a	-	1/3
Equeisetin	1.37	1.37	n.a	-	1/3
Nonactin	0.42	0.42	0.10	-	2/3

Table 4 Building material - Sand

Building material -Wall paper (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Beauvericin	70.24	70.24	n.a	-	1/6
Chaetoglobosin A	4001.89	3.14	5655.521	-	3/6
Chloramphenicol	9.16	9.16	0.06	-	2/6
Emodin	0.86	0.86	0.77	-	2/6
Enniatin B	0.44	0.44	n.a	-	1/6
Equisetin	3.98	3.98	n.a	-	1/6
Meleagrin	30.90	4.48	38.97	-	3/6
Monactin	0.30	0.30	0.17	-	2/6
Mycophenolic acid	66.72	66.72	n.a	-	1/6
Roquefortine C	7.32	7.32	2.89	-	2/6
Stachybotrylactam	76.47	76.47	73.53	-	2/6
Sterigmatocystin	0.78	0.78	n.a	-	1/6
Valinomycin	0.98	0.98	0.87	-	2/6

Table 5 Building material -Wall paper

Building material-Gypsum board (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Altenusin	8449.64	9296.20	3385.88	0.80	10/21
Aspyrone	64.40	64.40	n.a	-	1/21
Chaetoglobosin A	660.00	660.00	n.a	-	1/21
Chloramphenicol	1.11	1.11	n.a	-	1/21
Emodin	5.04	4.75	3.01	0.09	17/21
Enniatin B	0.60	0.54	0.27	-1.50	6/21
Enninatin B3	0.46	0.44	0.36	-	3/21
Macrosporin	1.24	1.23	0.20	-	3/21
Meleagrin	11.00	11.00	n.a	-	1/21
Nonactin	1.28	1.10	0.74	-1.61	4/21
Roquefortine C	5.22	5.22	n.a	-	1/21
Stachybotrylactam	3.81	3.81	n.a	-	1/21
Valinomycin	4.54	4.54	2.51	-	3/21

Table 6 Building material-Gypsum board

Comparison of dust sampling methods (swab and vacuum sampling)

Dust swabs – Moisture damaged buildings (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
3-NPA	1.20	1.27	0.55	-1.90	6/133
Alamethicin F30	8.20	8.20	n.a	-	1/133
Beauvericin	0.03	0.02	0.02	-1.97	6/133
Emodin	0.18	0.15	0.12	-1.76	26/133
Enniatin B	0.21	0.01	0.38	-1.82	33/133
Enniatin A	0.40	0.40	n.a	-	1/133
Enniatin A1	0.43	0.43	n.a	-	1/133
Enniatin B1	1.05	0.75	0.93	-1.85	12/133
Equisetin	1.30	0.34	1.36	-	3/133
Meleagrin	2.46	2.46	1.94	-	2/133
Penicillic acid	20.00	20.00	n.a	-	1/133
Physcion	10.13	8.00	7.90	-1.50	23/133
Sterigmatocystine	0.24	0.24	n.a	-	1/133

Table 7 Dust swabs – Moisture damaged buildings

Dust swabs - Non damaged buildings (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
3-NPA	0.62	0.69	0.22	-1.87	5/70
Alamethicin F30	2.59	2.59	2.17	-	2/70
Beauvericin	0.06	0.06	n.a	-	1/70
Emodin	0.16	0.14	0.07	-1.88	7/70
Enniatin B	0.01	0.01	0.01	-1.94	11/70
Meleagrin	0.82	0.81	0.18	-	3/70
Penicillic acid	1.70	1.70	n.a	-	1/70
Physcion	2.30	2.30	n.a	-	1/70

Table 8 Dust swabs - Non damaged buildings

Surface swabs - Moldy spots/surfaces (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Altertoxin	1.54	1.54	n.a	-	1/22
Beauvericin	2.66	2.66	n.a	-	1/22
Emodin	0.98	0.80	0.93	-	4/22
Enniatin B	1.53	0.04	3.16	-1.36	11/22
Enniatin B1	6.70	3.53	7.97	-1.38	5/22
Griseofulvin	0.15	0.15	n.a	-	1/22
Meleagrin	360.92	101.70	510.03	-	4/22
Physcion	6.24	6.24	n.a	-	1/22
Roquefortine C	5.04	1.75	5.76	-	3/22
Stachybotrylactam	41.00	41.00	n.a	-	1/22
Sterigmatocystine	2.44	2.44	n.a	-	1/22
Tentoxin	0.18	0.18	n.a	-	1/22
Valinomycin	0.01	0.01	n.a	-	1/22

Table 9 Surface Moldy spots/surfaces

Surface swabs - Non damaged area (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
3-NPA	1.32	1.32	n.a	-	1/10
Chaetoglobosin A	5120.00	5120.00	n.a	-	1/10
Enniatin B	0.02	0.02	n.a	-	1/10
Emodin	0.39	0.39	0.26	-	2/10
Meleagrin	3.56	3.56	n.a	-	1/10
Physcion	5.90	5.90	n.a	-	1/10
Sterigmatocystine	0.16	0.16	n.a	-	1/10

Table 10 Surface swabs - Non damaged area

Vacuum collected dust sample – Moisture damaged buildings (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Altenuen	744	744	n.a	-	1/76
Alternariol	6.22	5.53	3.06	-1.63	10/76
Alternariol monomethyl ether	1.84	2.04	0.52	-1.76	8/76
Andrastin A	200.40	187.20	91.11	-	3/76
Averantin	0.37	0.37	0.02	-	3/76
Averufin	0.37	0.32	0.27	-1.84	8/76
Beauvericin	3.37	0.81	13.30	-0.73	48/76
Chaetoglobosin A	1856	1856	n.a	-	1/76
Chlamydosporol	9.38	8.62	1.64	-	4/76
Chloramphenicol	31.67	16.54	68.66	-0.59	35/76
Cyclopeptine	4.36	4.36	3.68	-	2/76
Emodin	60.10	33.64	155.97	0.64	60/76
Enniatin A	3.29	0.80	9.26	-1.53	18/76
Enniatin A1	2.62	1.54	2.94	-1.49	17/76
Enniatin B	3.44	2.17	3.94	0.09	71/76
Enniatin B1	4.02	3.07	3.58	-1.05	29/76
Enniatin B2	0.68	0.36	0.50	-	3/76
Equisetin	12.95	6.26	14.06	-1.63	10/76
Griseofulvin	87.57	51.20	62.37	-	3/76
Mycophenolic acid	24.32	24.32	n.a	-	1/76
Norsolorinic acid	0.01	0.01	0.01	-	3/76
Nonactin	1.43	1.43	0.00	-	1/76
Physcion	6277.29	205.76	22106.93	-0.50	25/76
Sterigmatocystin	4.17	3.27	3.14	-1.80	6/76
Valinomycin	4.78	4.63	1.90	-1.79	6/76
Viridicatin	45.68	45.68	n.a	-	1/76

Table 11 Vacuum collected dust sample – Moisture damaged buildings

Vacuum collected dust samples - Renovated buildings (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Alternariol	11.45	5.55	10.29	-1.52	5/31
Alternariol monomethyl ether	3.95	2.55	3.20	-1.35	8/31
Andrastin A	88.00	88.00	n.a	-	1/31
Averantin	0.71	0.70	0.25	-1.58	7/31
Averufin	2.85	1.35	3.01	-1.56	6/31
Beauvericin	1.69	0.97	2.06	-0.53	22/31
Chlamydosporol	13.88	14.24	7.07	-1.84	7/31
Chloramphenicol	2.78	1.82	2.79	-1.18	11/31
CJ21058	4.98	2.13	6.14	-1.61	5/31
Cytochalasin C	259.36	259.36	230.24	-	2/31
Emodin	31.07	25.15	16.67	-0.12	17/31
Enniatin A1	2.43	1.97	1.75	-0.73	17/31
Enniatin B	5.87	5.26	5.14	0.58	31/31
Enniatin B1	5.26	4.23	3.69	0.20	26/31
Mehtylsulochrin	2.01	2.01	0.51	-	2/31
Mevinolin	156.13	103.20	159.12	-	7/31
Nonactin	0.25	0.25	n.a	-1.08	1/31
Norsolorinic acid	1.77	0.71	2.23	-	7/31
Physcion	382.26	168.00	469.23	-1.56	8/31
Sterigmatocystine	9.09	2.58	12.43	-0.87	8/31
Valinomycin	3.80	3.80	0.8226	-1.31	2/31
Viridicatin	11.60	11.60	n.a	-	1/31

Table 12 Vacuum collected dust samples - Renovated buildings

Vacuum collected dust samples - Control buildings (concentration in µg/kg)					
Metabolite	**Average**	**Median**	**STDEVP**	**Mean** (Log_{10})	**No. positives**
Alternariol	48.80	48.80	n.a	-	1/8
Alternariol monomethyl ether (AME)	11.53	11.53	9.91	-	2/8
Averentin	0.60	0.60	n.a	-	1/8
Averufin	0.71	0.71	n.a	-	1/8
Beauvericin	1.55	1.28	0.83	-0.93	4/8
Chlamydosporol	4.43	4.43	n.a	-	1/8
Chloramphenicol	7.27	7.27	5.88	-	2/8
Emodin	17.61	17.69	1.99	-0.37	4/8
Enniatin A1	1.13	1.13	0.12	-	2/8
Enniatin B	2.36	1.50	2.25	0.22	8/8
Enniatin B1	3.80	3.15	1.94	-0.42	5/8
Equisetin	67.97	57.77	62.89	-0.30	4/8
Norsolorinic acid	0.32	0.32	n.a	-	1/8
Physcion	1548.00	1548.00	1418.40	-	2/8
Sterigmatocystine	3.45	3.45	n.a	-	1/8

Table 13 Vacuum collected dust samples - Control buildings

2.13 Discussion

The metabolites found in different indoor matrices are given in tables (1-13). Approximately 2000 samples were screened for the presence of 250 target metabolites. A total of 389 (254 building materials and 135 methanol swabs) samples were positive for at least one of the target microbial metabolites. These samples were considered for statistical analysis. Among the evaluated matrices MMF (man made fiber) and sand from moisture damaged buildings contained highest (n=29) and least (n=3) number of metabolite respectively. Moreover the SFD, wood and wall paper from moisture damaged were positive for 26, 20 and 13 different microbial secondary metabolites respectively. Stachybotrylactam a metabolite of *Stachybotrys chartarum* and an indicator for the presence of high R^H was observed in gypsum board, wood, wall paper and swabs (moldy surfaces). The average concentration was found to be 30.5 µg/kg. The other frequently found metabolite were beauvericin (n=93, *Beauveria bassiana, Fusarium species*), equisetin (n=35, *Fusarium spp*), alternariol (n=22, *Alternaria spp*) and alternariolmonomethylether (n=25, *Alternaria spp.*). Indoor metabolites had inconsistent distribution on different matrices. This could be reasoned as a consequence of cross contamination due to air currents (mycelia and spore deposition) and also versatility of indoor fungi producing some of the common metabolites. Hence metabolite detected could not be designated as matrix specific marker. The clinical relevance of different metabolites and species found in these samples are discussed in Chapter 1.

Since variances of groups were very heterogeneous we decided to use Welch‘s t test, as most relevant statistical test for our purpose. The metabolites found significant by Welch‘s t-test in each group are highlighted in the tables with normalized mean values (Mean $\log_{10}$). The presence of enniatins (particularly Enniatin B, B1) was found significant (Welch t test) in all buildings, building materials and also in respective dust samples. Emodin, a common metabolite of *Penicillium*, *Aspergillus* and *Cladosporium* was significant in all sample groups (matrices) with the exception of chipboard. However sterigmatocystine, another Aspergillus metabolite (tertiary colonizer) and a precursor of aflatoxins, was found significant in SFD and wood materials of moisture damaged buildings. Physcion, another common metabolite of Eurotium and Aspergillus was found predominant (significant) in moisture damaged and renovated building SFD, in dust swabs of moisture damaged buildings and MMF. The significance of chaetoglobosin A

(*Chaetomium* metabolite) on wood material is consistent with earlier reports –*Chaetomium* as prolific wood degrading microbes"(*174*). The occurrence of chaetoglobosin A on another cellulose rich matrix, gypsum board (but no Welch's T-test significance) further justifies matrix specific infestation of *Chaetomium* species. Similarly the *Alternaria* metabolites alternariol and alternariol mono methyl ether (AME) were significant in SFD matrix of moisture damaged and renovated buildings. Bacterial metabolites chloramphenicol (moisture damaged and renovated) and valinomycin (moisture damaged) were significant in SFD matrix of moisture damaged and renovated buildings. This is consistent with the reports, for the co-occurrence of *Streptomycetes* (producers of valinomycin and chloramphenicol) and tertiary colonizing fungal flora in water damaged buildings (*175*). Recent report from Täubel et al. 2011 (*176*) has also confirmed for bacterial and fungal species releasing bioactive substances to their immediate surroundings.

"Attitude is a little thing that makes a big difference."
-Winston Churchill

Chapter 3

Microbial and anthropogenic volatile organic compound evaluation by GC-MS

“Efforts and courage are not enough without purpose and direction.”
-John F. Kennedy

3.0 Volatile organic compounds in indoor environments

The air quality in indoor environments is directly related to spectrum of volatile organic compounds (VOCs) present in the building. However in modern buildings additional factors such as efficiency of HVAC (Heating, Ventilation and Air Conditioning) systems also determine the quality of indoor air. The volatile compounds in indoors primarily originate from the solvents, cleansing agents, consumer products, combustion of fuel and as well due to microbial metabolism. Moreover the indoor matrices (carpets, furniture, decorative, paints) under the influence of light, fluctuating temperature and humidity may also release volatiles to variable extents. Similarly VOCs also constitutively emanate from building surfaces from the commonly used building materials such as mortar (MR) and carton gypsum board (CG). Nevertheless the volatile pattern varies under non obvious water damage conditions and in the events of water incursion. The building destructive fungi *Serpula lacrymans* and *Meruliporia incrassate* degrade building materials by enzymatic and non enzymatic processes upon invasion into buildings. These processes contribute for the non conventional pattern of (M)VOCs in indoor environments. However prevalence of higher concentration of VOCs (compared to residential indoors) that could be related solvents and combustion of fuels are common observation in the industries manufacturing varnishes, paints, textiles, chemicals and alloys. Nevertheless evaluation of indoor air quality for VOCs is essential for ensuring the well being of inhabitants and also for the prevention of occupational hazards.

The evaluation of VOC is reported to be beneficial for the detection of concealed microbial growth in buildings (*177-178*), food quality assurence and chemotaxonomy (*179-180*). For all the above purposes application of gas chromatography (GC) couple to mass spectrometry or GC in combination with different detectors are common practice. The working principle, specific advantages and challenges, various parameters and data treatment associated with GC-MS are detailed in this section.

3.1 Analytical methods for volatile evaluation

Gas chromatography-mass spectrometry (GC-MS) is a technique that combines the features of gas - liquid chromatography and mass spectrometry. The early reporting of feasibility and separation of volatiles is credited to James AT et al. (1952) for the separation of fatty acids (*181*). The first successful coupling of gas–liquid partition chromatography to time of flight mass spectrometer was demonstrated by Gohlke RS et al. in his landmark GC-TOF-MS instrumentation (*182*). The technology enabled analysis of a wide range of molecules such as ketones, aldehydes, alcohols, heterocyclic compounds, isocyanates, isothiocyanates, sulfides, lipids, and hydrocarbons (*149, 183*). In the course of evolution of GC-MS technology several types of mass spectrometers became available varying in the type interfaces, sensitivity and precision (Fig 10). Single quadrupole mass spectrometers with electron impact (EI) ionization are the most widely used type of instrumentation today (Fig 12).

In principle, samples to be separated are injected through the GC inlet (additional vaporization for liquid samples) and mobilized to the chromatographic column by the carrier gas (Fig 11). In all GC experiments one of the gases as hydrogen, helium and nitrogen are engaged, as carrier gas (*184*). The sample molecules undergo fractionation depending upon their extent of interactions with the stationary phase. Conventionally capillary GC columns are maintained under gas flow rates of ~ 1 to 2 mL/min. The present day mass spectrometers are devised to support higher gas flow rates, such as corresponding to pumping a rates of 300 L/sec (*185*). Likewise the separation in GC is also influenced by stationary phase of the capillary column. GC columns with variable polarities, lengths and film thickness are marketed commercially under different trade names. Hence the selection of columns should be made diligently. Some of the general purpose columns with non polar phases are poly-dimethylsiloxane (DB-1 or equivalent) or poly-phenyl (5%) / dimethlysiloxane (95%) (DB-5 or equivalent) (*186*).

Since GC-MS is a well known and established analytical technique for decades, only brief descriptions of critical steps are given here. As shown in the illustration diagram GC-MS instrumentation consists of four main parts (Fig 10 and 11). Most of the present day

instrumentations are comparable in terms of chromatographic capabilities, oven programming and separation efficiency of the volatile components. The advent of new interfaces/effective vacuuming systems has enabled to combine GC to various forms of MS ionization sources and detectors making customization almost limitless.

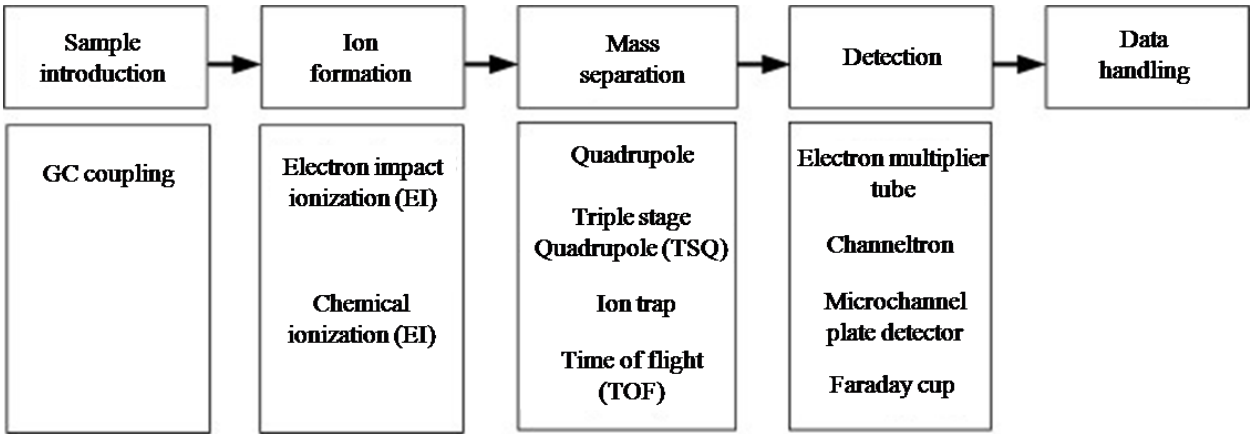


Fig 10. Overview on different GC-MS instrumentation types Wittmann et al. 2007

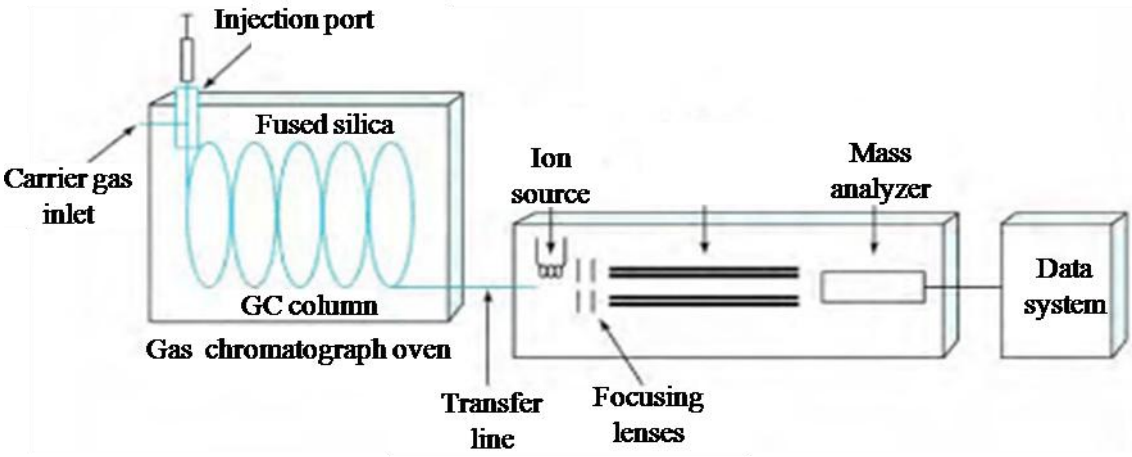


Fig 11. GC-MS instrumentation and set up-schematic

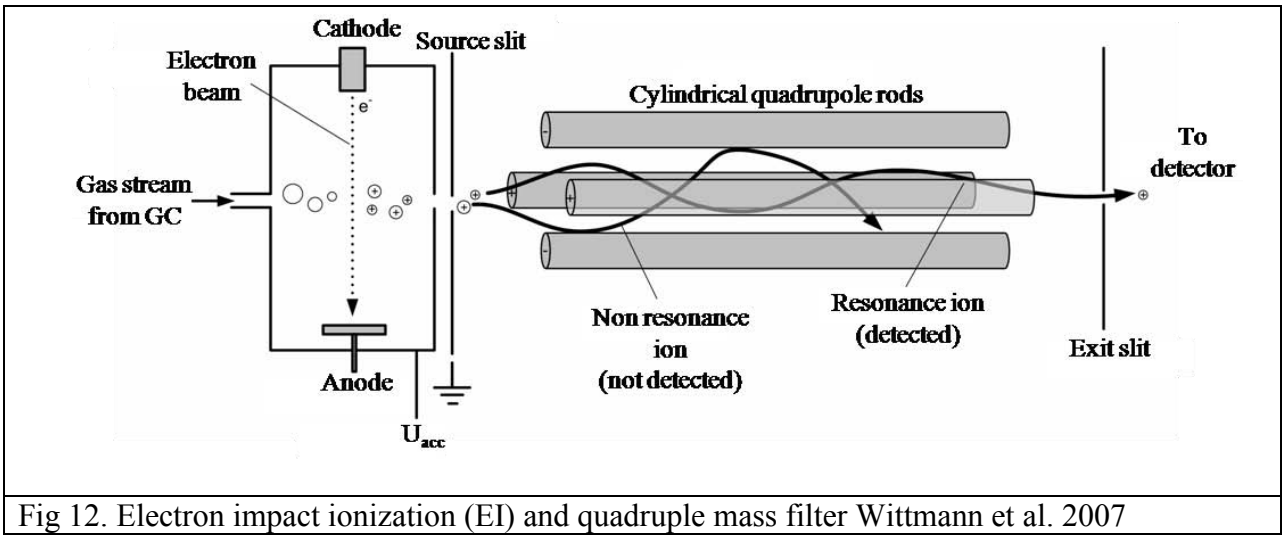


Fig 12. Electron impact ionization (EI) and quadruple mass filter Wittmann et al. 2007

3.2 Sample preparation and introduction

Properties of analytes such as volatility and polarity determine the extent of ionization when GC coupled to MS. The goal of any sample preparation is to yield analyte(s) of interest in a defined form that is amenable for GC-MS measurement. Until Janusz Pawliszyn introduced (1989) the solvent free sampling technique, GC sample preparation was solely dependent on organic solvent based extraction and derivatization procedures. The routine solvent extraction methods used even today in association for GC are soxhlet extraction and liquid-liquid extraction. These procedures are advantageous for monitoring preselected handful number of target analytes. Since the goal of our study was to profile all or most of the volatile molecules adsorbed to SFD surface, we decided to use Solid Phase Micro Extraction (SPME) procedure.

3.2.1 Solid phase microextraction (SPME)

Solid phase micro extraction (SPME) is a rapid, easy, solvent free and sensitive sampling technique. This method was envisaged by Janusz Pawliszyn to match with high-speed GC. The technique is based on the extraction of the analyte by the polymeric phase coated on a fused-silica fiber. The advantage of SPME over the conventional extraction methods is that no solvent has to be added, the extracts do not have to be concentrated prior to analysis and that losses of low boiling volatiles are prevented (*187*). The extraction process is initiated as soon as the polymeric coating is exposed to the sample matrix and reaches completion once the analyte concentration establishes distribution equilibrium between the sample matrix and the fiber

coating (*188*). For our study, following SPME-fibers with different stationary phases were tested. For all other evaluation purposes, pre-optimized combination of fiber, temperature, extraction time and sample volumes were used.

Fiber	Interaction	Polarity
100 µm polydimethylsiloxane (PDMS)	Absorption	Nonpolar
65 µm polydimethylsiloxane/divinylbenzene (PDMS/DVB)	Adsorption	Bipolar
85 µm carboxene/polydimethylsiloxane (CAR/PDMS)	Adsorption	Bipolar
85 µm polyacrylate (PA)	Absorption	Polar
50/30 µm divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS)	Adsorption	Bipolar

3.3 Gas chromatography & capillary column parameters

Selection of appropriate column determines the separation characteristics. A non-polar column is the recommended starting point for the analyses of non-polar compounds and likewise polar columns for the separation of polar compounds. Moreover, selection of capillary columns is accomplished considering four associated factors: stationary phase, column inner diameter (I.D), film thickness and column length (*189*). Typical construction of capillary column is illustrated in Fig 13.

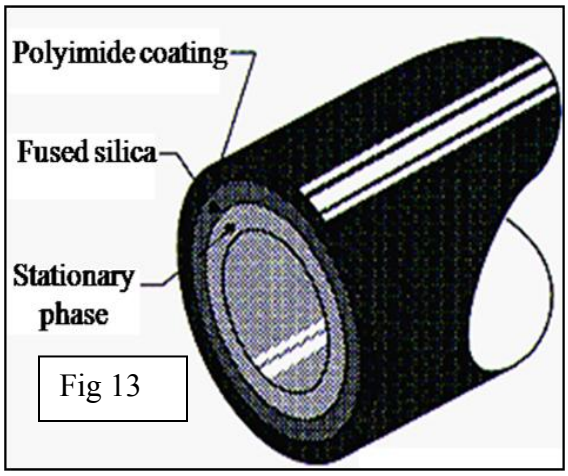


Fig 13

3.3.1 Column length

Determining an optimal column length is a compromise between speed and head pressure on one side, and resolution on the other side. The column length to resolution is correlated as, ~~–a~~n increase in resolution is directly proportional to the square root of the column length". Hence column length can regarded as the last parameter in method optimization. However column length still plays a critical role determining the measurement time. The length of the column is proportional to time taken for separation of mixture of volatile organic molecules. In cases such

as the separation of relatively simple samples (few volatile organic molecules) a column with shorter length is not only fit for the analytical purpose but also ideal for optimization of measurement time. Shorter columns (< 15m) are more suitable for separation of volatile mixtures consisting of chemically distinct structures or in general for screening purposes (*189*).

During random trails we realized the complexity of volatile pattern in SFD samples was far higher compared to any of the biological samples. Moreover the volatile mixture also consisted of many chemical components with structural similarity (publication 3, Fig 2). Considering the complexity of volatile mixture we decided to use 30m long HP-5MS and Optima® wax columns. The advantage of using column pairs is detailed in compound identification and also given in publication 3.

3.3.2 Column inner diameter (I.D.)

Commercial columns are available in a range of column inner diameter (~ 0.1-0.5 mm) and phase polarities. The diameter is related to two conspicuous aspects of GC namely efficiency and the sample loading capacity. The efficiency of a capillary column is expressed in terms of plates per meter (N/m). The columns with higher efficiencies are generally with lower I.D or plates per meter (N/m). Since the efficiency and sample capacity (or column capacity) are inversely related, ideal column diameter is determined considering the analytical purpose. Hence for the analyses of mixture of trace analytes or for the separation of co-eluting substances narrow I.D (as 0.10 or 0.18 mm I.D.) capillary columns are used. On the other hand for the analysis of enriched samples, samples predominantly containing target analytes and for preparative purposes wide bore columns (0.53 mm I.D.) may be more appropriate (*189*).

Since samples of our study were environmental samples (highly dissimilar), approximation of the concentration of individual volatiles were not feasible. Hence, instead of experimenting with different column I.D to achieve optimal chromatographic separation, we decided to optimize other method parameters as sample volume, extraction time and temperature etc. (publication 3, supplementary table 3).

3.3.3 Stationary phase

The selection of GC stationary phase is made based on the chemical principle ―likes dissolves in likes.‖ On this bedrock manufacturers have commercialized various stationary phases which can separate co-eluting compounds by altering polarity of stationary phases thereby modifying analyte-phase interaction. Non-polar compounds as n-alkanes are readily separated on non-polar capillary columns such as HP-5MS (Agilent) or SLB-5ms, SPB-5 (SUPELCO Analytical). The nature of interaction between analyte and non-polar stationary phase are dispersive. The extent of intermolecular attractions is generally proportional to size of the molecule. Contrasts to non-polar compounds, molecules as alcohols, amines, carboxylic acids, diols, esters, ethers, ketones and thiols with variable polarity are classified either as intermediate polar or polar molecules. The capillary columns with intermediate polar or polar phases can separate respective compounds. The separations of these molecules are the net result of dispersive and somewhat stronger interactions such as dipole-dipole or π-π electron interactions with the phase. These intermediate polar or polar compounds are claimed for effective separation on Optima® Wax (Agilent), Omegawax, SUPELCOWAX 10 and SPB-20 (SUPELCO Analytical) commercial columns. Molecules with high degree of unsaturation are an exception to ―likes dissolves likes‖. Yet another factor to be considered when the GC is coupled to MS is column bleeding (*184*). Column bleed is the continuous elution of the components produced from normal degradation of the stationary phase. A sudden or rapid increase in column bleed is usually regarded an indicator of column damage or a problem in the GC system. Prolonged heating of a column above its upper temperature limit, constant exposure of the column to oxygen (usually via a leak), or repeated injection of damaging compounds are the common causes for the problem. This aspect becomes critical as column bleed may show up in the MS spectra and increase the noise and complicate identification of the target. Hence bonded phases are recommended in combination with MS. Commercially they are marketed with an addition designation of MS (*189*).

As mentioned earlier, in our study column pairs, HP-5MS and Optima® wax columns were employed (in separate GC runs) for separation of volatiles present in every SFD sample. The reasoning behind this approach was to clearly separate co-eluting molecules and constitutional isomers and avoid ambiguity in identification. This approach was particularly advantageous for

use in distinguishing ψ-cumene, mesitylen and hemimellitene as three different components (publication 3).

3.3.4 Phase or film thickness

Similar to column diameter decreasing film thickness results in sharper peaks, increased signal-to-noise and reduced sample capacity. Capillary columns with thinner film thickness are suited for separation of analytes with higher boiling point (>300 °C). The columns with thinner films of ~ 0.10 to 0.25 μm are generally used in trace analyses of pesticides, PCBs, FAMEs (fatty acid methyl esters), phthalate esters, and other semi volatile compounds (*184*).The reduced film thickness increases analyte interaction with the tubing wall (fused silica) and reduce retention time and elution temperatures. The shorter retention time and low temperature elution are the characteristics of columns with thinner film thickness. The columns with 1 to 5 μm film thickness are ideal for the separation of analytes with low boiling points, such as volatile organic compounds and gases (*189*). Hence any of the consideration is specific to a particular application.

3.4 Carrier gas or mobile phase

The carrier gas is the means to move analytes through the column. Ideally a mobile phase gas should be inert to solute and stationary phase and also must be free of detectable contaminants. The choice of a carrier gas is simple, nitrogen (N_2), helium (He) or hydrogen (H_2). Predominantly used gases are nitrogen or helium because of their inherent non explosive nature. The other reason could be the costs associated with these gases. Nitrogen is approximately 2.5 times more economical compared to helium (*190*). Under appropriate safety measures hydrogen can also be used as mobile phase. In cases of portable on-site chromatographs or residual gas analyzers (RGA) air is used as carrier gas.

3.4.1 Advantages of using different carrier gases

The nature of the carrier gas strongly affects the height equivalent to a theoretical plate (HETP) of the column. The chromatography investigation of Korolev et al. 2006 reported for a decrease in retention time and HETP for lighter hydrocarbons (C_1-C_4) in the order of He > H_2 > N_2 > CO_2

~ N_2O (*191*). As a function of lower diffusivity, chromatography using nitrogen is relatively slower compared to hydrogen and helium. This is best explained by van Deemter equation (*190*). However for cost conservation nitrogen is an obvious choice over He and H_2. For hydrogen though it has half the viscosity of helium and nitrogen and better efficiency and speed is not a popular choice. This is mainly due to its reductive properties and associated higher costs.

3.5 Ovens and temperature maintenance

Maintenance of analyte in vapor state is a primary requisite for GC-MS. Conventionally volatile analytes intended for GC separation are introduced either as liquids (additionally vaporization) or vapor adsorbed on to the fiber surfaces. The core of the GC instrumentation, columns is affixed in ovens devised with temperature regulation. The GC ovens support the maintenance of wide range of temperature conditions between 40 to 350 °C. The modern instrumentation with instant ramping abilities allows user to run GC under both isothermal and as temperature programmed conditions. This is of utmost advantage in separations of mixture of chemicals with different vapor pressures in a single GC run (*192*).

3.6 GC-MS interfaces

In early days GC-MS had difficulties in reducing GC effluent pressure (~ 760 torr) to required pressure 10^{-6} to $l0^{-5}$ torr. The primitive approach was to split a small fraction (1 to 5%) of the GC effluent entering into the mass spectrometer (*185*). Subsequent advancement in technology led to invention of carrier gas separators. The new interfaces acted more like an enrichment method increasing concentration of the organic compounds in the carrier gas stream. They allowed all or most of the analyte molecules from the GC into the mass spectrometer. Some of the earlier gas separator interfaces were effusion separators (Watson-Biemann), jet separators (Ryhage-Stenhagen), and semi permeable membranes (*193*). Modern GC-MS set up mostly rely on maintenance of required ion source pressure appropriate for generating electron ionization (EI) spectra. The maintenance of ion source pressure (10^{-6} to 10^{-5} torr) is achieved by employing appropriate mobile flow rates of few ml/min enabled by capillary column dimension (e.g. 25 to 30 m long by 220 to 250 μm I.D) in combination with efficient gas pumping system (*194*). For

most combinations of modern mass spectrometers with capillary GC columns, gas flow rates of 1 to 2 mL/min, and pumping speed of at least 300 L/sec are recommended (*185*).

3.7 Ionization methods used in combination with GC

The selection of ionization method plays a critical role in determining the efficiency of the GC-MS method. In addition to the data quality it also determines method characteristics such as selectivity, sensitivity, and limits of detection of an analytical method. The secondary considerations in this regard are polarity, derivatization, purpose and extent of desired fragmentation of a target molecule. The two most commonly used ionization techniques in combination with GC-MS are EI or CI. For our study purpose we used electron ionization (EI).

3.7.1 Electron ionization (EI)

Electron ionization or electron impact ionization (EI) is a well characterized ionization method. The ionization is accomplished by passing a beam of electrons through the column elute. An electron upon collision with a neutral analyte molecule knocks off another electron, resulting in a positively charged radical ion. EI generally employs an ionization potential of -70 ev. This voltage is regarded as the maximum energy required for the ionization of all volatile molecules amenable for GC-EI-MS. Moreover depending upon the internal energy of the molecule EI produce either a molecular radical ion which has the same molecular weight and elemental composition as the analyte, or produces a fragment ions which corresponds to a different fractions of the analyte molecule (*195*). Decreasing the electron energy can reduce fragmentation, but it also reduces the number of ions formed.

3.7.2 Chemical ionization (CI)

Chemical ionization is based on ion-molecule reactions. Primarily gases such as methane, isobutane, or ammonia at high pressure (~1 Torr) are subjected to EI to produce reagent or reaction gas. Subsequently the reagent gas reacts with neutral analyte gaseous molecules (100:1) producing spectra of molecular ions characteristic of the analyte molecule (*195*). An advantage of CI over EI is that, it predominantly produces molecular ions compared to fragment ions. This is often more useful for elucidation of structural details of compounds in addition to compound identification. (*196*). Almost all neutral analytes form positive ions. Negative chemical ionization

(NCI) is more pronounced for compounds containing acidic groups or electronegative elements such as halogens. NCI is more popular in environmental analysis for the detection PCBs, flame retardants and pesticides (*197*).

3.8 Signal amplification and detection

The signal amplification and sensitivity detecting signals close or below the LOD are two important issues. In addition to analyte abundance the limit of detection of a method may also partly depend on ion detection device associated with chromatographic system. In principle each detector generates signals from incident ions. The incident ions may either generate secondary electrons which are later amplified or may induce current generation by the mobility of charges. Technological evolution has offered a wide range of detection instrumentation. In addition to high resolution and speed, GC offers advantage of interfacing with wide variety of detection methods, which can be broadly classified into ionization detectors and optical detectors (*198*). The list begins with earliest photographic plates, Micro channel plates (MCP), Electron multiplier tubes (EMT) to present day popular Channeltron. The modern instruments can generate reliable signals with as little as 1-10 primary ions, which are later amplified to ~ $l0^6$ electrons (*199*).

3.8.1 Electron multiplier tube (EMT)

Electron multiplier tube **(EMT)** belongs to class of vacuum tube detectors. The EMTs are based on secondary emission principle. Secondary emission is the process wherein an electron strikes a metal surface and ejects other electrons from the metal at the expense of some of its kinetic energy. In EMTs the particles to be detected is primarily converted to electrons before amplification which is generally performed by conversion dynodes. However, the amplification is due to biased dynodes that eject secondary electrons when they are struck by an ion. These secondary electrons are accelerated from one dynode to another and each secondary electron causes the emission of several other electrons. In order to have a measurable effect, the initial electron needs to induce release of a total number of electron counts between 10^4 and 10^5. A ―Channeltron" is a special type of EMT which is not sealed in vacuum; instead, the secondary

electrons are excited at the input by direct ion or an electron impact. The Channeltrons are widely used in ion traps and quadrupole instruments.

3.9 GC-MS data

The computers aided with dedicated software record the information pertaining to the MS generated ions (i.e. m/z values) and corresponding abundance and form the GC-MS data. The mass chromatograms are recorded either in full scan mode (Total ion current [TIC]) or Selected ion monitoring mode (SIM). These are the two predominantly used scan modes in quadrupole mass spectrometers (*200*).

3.9.1 Total ion current (TIC)

Conventionally the GC-MS data is regarded as the sum of its ion chromatograms, which is designated as total ion current (TIC) (Fig 14). Mathematically, a TIC chromatogram from a quadrupole mass analyzer is obtained by summing the complete GC-MS data matrix along its columns (*200*). Total ion chromatogram is a preferential choice when user anticipates a set of target molecules in a certain m/z value range or indecisive of exact mass of a molecule. As a consequence the detector is set to scan a range of masses (lowest to highest 8-10 fold). Hence TIC can be defined as sum of all ions in a particular mass range. The data generated in full scan mode is perceived preliminary and which also warrants for additional confirmation experiments. The drawback of this analysis is requirement of higher duty cycle accompanied by compromised sensitivity or sensitivity loss in the event of broad scan range.

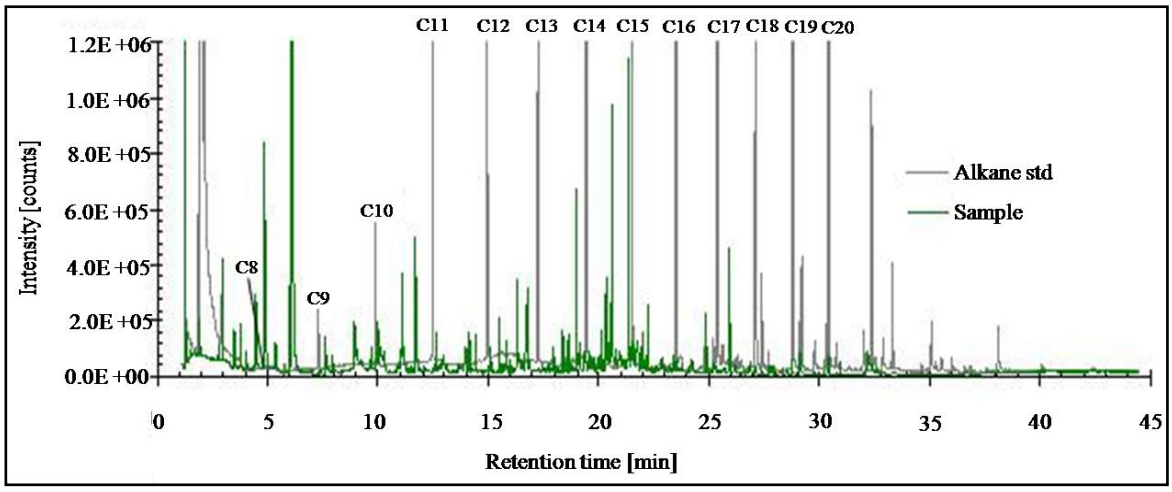


Fig 14. GC-MS TIC chromatogram with Alkane standard overly

3.10 GC-MS data processing

In case of samples containing structurally similar volatile components (e.g. environmental and food samples) chromatography may fail to achieve a base peak separation for all components. Instead a signal overlap is a generally observed feature. The other reason for signal overlap and limited chromatographic resolution could be attributed to dynamic interaction of analyte with stationary phases during diffusion processes of analytes along the column. The above mentioned are couple of factors responsible for characteristic and complex peak shapes, complicating analysis. These inherent challenges can be minimized by peak deconvolution and modeling with exponentially modified Gaussian function (*201*).

Moreover the electronic noise and chemical noises are typical in GC-MS spectra. Irrespective of the source of noise, the net effect may result in deterioration of the signal quality. Specifically this is due to the increased background, lower signal-to-noise ratio, skewed peak shapes and distorted signal baseline. The electronic noise or true noise refers to random fluctuations that originate from the limitations in instrument electronics. On the other hand, chemical noises are due to extraneous chemical components introduced in the system unintentionally. The chemical noises may also arise from derivatizing agents or due to the column bleeding. Hence in a true sense chemical noises are regarded as unwanted signals originating from chemical components introduced as a part of the experimental process (*200*).

3.10.1 Deconvolution

The problem of extraction of pure component signals in the instances of incomplete chromatographic separation is often referred to as "peak deconvolution". However this should not be mistaken for inversion of a convolution process employed in signal processing (*200*). For our purpose, the free software package Automated Mass Spectral Deconvolution and Identification System (AMDIS, NIST Gaithersburg, MD) was used as described earlier (*202-203*). The AMDIS software versions 32 V2.1 and above are competent to handle data generated on different makes of GC-MS. The process of deconvolution mainly involves four major steps noise analysis, component perception, spectrum deconvolution and compound identification. Moreover, Lu and coworkers in their report commented on the performance comparability of

AMDIS to other available commercial software packages for GC–TOF–MS data (time of flight) (*204*). However, reports emphasizing false positives and the necessity of secondary verification of AMDIS results with other data evaluation tools are also not uncommon (*205*).

3.11 Criteria for component recognition and identification

Mass spectral matching is useful and widely practiced criteria for metabolite identification. However, mass spectral matching alone some time may be insufficient for unambiguous and reproducible identification of components. The major obstacles for this are the presence of multiple structural isomers (with very similar mass spectra) in biological and environmental samples. As a consequence ideas such as Retention index (RI) based on retention tims of *n*-alkanes was put forth. Currently the RI information based on *n*-alkanes is used as additional criteria for spectral matching and identification. This led to inclusion of RI information along with mass spectra while building target libraries. These libraries are referred as mass spectral and retention index data bases or libraries (MS/RI library) (*206*). The criteria considered during our study for the confirmation of identity of components is described in attached publication 3 (*207-208*). Briefly, in our study a component was considered as valid information, once its occurrence was found on two columns (inverse polarities). The set of three criteria for confirmation were spectral match factor, retention index and spectral similarity to pure standards.

3.11.1 Library search

One approach to aid in identification of compounds in mixtures relies upon comparing mass spectra to precompiled spectral libraries (*200*). This approach is with limited success due to the limited scope of already available spectral libraries and other technical limitations. The dedicated mass spectral libraries are desirable, but their constructions are elaborate processes considering the necessity of curation (manual examination of spectra before inclusion). The reasons for this can be attributed to multiplicity of mass spectra for any particular compound in commonly relied parent spectral libraries e.g. NIST and Wiley. However in cases of signal overlap the library search fails to extract and match to any of the components from the mixture or may lead to erroneous identification. These difficulties warrants for more reliable criteria to achieve explicit identification of components. In spite of limitations listed above the mass spectral library search is widely practiced for automated (optimization of time) analysis of large data sets.

3.11.2 Retention index (RI)

The RI is a system independent and a reproducible parameter, which can be used for identification of components. The RI system has essentially eliminated the necessity of maintaining strict chromatographic conditions for the comparison of spectra/data generated by various users. Retention indices are relative retention times normalized to retention times of adjacently eluting n-alkanes. The elapse in retention time of two consecutive n-alkanes is divided into 100 parts; hence by definition the retention index of an alkane with n carbon atoms per formula unit is defined as 100 n. As a primary requirement for using RI, the expected retention time range of all target compounds should be ideally encompassed within the retention time window of used n-alkane standard series. Two commonly used index systems are proposed by Kováts - for isothermal conditions, and van den Dool and Kratz - for temperature programmed gas chromatographic (TPGC) runs respectively. Example: for monoterpenes and sesquiterpenes a range of C_8 to C_{20} is generally considered adequate. For oxygenated sesquiterpenes and diterpenes require higher alkanes up to C_{26}. In our study we used n-alkanes C_5 to C_{27} for RI calibration purpose. The source of the alkane standards and concentrations of individual mixtures are described in publication 3. Throughout our investigation van den Dool and Kratz indices were adopted (*209*). The retention index for TPGC is determined by the formula $\mathbf{RI = 100[n+ (t_{Ri} - t_{Rn})/ t_{R(n+1)}- t_{Rn})]}$. In the formula $t_{Ri,}$ $t_{Rn,}$ $t_{R(n+1)}$ and n represents retention times of metabolite, earlier eluting alkane, later eluting alkane and number of C atoms of earlier eluting alkane respectively.

The entire work flow related GC-MS is illustrated in below seen image Fig 15.

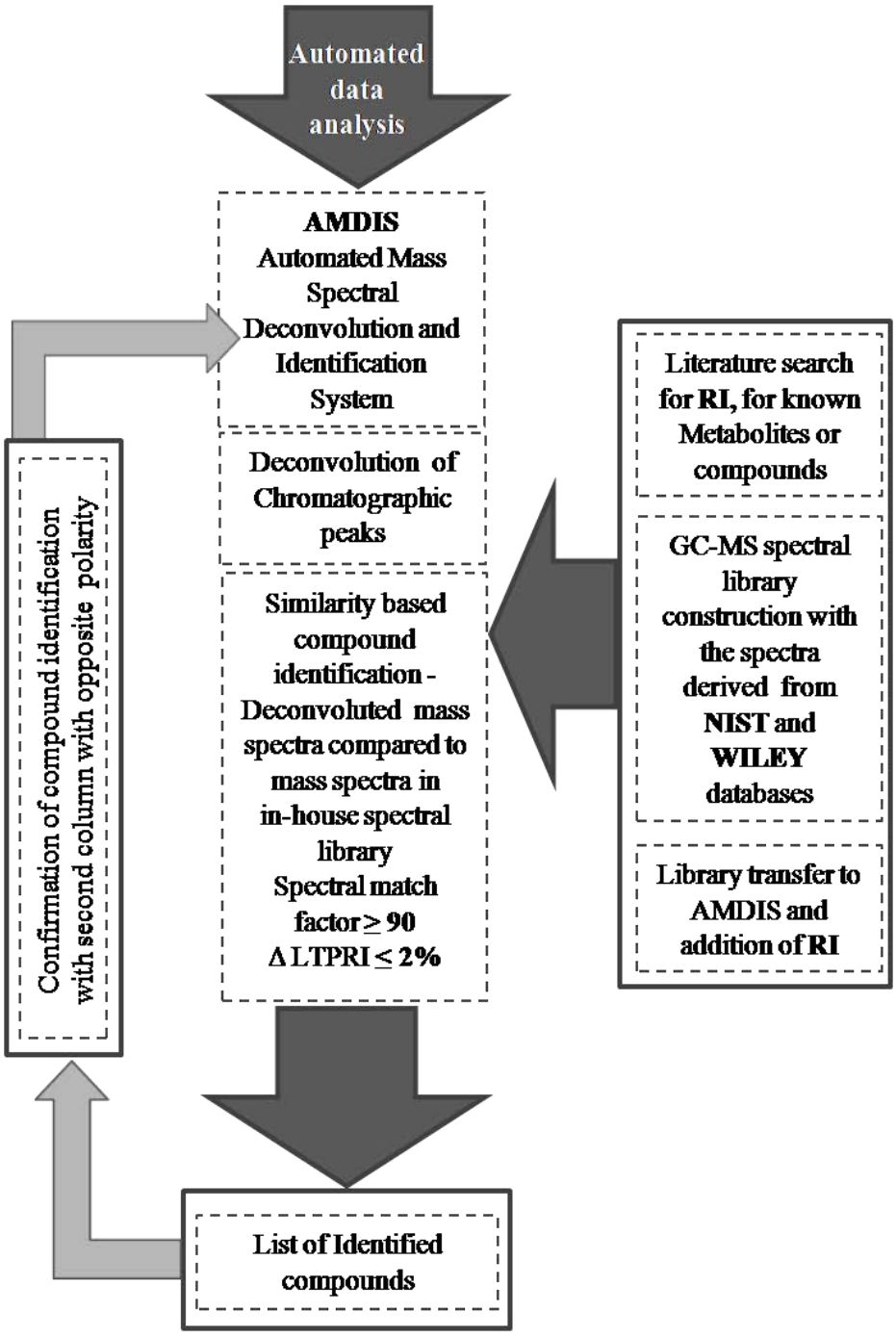


Fig 15 GC-MS work flow

3.12 Statistical evaluation of GC-MS data

Multivariate statistical analysis (MVA) is essential for analysis of large data sets with multitudinous variables. The results of non supervised (cluster/group information from the samples were not taken into consideration/included in machine learning) multivariate Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) are generally useful for cross verification purposes. In our study, GC-MS method parameters and repeatability of method were evaluated using MVA.

The purpose of PCA was to identify factors causing distinctions between various SFD samples. However HCA was employed for verification of repeatability of the SPME method, stability of instrumentation over longer sequences (measurement time) and also for component distribution similarity among different groups of samples. Statistical software Unscrambler® and R package (R 2.12.0) were used for PCA and HCA respectively. The data was leverage corrected and centered before subjecting to PCA and for the HCA –Euclidean" distance and –Ward" linkage methods (between groups) were used.

3.13 Spectconnect

The salient feature of the software Spectconnect is its non dependency on any of the reference libraries. The freely available software Spectconnect (http://spectconnect.mit.edu.), can be used for enumeration and tracking of unidentified metabolites in various samples. The working principle, algorithm, data extraction procedures followed by Spectconnect, are described elsewhere in detail (*210*). Appertain to data validation we demonstrated usability of AMDIS deconvoluted chromatograms in combination with Spectconnect. Moreover this software can also be used for systematic detection of conserved components across replicates of a sample or between sample groups. In our study systematic conserved component identification was done for verifying the sample homogeneity, method performance evaluation and source recognition/apportionment. Criteria for picking conserved components were kept stringent and are described in attached publication 3. Statistical software Unscrambler® (*211*) and R package (R 2.12.0) were used for multivariate statistics PCA and HCA respectively. The present work

resulted in the first report of application of Spectconnect for both analytical method validation and comparison of samples without compound identification.

“Dream is not what you see in sleep, dream is the thing which does not let you sleep.”
- Dr. A P J Abdul Kalam

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List of figures and tables

Conclusion

“Stay hungry stay foolish”
-Steve jobs

The objective of this thesis was to develop and validate analytical procedures suitable for indoor safety assurance. The details related to LC-MS/MS method expansion, validation and application to Slovakian samples are compiled in publication 1 (Vishwanath et al. 2009). We optimized extraction procedures and analytical methods for multi mycotoxin determination in wide range of indoor matrices. Also we compared extraction efficiencies of three extraction solvents. Among the three solvents acidified mixture of acetonitrile water was most appropriate with good extraction recovery (R_E 70-120%) for the majority of target analytes (results in list of publication 1).

The primary task of identification of buildings with water damage was done by civil engineers and building safety experts. The selection was mainly based on visual observation and questionnaires regarding health status of occupants. The sampling sites were Slovakia, Spain, Finland, The Netherlands and Austria. A wide range of mold affected and relevant materials with different physical and chemical characteristics were collected from these dwelling for investigation. The larger data set was sorted into positive and negative lists. The list consisting of samples positive for at least one of the target metabolite were considered for statistical analysis (Welch‘s T-test). This was done in order to verify microbial specificity to indoor matrices. Among the many matrices SFD was found to be significant for the presence of six fungal and two bacterial metabolites (Enniatins (B, B1), sterigmatocystine, physcion, alternariol, alternariol mono methyl ether, valinomycin and chloramphenicol). The significance of *Chaetomium* species was limited for wood and other cellulose rich materials (chaetoglobosin A). The other matrices building materials and swab samples shared a comparable significance pattern. The results and discussion of the Welch‘s T-test are given chapter 2 under the section title ―unpublished data”.

Similarly extraction efficiencies of microwave extractor, ultrasonic tweeter and orbital shaker were compared for extraction with acidified mixture of acetonitrile water. However there was no significant improvement in extraction efficiencies upon extraction using microwave and ultrasonic tweeter. Moreover co-extraction of matrix with more intensive extraction procedures such as microwave extractor and ultrasonic tweeter cannot be completely ruled out. Considering extraction time, thermal degradation of analytes and processivity the orbital shaker was found

most appropriate. Hence orbital shaker was employed for extraction throughout the research work (data not shown, manuscript in progress).

However even after the completion of method validation we continued with our method expansion, since additional reference substances became commercially available. As of now the method is capable of scanning > 250 target metabolites belonging to various fungal and bacteria genera. The effectiveness of the method expansion was observed during investigation, by the detection of co-occurring bacterial metabolites with other indoor mycotoxins in the samples procured from moisture damaged buildings (results in publication 2). Moreover additional 20 fungal and 5 bacterial metabolites were detected in naturally infested building materials and SFD samples and described in attached publications (results in publication 2 and 3) after publication 1 had been accepted.

In one of the collaborative studies (University of Nitra, Slovakia), we detected the prevalence of species *Engyodontium album* and *Acremonium* along with species of *Fusarium*, *Pencillum*, *Chaetomium* and *Cladosporium* in water damaged buildings of Slovakia. Species *Engyodontium album* and *Acremonium* which were not detectable with our LC-MS/MS target method (due to non availability commercial standards) was recognized by ancillary culture based methods (results in list of publication 1). This was an important finding as *Engyodontium album* is less frequently reported in indoor evaluation studies. Interestingly the earliest report of the *Engyodontium* species can also be traced back to Slovakia, as causatives for keratinolysis among children (*115*). The targeted approaches as LC-MS/MS when supplemented by other techniques as GC-MS, microbial culturing and molecular diagnostics (qPCR) can be highly advantageous for comprehensive evaluation of indoor environments.

In indoors every matrix undergoes degradation producing micro particles and ultimately leading to formation of settled floor dust (SFD). Depending upon the particle aerodynamic diameter particles are classified as aerosols and sedimentary particles. The particles of dust with their unique surface properties adsorb volatiles prevailing in air. Taking this into account we decided to evaluate settled floor dust to investigate various (M)VOCs occurring in widely differing indoor environments. We procured samples from buildings with water damage, new constructions and also from indoor environments used for waste sorting and disposal (recyclable

wastes). For the investigation of (M)VOCs we used a HS-SPME-GC-MS technique along with in-house developed mass spectral library. With our effort we could detect the presence of 155 (both identified and annotated) volatile organic compounds (results in list of publication 3). To the best of our knowledge 30 of the 71 identified volatile organic compounds (VOCs) are new reportings in SFD matrix. Issues like matrix homogeneity and method reproducibility were validated in a theoretical fashion using ―AMDIS and Spectconnect". Similarly we demonstrated the feasibility of source identification/apportionment based on volatile pattern hidden in settled floor dust without any requirement of component identification. Principal component analysis (PCA) of peak areas of microbial volatile organic compounds (MVOCs) resulted in identification of nonanal as potential MVOC marker. To the best of our knowledge this is the first comparative study of individual SFD samples derived from widely differing indoor environments (in their purposes) using both LC-MS/MS and GC-MS methods. From the analytical point of view we demonstrated a new way of validation of GC-MS based methods (results in publication 3). The sensory-irritation effects due to volatiles and effects of mycotoxins are known for synergistic modes of action (*32, 11*). Considering these earlier reports our study can be regarded as a comprehensive evaluation. The summary of results of mycotoxin and volatile in dust samples are compiled in publication 3.

During the course of dissertation research the presence of (M)VOCs was confirmed and concentration of non volatile microbial metabolites in indoor environments was quantified. This information is beneficial envisaging probable health threats. However these confirmatory evidences still may require supplementary data to clarify all open questions related SBS and BRI.

The toxin prevalence studies supplemented with clinical correlation are advantageous identifying etiological agents for diseases like sick building syndrome and building related illnesses. We are optimistic about the outcome of ongoing research project (HITEA till 2013) which is intended for providing a comprehensive picture of microbes, metabolites relating to (observed) ill health symptoms among pupils and staff in schools. Similarly, the aspect of the project to monitor renovation of water damaged buildings can be useful in formulating criteria for building restoration purposes. The prevalence data from large screening studies like ours can be the early

start point for systems biology/toxicology approach to decipher complex net work behind the SBS and BRI.

In addition to all the academic goals, the FFG initiative also enabled production and commercialization of 25 indoor essential analytical standards for mass spectrometry and research purposes with our industrial collaborators. The ready availability of standards can speed up the toxicity testing studies in addition to shedding more light on indoor related problems. The validated method (LC-MS/MS) was transferred to industrial partners as according to their specific needs. This dissemination of the technology or method also regarded beneficial, making testing facility widely accessible.

“An unexamined life is not worth living”

-Plato

Original works

1. **Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry**.

   Vishwanath V, Sulyok M, Labuda R, Bicker W, Krska R**. Anal Bioanal Chem**. 2009 Nov; 395(5):1355-72.

2. **Co-occurrence of bacterial toxins and mycotoxins in moisture damaged indoor environments**.

   Täubel M, Sulyok M,**Vishwanath V**, Bloom E, Turunen M, Järvi K, Kauhanen E, Krska R, Hyvärinen A, Larsson L and Nevalainen A. Täubel M, Sulyok M, **Vishwanath V**, Bloom E, Turunen M, Järvi K, Kauhanen E, Krska R, Hyvärinen A, Larsson L, Nevalainen A. **Indoor Air**. 2011 Oct; 21(5):368-75.

3. **Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC-MS/MS and GC-MS methods**.

   Vishwanath V, Sulyok M, Weingart G, Kluger B, Täubel M, Mayer S, Schuhmacher R, Krska R. **Talanta**. 2011 Sep 30; 85(4):2027-38.

Peer reviewed conference proceedings

4 **Occurrence of toxic bacterial and fungal metabolites on mold damaged building materials.** Sulyok M, Täubel M, Vishwanath V, Peitzsch M, Kauhanen E, Krska R, Larsson L, and Nevalainen A, Healthy Buildings, Syracuse, USA, 14.09.2009.

5 **Microbial toxins in surface swab and dust samples from school buildings in three European countries.** Peitzsch M, Suylok M, Täubel M, Vishwanath V, Jacobs J, Borras A, Krop E, Vepsäläinen A, Zock JP, Hyvärinen A, Heederik D, Nevalainen A, and Larsson L. Indoor Air Conference, Austin, Texas, 09.06.2011

6 **An Intervention Study In Moisture Damaged Homes – preliminary results before renovation.** Hyvärinen A, Täubel M, Sulyok M, **Vishwanath V**, Turunen M, Järvi K, Patovirta RL, Tuomainen TP, Pirinen J, Krska R, Pekkanen J and Nevalainen A. Indoor Air Conference, Austin, Texas, 09.06.2011

Other publications

- **Determination of moulds and their metabolites in indoor environments**. **Vishwanath V**, Sulyok M, Krska R, Schuhmacher R, Proceedings, MOLD-MEETING, Mikrobiologie und Molekularbiologie, **ALVA-Mitteilungen**, Heft 7, 2009

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ORIGINAL PAPER

Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry

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Abstract This paper describes the application of a previously published multi-mycotoxin method for food and feed matrices based on liquid chromatography/electrospray ionization-tandem mass spectrometry (HPLC/ESI-MS/MS) to the analysis of microbial metabolites in indoor matrices. The range of investigated analytes has been extended by 99 fungal and bacterial metabolites to cover now 186 compounds overall. The method is based on a single extraction step using an acidified acetonitrile/water mixture (which has been determined to be preferable to methanol and ethyl acetate) followed by analysis of the diluted crude extract. The analytical signal of one third of the investigated analytes was reduced by more than 50% due to matrix effects in a spiked extract of house dust, whereas the other investigated materials were less critical in that aspect. For determination of method performance characteristics, a spiked reference material for house dust was chosen as a model sample for an extremely complex matrix. With few exceptions, coefficients of variation of the whole procedure of <10% and limits of detection of <50 μg kg^{-1} were obtained. The apparent recoveries were below 50% for half of the investigated analytes due to incomplete extraction and/or detection-related matrix effects. The application of the method to 14 samples from damp buildings revealed the presence of 20 different analytes at concentrations of up to 130 mg kg^{-1}. Most of these compounds have never been identified before in real-world samples, although they are known to be produced by indoor-relevant fungi. This underlines the great value of the described method for the on-site determination of microbial metabolites.

Keywords Liquid chromatography · Tandem mass spectrometry · Mycotoxins · Bacterial metabolites · Damp buildings · Indoor molds

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Introduction

Molds and microbes in general are ubiquitous in nature and constitute a vital part of ecological systems. Yet, their manifestation in the indoor environment is usually undesired and under normal conditions, bacteria and fungi do not notably grow in building materials or on indoor surfaces [1]. This is mainly due to a lack of adequate moisture, as water activity is a key parameter especially for fungal growth [2]. Both, acute water damage of buildings (due to construction flaws, plumbing leakage, flooding, etc.) as well as moisture accumulation due to today's energy-effective way of construction (thermal insulation), insufficient airing and insufficient maintenance of plumbing, heating, and air-conditioning systems lead to conditions that favor microorganisms specialized in growing in damp indoor environments [1, 3]. As far as molds are concerned, increasing water activity shifts the fungal spectrum from

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the common airborne genera found in indoor environment (such as *Penicillium*, *Aspergillus*, and *Cladosporium* [4]) to water-damage molds. The latter includes many of the most toxic fungal species such as *Stachybotrys chartarum*, *Chaetomium globosum* and *Memnoniella echinata* as well as species of *Trichoderma* [2].

The incidence of indoor molds has been epidemiologically linked to a variety of symptoms, e.g., respiratory infections and allergic rhinitis [5], inflammations [6], increased risk of developing asthma in young children [7], allergies [8] and irritation of the eye [9]. The suspected etiological agents are allergenic fungal proteins, cell wall components such as β-1,3-D-glucans, microbial volatile organic compounds (MVOCs) as well as mycotoxins and other fungal secondary metabolites [2].

In contrast to mold-related health effects that can be characterized by parameters that are amenable to clinical diagnostics (such as immune response), the contribution of toxic effects caused by exposure to specific fungal metabolites is still a matter of intense debate [10]. Experiments in animal modesl suggest that the inhalation of mycotoxins may be much more toxic than ingestion [11]. Furthermore, in real-world inhalative exposure scenarios, the observed biological effects may not only be due to inhalation of mycotoxins but they may be the overall result of co-exposure to other microbial contaminants or volatile chemicals such as cleaning agents [12]. Thus, specific toxic effects caused by exposure to fungal metabolites are much more difficult to pin down and may amount to a crucial problem in risk assessment of indoor mold growth.

In addition to this, the controversy is stirred up by the lack of a sound exposure assessment of indoor mycotoxins due to methodological gaps. Methods for the determination of related fungal species (e.g., by classical taxonomy, PCR-based methods or chemical analysis of marker compounds such as β-1,3-D-glucans or ergosterol) are insufficient as the occurrence of certain molds does not allow the prediction of the occurrence of their metabolites [13]. On-site determination of fungal metabolites in dust and other indoor matrices is thus inevitable to elucidate the actual role of these compounds.

From an analytical point of view, indoor materials (especially dust) are very complex matrices. For this reason, most of the published methods rely on a chromatographic separation (mostly HPLC) and a subsequent mass-spectrometric (MS) detection. Dedicated methods for the determination of single compounds (or a single compound class) in damp buildings have been developed for trichodermin and macrocyclic trichothecenes (based on their conversion to trichodermol and verrucarol, respectively, and subsequent analysis by GC-MS/MS) produced by *S. chartarum* [14, 15], for satratoxins G and H [16, 17] and for the *Aspergillus versicolor* metabolite sterigmatocystin [16, 18]. LC-MS/MS-based quantitative multi-analyte methods dedicated to indoor analysis have been developed for building materials [13, 19] and for fungal cultures and spiked cellulose filters [20]. However, the results obtained by the former method have been questioned by others as no fungal producers were present in the samples and the list of detected compounds was somewhat inconsistent [2], whereas the latter method has not been applied to real-world samples.

It is remarkable that in all these references, comparatively low recoveries are reported. However, no clear statement is given whether this is related to mass-spectrometric detection (due to matrix-induced signal suppression) or due to analyte losses caused by incomplete extraction or by the chosen clean-up procedure (although the recovery of 74±20% reported for the determination of ochratoxin A in dust by HPLC-FLD [21] suggests that these difficulties cannot solely related to the detection).

In this work, we report on the application of an HPLC-MS/MS-based method that has initially been developed for the multi-mycotoxin determination in food and feed [22, 23]. The list of analytes has been further extended to 159 fungal and 27 bacterial metabolites, most of which have never been addressed before by an analytical method dedicated to the analysis of indoor materials (the most comprehensive method for screening of fungal metabolites [24] that covers 474 compounds has, to the best of our knowledge, not been applied to the analysis of naturally infected indoor samples). A number of bacterial metabolites (most of them produced by *Streptomyces* species) have been included since data on their occurrence in the indoor environment is extremely scarce and is restricted to the finding that a strain of *Streptomyces griseus* isolated from an indoor environment was capable of producing the antibiotic valinomycin [25]. Furthermore, the simultaneous determination of fungal and bacterial metabolites is needed to support investigations on biological effects due to co-exposures to fungal and bacterial species (such as the increase of inflammatory responses of mouse macrophages after the simultaneous exposure of *Streptomyces californicus* and *S. chartarum* [26]) on a metabolic level. It was our goal to prove the applicability of the LC-MS/MS-based multi-analyte method to crude extracts of indoor-relevant materials including dust on the basis of a model sample that was spiked at multiple levels in order to determine the method performance parameters and to investigate matrix effects and recoveries of the extraction step. Furthermore, the method was applied to a small set of moldy real-world samples in order to obtain a preliminary picture on the pattern of microbial metabolites that are produced in damp indoor environments.

Experimental

Chemicals and reagents

Methanol, acetonitrile (both LC gradient grade) and ethyl acetate p.a. were purchased from J.T. Baker (Deventer, The Netherlands), ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

Standards of fungal and bacterial metabolites were obtained either as gifts from various research groups or from the following commercial sources: Biopure Referenzsubstanzen GmbH (Tulln, Austria), Sigma-Aldrich (Vienna, Austria), Iris Biotech GmbH (Marktredwitz, Germany), Axxora Europe (Lausanne, Switzerland) and LGC Promochem GmbH (Wesel, Germany). Stock solutions of each analyte were prepared by dissolving the solid substance in acetonitrile (preferably), acetonitrile/water 1:1 (*v*/*v*), methanol, methanol/water 1:1 (*v*/*v*) or water. Twenty-three combined working solutions were freshly prepared prior to the spiking experiments by mixing the stock solutions of the corresponding analytes, followed by a further dilution in neat solvent. All solutions were stored at −20 °C and were brought to room temperature before use.

Samples

For the comparison of extraction efficiencies of different solvents and initial investigations on matrix effects, dust from the shelf tops of offices in our department was collected using a common vacuum cleaner attached to a sampling nozzle containing a Whatman No. 4 25 μm filter paper (ALK-Abelló, Linz, Austria). Method validation was carried out by spiking a standard reference dust material (SRM 2583, National Institute of Standards and Technology, Gaithersburg, Maryland). SRM 2583 is certified for five trace elements and is composed of dust collected from vacuum cleaner bags used in routine cleaning of interior dwelling spaces. Moldy indoor samples (carton-gypsum board and splints scraped off from walls, coarse-soil-containing wooden particles, paper cover taken from the underneath of a carpet) were collected in damp buildings in Slovakia and Austria. Isolation and identification of the corresponding fungi were carried out by dilution-plating method on Dichloran Rose Bengal Chloramphenicol agar (DRBC) and in some cases also on a malt extract isolation medium. Fungal identifications to appropriate species level (or genus level) were done based on phenotypic traits of the individual isolates/strains according to methodologies given in Refs. [27, 28].

Sample preparation

SRM 2583 reference dust (0.05 g) was spiked with appropriate amounts of the 23 combined working solutions at ten different concentration levels (each in triplicate). The spiked samples were later dried overnight at 40 °C to allow the evaporation of the solvent and to establish equilibration between the analytes and the matrix. Since dust was found to absorb a significant amount of solvent, the proportion of extraction solvent (acetonitrile/water/acetic acid 79:20:1, *v*/*v*/*v*) had to be increased (in comparison to our previous method dedicated to food and feed matrices) to 400 μl, resulting in a sample-to-solvent ratio of 1:8 (*w*/*v*). The samples were extracted for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and subsequently centrifuged for 2 min at 3,000 rpm (15 cm radius) on a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA). Aliquots of 100 μL of raw extract were transferred into autosampler vials equipped with glass microinserts using Pasteur pipettes and were diluted with the same volume of dilution solvent (acetonitrile/water/acetic acid 20:79:1, *v*/*v*/*v*). The diluted extracts were vortexed and 5 μL were injected without further pretreatment.

The same extraction procedure was applied to the moldy building materials with the sample-to-solvent ratio varying between 1:4 (*w*/*v*) and 1:8 (*w*/*v*) and to the blank building materials (the sample-to-solvent ratio was 1:4 (*w*/*v*) for these samples). For the comparison of the extraction efficiencies of ethyl acetate, methanol, and acetonitrile/water/acetic acid (79:20:1, *v*/*v*/*v*), 250 mg of spiked dust were extracted in duplicate using 2 mL of the respective solvent.

Instrumental parameters

Detection and quantification was performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C_{18}-column, 150×4.6 mm i.d., 5 μm particle size, equipped with a C_{18} security guard cartridge, 4×3 mm i.d. (all from Phenomenex, Torrance, CA, US). Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (*v*/*v*/*v*; eluent A) and 97:2:1 (*v*/*v*/*v*; eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% within 12 min, followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1000 μL min^{-1}.

ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs

per sample by scanning two fragmentation reactions per analyte. The sMRM detection window of each analyte was set to the respective retention time ±24 s and the target scan time was set to 1 s. The settings of the ESI-source were as follows: source temperature 550 °C, curtain gas 10 psi (69 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 50 psi (345 kPa of nitrogen), ion source gas 2 (drying gas) 50 psi (345 kPa of nitrogen), ion-spray voltage −4,000 V and +4,000 V, respectively, collision gas (nitrogen) high. The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (diluted in a 1+1 mixture of eluent A and B) into the MS using a 11 Plus syringe pump (Harvard Apparatus, Holliston, MA, US) at a flow rate of 10 μL/min—see Table 1 for the corresponding values.

Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid, that each exhibit only one fragment ion), which yields 4.0 identification points according to commission decision 2002/657/EC [29]. In addition, the LC retention time and the intensity ratio of the two sMRM transitions have to agree with the related values of an authentic standard within 0.1 min and 30% rel., respectively.

Method performance characteristics in house dust

For the determination of the performance characteristics of the extended method SRM 2583 was spiked at ten different concentration levels (each in triplicate) with relative concentrations of 1:2:4:10:20:40:100:200:400:1,000. For external calibration, the 23 combined working solutions were mixed and further diluted in acetonitrile/water 50:50 (*v*/*v*, acidified with 1% acetic acid). Blank extracts were diluted 1+1, as described in "sample preparation" and fortified for matrix-matched calibration. The concentrations of the analytes in the external standards and the matrix-matched standards were matched at each level to the expected concentrations in the final diluted extract of the spiked samples. Linear, 1/*x* weighted calibration curves were constructed from the data obtained from the analysis of each sample type (spiked sample, liquid standard, spiked extract) using the Analyst® software version 1.5. To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated to yield the apparent recovery (R_A), the signal suppression/enhancement (SSE) due to matrix effects and the recovery of the extraction step (R_E) as follows:

$$R_A(\%) = 100 \times \text{slope}_{\text{spiked samples}} \Big/ \text{slope}_{\text{liquid standards}} \quad (1)$$

$$\text{SSE}(\%) = 100 \times \text{slope}_{\text{matrix-matched standards}} \Big/ \text{slope}_{\text{liquid standards}} \quad (2)$$

$$R_E(\%) = 100 \times \text{slope}_{\text{spiked samples}} \Big/ \text{slope}_{\text{matrix-matched standards}} \quad (3)$$

The coefficients of variation (CVs) of the whole method were calculated using Validata®, a Microsoft Excel macro [30] from linear, 1/*x* weighted calibration curves obtained after the analysis of the spiked samples. This software tool was also used for the calculation of the repeatability of the method at the lowest and the highest concentration level and of the 95% confidence interval of the slopes of the calibration functions of the three sample types. From the latter, the 95% confidence interval of R_A, SSE, and R_E were calculated according to the law of error propagation. The limits of detection (LODs) were calculated at the lowest evaluable concentration levels both of spiked samples as well as of liquid standards as concentrations corresponding to a signal-to-noise ratio (S/N) of 3:1 by applying the "S-To-N" script of Analyst® 1.5.

To estimate the extent of signal suppression/enhancement in case of building materials, the diluted extracts were fortified with a multi-analyte standard on one concentration level and the resulting peak areas were compared to the corresponding peak areas of the liquid standards. The extraction efficiencies of the three investigated solvents were determined by comparing the peak areas of the collected dust spiked before extraction to the related values of the related blank extract spiked after extraction.

Results and discussion

Extension of the LC-MS/MS protocol

We decided to further extend our LC-MS/MS protocol since a couple of metabolites produced by indoor-relevant fungal and bacterial species were not included in the list of target analytes of our previous methods dedicated to food and feed analysis [22, 23], e.g., stachybotrylactam and satratoxins (produced by *S. chartarum*), roquefortine C (produced by *Penicillium chrysogenum*), fumigaclavin (produced by *Aspergillus fumigatus*), austdiol and austocystin A (produced by *Aspergillus ustus*), altertoxin-I (produced by *Alternaria tenuissima*), calphostin C (produced by *Cladosporium cladosporoides*), K-252a and K-252b (produced by *Nocardiopsis* species), and valinomycin and other *Streptomyces* metabolites. As expected, not all of these compounds are perfectly compatible to the chosen LC-MS/MS conditions (that we did not want to change as our long-term goal is to establish one single method that is able to deal with almost all of our applications): ascomycin, cyclosporins C and D, FK 506, HC-toxin, rapamycin and tenuazonic acid exhibited broad peaks, whereas austdiol, cephalosporin C, cochliodinol, penicillin G, and tetracycline were not long-term-stable

Table 1 List of analytes together with optimized ESI-MS and ESI-MS/MS parameters

Analyte	Retention time (min)	*m/z* precursor ion	DP[a] (V)	*m/z* product ions[b]	Rel. int.[c]	Collision energy (V)[b]	Cell exit potential (V)[b]
AAL TA Toxin	11.7	522.3 $[M+H]^+$	71	328.5/292.4	0.89	35/41	20/16
3-Acetyldeoxynivalenol	10.4	397.3 $[M+Ac]^-$	−40	59.2/307.1	0.16	−38/−20	−8/−7
15-Acetyldeoxynivalenol	10.4	339.1 $[M+H]^+$	61	321.3/137.2	0.69	13/17	18/8
Actinomycin D	15.2	1,253.6 $[M-H]^-$	−155	329.3/698.3	2.09	−62/−74	−21/−17
Aflatoxin B1	12.1	313.0 $[M+H]^+$	76	285.2/128.1	0.65	33/91	16/10
Aflatoxin B2	11.8	315.1 $[M+H]^+$	66	287.2/259.2	0.85	37/43	18/18
Aflatoxin G1	11.5	329.0 $[M+H]^+$	56	243.2/200.0	0.60	39/59	14/12
Aflatoxin G2	11.1	331.1 $[M+H]^+$	81	313.2/245.2	0.69	35/43	18/14
Aflatoxin M1	11.2	329.1 $[M+H]^+$	61	273.2/229.1	0.49	35/59	16/12
Aflatoxin M2	10.7	331.0 $[M+H]^+$	66	273.1/285.2	0.43	31/33	16/14
Agroclavine	8.9	239.1 $[M+H]^+$	56	183.2/208.2	0.88	27/27	10/12
Alamethicin F30	15.9	775.5 $[y7^d+H]^+$	81	282.3/197.2	0.83	55/71	20/28
Altenuene	12.1	293.2 $[M+H]^+$	36	257.2/275.2	0.39	21/15	16/16
Altenusin	12.4	289.0 $[M-H]^-$	−60	230.0/245.2	0.27	−30/−24	−11/−1
Alternariol	13.9	257.0 $[M-H]^-$	−70	212.9/214.9	0.85	−34/−36	−11/−11
Alternariolmethylether	15.1	271.1 $[M-H]^-$	−65	256.0/227.0	0.16	−32/−50	−13/−9
Altersolaniol A	11.3	319.0 $[M-H]^-$	−45	301.2/282.9	0.77	−20/−28	−13/−13
Altertoxin-I	13.2	351.1 $[M-H]^-$	−75	263.2/315.3	0.76	−44/−24	−11/−7
2-Amino-14,16-dimethyloctadecan-3-ol	15.7	314.3 $[M+H]^+$	41	296.2/125.2	0.014	25/25	18/6
Anisomycin	7.6	266.3 $[M+NH_4]^+$	56	121.3/78.3	0.11	33/85	8/12
Apicidin	15.0	622.4 $[M-H]^-$	−105	462.2/209.0	0.14	−32/−68	−11/−9
Ascomycin	15.4	809.6 $[M+NH_4]^+$	81	774.6/564.6	0.46	25/35	12/16
Asperlactone	7.0	185.2 $[M+H]^+$	51	141.2/113.2	0.29	11/13	12/6
Asperloxin A	13.1	394.1 $[M+H]^+$	71	123.1/95.1	0.57	33/61	8/16
Aspinonene	5.3	206.1 $[M+NH_4]^+$	21	127.2/81.0	0.42	11/25	8/14
Aspyrone	8.2	185.1 $[M+H]^+$	51	125.0/139.2	0.53	13/11	10/8
Asterric acid	13.1	347.0 $[M-H]^-$	−55	149.1/165.9	0.11	−20/−26	−9/−11
Atpenin A5	15.3	363.9 $[M-H]^-$	−45	328.1/292.3	0.75	−14/−22	−7/−7
Aurofusarin	14.5	571.2 $[M+H]^+$	116	556.3/485.3	0.30	35/53	16/12
Austdiol	8.7	237.0 $[M-H]^-$	−50	147.0/145.0	0.23	−30/−38	−1/−1
Austocystin A	15.1	373.2 $[M+H]^+$	76	312.2/283.2	0.59	39/59	18/16
Avenacein Y	13.5	319.2 $[M+H]^+$	36	287.2/175.1	0.92	27/49	16/10
Bafilomycin A1	16.1	645.6 $[M+Na]^+$	126	443.5/327.4	0.05	43/57	26/16
Beauvericin	15.8	801.5 $[M+NH_4]^+$	86	244.2	2.29	47/73	12/10
		806.5 $[M+Na]^+$	161	384.4			
Brefeldin A	13.2	281.0 $[M+H]^+$	36	245.3/263.3	0.72	11/9	14/14
Calphostin C	15.4	789.3 $[M-H]^-$	−105	459.3/108.1	0.53	−56/−84	−9/−5
Cephalosporin C	6.4	416.3 $[M+H]^+$	46	143.0/185.2	0.75	23/25	12/16
Cerulenin	12.9	224.1 $[M+H]^+$	21	179.1/206.3	0.90	10/12	11/9
Chaetocin	13.9	695.0 $[M-H]^-$	−50	631.0/567.1	0.34	−18/−26	−9/−15
Chaetoglobosin A	14.5	529.4 $[M+H]^+$	76	130.2/511.3	0.29	59/15	8/14
Chanoclavine	7.8	257.1 $[M+H]^+$	46	168.2/226.2	0.92	27/17	10/14
Chetomin	14.5	711.2 $[M+H]^+$	66	298.2/348.2	0.53	25/21	10/18
Chloramphenicol	10.9	320.9 $[M-H]^-$	−50	151.9/256.9	0.54	−24/−18	−7/−13
Chromomycin A3	15.0	1,181.6 $[M-H]^-$	−200	1,033.4/269.2	0.79	−60/−106	−15/−19
Citrinin	14.6	251.0 $[M+H]^+$	26	233.0/205.2	0.12	25/39	14/12
Citreoviridin	14.4	403.4 $[M+H]^+$	61	139.2/297.2	0.70	33/23	8/18
Cochliodinol	15.9	505.1 $[M-H]^-$	−120	224.0/477.4	0.90	−62/−48	−11/−11
Curvularin	12.9	291.1 $[M-H]^-$	−60	122.9/189.9	0.74	−38/−40	−7/−9

Table 1 (continued)

Analyte	Retention time (min)	*m/z* precursor ion	DP[a] (V)	*m/z* product ions[b]	Rel. int.[c]	Collision energy (V)[b]	Cell exit potential (V)[b]
Cycloaspeptide A	14.3	642.3 $[M+H]^+$	91	150.1/134.1	0.57	45/63	12/8
Cycloheximide	11.5	282.2 $[M+H]^+$	46	247.2/246.1	0.95	23/21	14/16
Cyclosporin A	16.2	1,200.8 $[M-H]^-$	−130	1,088.8/893.9	0.07	−48/−68	−15/−11
Cyclosporin C	16.1	610.2 $[M+2H]^{2+}$	76	100.1/156.2	0.08	127/119	6/12
Cyclosporin D	16.5	609.3 $[M+2H]^{2+}$	66	100.2/156.2	0.94	129/127	16/10
Cyclosporin H	16.1	602.3 $[M+2H]^{2+}$	71	100.3/156.3	0.46	67/55	6/12
Cytochalasin A	14.2	478.2 $[M+H]^+$	71	460.5/120.2	0.09	23/38	12/8
Cytochalasin B	13.5	480.2 $[M+H]^+$	51	462.5/444.5	0.63	23/23	10/12
Cytochalasin C	13.7	525.2 $[M+NH_4]^+$	31	430.5/490.5	0.41	23/17	12/14
Cytochalasin D	13.2	525.2 $[M+NH_4]^+$	31	430.5/490.5	0.53	23/17	12/14
Cytochalasin E	14.0	513.3 $[M+NH_4]^+$	41	416.4/434.5	0.64	19/17	12/12
Cytochalasin H	13.5	494.2 $[M+H]^+$	26	416.5/434.5	0.57	19/11	12/12
Cytochalasin J	13.0	452.2 $[M+H]^+$	31	434.5/416.5	0.75	13/21	12/12
Deepoxydeoxynivalenol	9.1	339.1 $[M+Ac]^-$	−40	59.1/248.9	0.10	−20/−18	−9/−17
Deoxynivalenol	7.6	355.1 $[M+Ac]^-$	−40	265.2/59.2	4.72	−22/−40	−13/−8
Deoxynivalenol-3-glucoside	7.6	517.3 $[M+Ac]^-$	−50	427.1/59.1	1.37	−30/−85	−11/−7
Diacetoxyscirpenol	11.9	384.2 $[M+NH_4]^+$	51	307.2/105.1	0.54	17/61	9/7
Dihydroergosine	11.1	550.2 $[M+H]^+$	96	270.1/253.0	0.46	47/43	16/16
Dihydroergotamine	11.3	584.3 $[M+H]^+$	81	270.3/253.2	0.70	43/47	16/14
Dihydrolysergol	7.2	257.1 $[M+H]^+$	81	167.2/154.2	1.00	55/55	10/8
Elymoclavine	7.2	255.1 $[M+H]^+$	61	181.2/180.2	0.97	41/57	10/10
Elymoclavine fructoside	6.5	417.2 $[M+H]^+$	61	255.2/237.3	0.34	29/33	18/20
Emodin	16.0	269.0 $[M-H]^-$	−70	224.9/240.9	0.32	−38/−38	−11/−13
Enniatin A	16.0	699.4 $[M+NH_4]^+$	76	210.1/228.0	0.31	43/47	12/18
Enniatin A1	15.9	685.4 $[M+NH_4]^+$	66	210.1/228.2	0.67	41/49	8/20
Enniatin B	15.6	657.5 $[M+NH_4]^+$	51	196.3/214.1	0.52	45/47	18/18
Enniatin B1	15.8	671.4 $[M+NH_4]^+$	81	196.0/210.0	0.73	43/41	12/12
Enniatin B2	15.4	643.5 $[M+NH_4]^+$	66	214.3/196.3	0.50	43/41	12/12
Enniatin B3	15.1	629.4 $[M+NH_4]^+$	46	196.3/214.3	0.67	41/41	10/12
Equisetin	16.3	372.2 $[M-H]^-$	−80	342.3/142.9	0.11	−34/−66	−7/−7
Ergine	6.4	268.1 $[M+H]^+$	56	223.2/208.2	0.74	29/35	14/10
Ergocornine	11.3	562.2 $[M+H]^+$	56	223.2/208.2	0.55	47/63	12/12
Ergocorninine	12.3	562.2 $[M+H]^+$	51	544.2/223.2	0.52	21/47	16/12
Ergocristine	12.0	610.4 $[M+H]^+$	56	592.4/223.2	0.84	21/47	18/12
Ergocristinine	13.0	610.4 $[M+H]^+$	46	592.5/223.3	0.51	21/47	18/12
Ergocryptine	11.8	576.4 $[M+H]^+$	51	223.3/208.2	0.57	47/61	14/16
Ergocryptinine	12.8	576.4 $[M+H]^+$	51	558.4/223.3	0.69	21/49	16/12
Ergometrine	7.2	326.2 $[M+H]^+$	66	223.2/208.2	0.39	39/35	14/14
Ergometrinine	8.5	326.2 $[M+H]^+$	66	208.2/223.2	0.55	35/39	14/14
Ergosine	11.1	548.4 $[M+H]^+$	56	223.2/208.2	0.32	45/57	14/12
Ergosinine	10.9	548.4 $[M+H]^+$	56	223.2/208.2	0.15	45/57	14/12
Ergotamine	11.4	582.2 $[M+H]^+$	66	223.2/208.2	0.31	47/59	12/14
Ergotaminine	11.2	582.2 $[M+H]^+$	66	223.2/208.2	0.17	47/59	12/14
Ergovaline	10.5	534.2 $[M+H]^+$	76	223.2/208.2	0.37	45/63	12/10
Festuclavine	9.0	241.2 $[M+H]^+$	76	154.2/168.2	0.67	47/39	10/10
FK 506	15.5	821.7 $[M+NH_4]^+$	81	768.8/109.2	0.53	29/107	12/6
Fumagillin	15.0	459.2 $[M+H]^+$	71	177.1/131.1	0.83	23/43	10/22
Fumigaclavin A	8.4	299.3 $[M+H]^+$	56	167.2/154.2	0.74	61/61	10/8
Fumitremorgin C	13.7	380.3 $[M+H]^+$	61	212.3/324.3	0.32	45/23	12/8

Table 1 (continued)

Analyte	Retention time (min)	*m/z* precursor ion	DP[a] (V)	*m/z* product ions[b]	Rel. int.[c]	Collision energy (V)[b]	Cell exit potential (V)[b]
Fumonisin B1	12.8	722.5 $[M+H]^+$	91	334.4/352.3	0.78	57/55	4/12
Fumonisin B2	13.9	706.3 $[M+H]^+$	96	336.3/318.5	0.47	59/51	8/2
Fumonisin B3	13.4	706.3 $[M+H]^+$	96	336.3/318.5	0.40	59/51	8/2
Fusarenon-X	8.9	413.3 $[M+Ac]^-$	−40	59.1/262.2	0.20	−44/−22	−9/−16
Geldanamycin	14.7	559.3 $[M-H]^-$	−65	280.0/516.3	0.95	21/23	16/14
Gibberellic acid	10.4	364.3 $[M+NH_4]^+$	36	239.2/221.2	0.52	23/35	14/12
Gliotoxin	12.2	327.1 $[M+H]^+$	31	263.2/245.2	0.61	15/25	16/20
Griseofulvin	13.1	353.2 $[M+H]^+$	51	165.2/215.2	0.91	27/27	10/12
HC-toxin	10.6	435.2 $[M-H]^-$	−95	184.0/113.1	0.21	−36/−52	−13/−3
HT-2 Toxin	13.0	442.2 $[M+NH_4]^+$	46	263.1	5.18	21/27	19/20
		447.4 $[M+Na]^+$	101	345.1			
Hydrolysed fumonisin B1	11.9	406.3 $[M+H]^+$	86	370.3/388.3	0.88	29/27	10/20
Ionomycin	16.4	707.5 $[M-H]^-$	−135	167.1/393.4	0.57	−68/−62	−13/−9
K252a	14.9	468.2 $[M+H]^+$	81	293.3/337.3	0.96	83/45	16/20
K252b	15.3	454.2 $[M+H]^+$	61	269.3/410.4	0.78	57/31	18/12
Kojic acid	3.3	143.0 $[M+H]^+$	56	69.2/113.2	0.26	23/31	10/10
Lysergol	7.2	255.1 $[M+H]^+$	61	240.2/197.2	0.78	29/33	14/12
Macrosporin	15.9	283.0 $[M-H]^-$	−65	268.1/224.8	0.17	−34/−50	−1/−19
Meleagrin	11.2	434.3 $[M+H]^+$	51	403.3/334.2	0.53	23/33	12/20
Methysergide	9.3	354.2 $[M+H]^+$	61	237.2/222.2	0.70	35/41	12/14
Mevinolin	15.6	405.4 $[M+H]^+$	46	199.2/173.3	0.64	17/29	14/10
Mithramycin	15.0	1,083.5 $[M-H]^-$	−105	935.3/269.1	0.89	−58/−98	−13/−15
Monactin	14.9	768.8 $[M+NH_4]^+$	66	185.2/111.3	0.48	49/81	16/18
Moniliformin	3.3	96.9 $[M-H]^-$	−70	41.2	–	−24	−5
Monoacetoxyscirpenol	11.0	342.2 $[M+NH_4]^+$	41	265.0/307.0	0.42	13/13	26/8
Mycophenolic acid	13.6	338.1 $[M+NH_4]^+$	31	207.2/303.2	0.87	33/19	16/18
Myriocin	14.7	402.4 $[M+H]^+$	46	104.0/267.4	0.68	31/27	6/16
Neosolaniol	9.3	400.2 $[M+NH_4]^+$	46	215.1/185.0	0.90	25/29	12/14
Nigericin	16.8	742.6 $[M+H]^+$	76	657.8/675.7	0.25	37/35	10/20
3-Nitropropionic acid	2.9	118.0 $[M-H]^-$	−35	46.0	–	−16	−3
Nivalenol	5.4	371.1 $[M+Ac]^-$	−45	281.1/59.1	1.55	−22/−42	−15/−7
Nonactin	14.8	754.6 $[M+NH_4]^+$	96	185.1/111.0	0.55	49/79	12/18
Ochratoxin A	14.6	404.0 $[M+H]^+$	61	239.0/102.0	0.53	37/105	16/14
Ochratoxin B	13.7	370.1 $[M+H]^+$	56	205.0/103.2	0.48	33/77	12/16
Ochratoxin α	13.2	254.9 $[M-H]^-$	−60	210.9/166.9	0.87	−24/−36	−11/−11
Oligomycin A	16.0	789.6 $[M-H]^-$	−115	533.5/109.1	0.89	−36/−76	−13/−17
Oligomycin B	15.8	803.5 $[M-H]^-$	−110	547.5/112.8	0.50	−36/−68	−15/−7
Oxaspirodion	10.3	251.1 $[M+H]^+$	56	161.1/133.1	0.42	15/27	10/8
Oxidized elymoclavine	5.1	259.1 $[M+H]^+$	61	184.2/156.1	0.40	27/29	8/10
Oxidized luol	5.8	291.5 $[M+H]^+$	41	259.3/201.2	0.73	25/37	22/12
Paspalin	17.1	422.3 $[M+H]^+$	36	130.1/103.0	0.13	27/101	16/8
Paspalinin	15.7	434.2 $[M+H]^+$	36	130.1/376.3	0.46	23/21	22/10
Paspalitrem A	16.5	502.3 $[M+H]^+$	46	198.2/154.2	0.78	27/97	12/8
Paspalitrem B	15.1	518.3 $[M+H]^+$	41	214.2/442.4	0.76	21/27	12/12
Patulin	5.5	152.9 $[M-H]^-$	−20	108.9/135.0	0.04	−12/−12	−9/−9
Paxilline	15.7	436.4 $[M+H]^+$	36	182.2/167.2	0.58	41/89	10/10
Penicillic acid	9.0	171.2 $[M+H]^+$	46	125.2/97.1	0.38	17/23	8/16
Penicillin G	12.9	335.1 $[M+H]^+$	36	160.2/176.2	1.00	21/19	14/8
Penicillin V	13.6	351.2 $[M+H]^+$	66	114.0/160.0	0.20	47/19	6/10

Table 1 (continued)

Analyte	Retention time (min)	*m/z* precursor ion	DP[a] (V)	*m/z* product ions[b]	Rel. int.[c]	Collision energy (V)[b]	Cell exit potential (V)[b]
Penitrem A	15.4	634.4 $[M+H]^+$	51	558.5/616.4	0.25	27/17	16/10
Pentoxyfylline	10.6	279.2 $[M+H]^+$	51	181.2/99.2	0.41	27/29	8/10
Physcion	16.8	283.0 $[M-H]^-$	−65	239.9/211.9	0.03	−36/−50	−11/−11
Pseurotin A	11.9	430.1 $[M-H]^-$	−35	269.9/308.0	0.60	−14/−12	−5/−7
Puromycin	9.9	472.4 $[M+H]^+$	46	150.2/164.2	0.28	41/55	8/10
Pyripyropene A	14.0	584.3 $[M+H]^+$	101	148.2/202.0	0.40	83/47	8/12
Radicicol	12.5	363.0 $[M-H]^-$	−70	182.9/275.1	0.30	−36/−32	−9/−5
Rapamycin	15.6	912.6 $[M-H]^-$	−145	166.8/101.1	0.71	−86/−88	−1/−1
Roquefortine C	12.3	390.2 $[M+H]^+$	61	193.2/322.3	0.43	39/29	10/18
Roridin A	13.7	550.4 $[M+NH_4]^+$	41	249.2/231.3	0.39	25/29	48/12
Rubellin D	14.9	541.1 $[M-H]^-$	−75	378.0/360.1	0.96	−28/−42	−9/−7
Satratoxin G	13.0	562.4 $[M+NH_4]^+$	41	249.2/231.3	0.61	21/25	14/20
Satratoxin H	13.2	546.4 $[M+NH_4]^+$	41	157.1/231.4	1.00	57/27	14/10
Secalonic acid D	15.2	639.3 $[M+H]^+$	91	561.4/589.4	0.50	37/33	8/8
Stachybotrylactam	15.1	386.3 $[M+H]^+$	101	178.2/149.9	0.17	51/61	10/8
Staurosporine	12.5	467.3 $[M+H]^+$	56	130.2/295.4	0.26	25/55	8/10
Sterigmatocystin	14.8	325.1 $[M+H]^+$	66	310.2/281.1	1.10	35/51	18/16
Sulochrin	11.9	333.2 $[M+H]^+$	26	209.1/136.2	0.13	17/59	12/8
T-2 Tetraol	5.5	316.2 $[M+NH_4]^+$	31	215.3/281.4	0.67	13/13	16/8
T-2 Toxin	13.6	484.3 $[M+NH_4]^+$	56	215.2/185.1	0.82	29/31	18/11
T-2 Triol	12.3	400.2 $[M+NH_4]^+$	41	215.2/281.3	0.34	17/13	12/16
Tentoxin	13.3	413.3 $[M-H]^-$	−75	141.0/271.1	0.58	−30/−24	−11/−15
Tenuazonic acid	12.1	196.0 $[M-H]^-$	−70	138.9/112.1	0.66	−28/−32	−7/−5
Territrem B	14.0	527.3 $[M+H]^+$	71	291.1/491.3	0.95	39/31	20/14
Tetracyclin	8.5	445.1 $[M+H]^+$	41	410.3/154.1	0.98	29/39	24/28
Trichostatin A	12.8	303.2 $[M+H]^+$	51	148.1/270.3	0.30	31/18	10/16
Tryprostatin	13.2	382.3 $[M+H]^+$	51	160.0/228.3	0.70	43/27	8/14
Valinomycin	17.0	1,128.8 $[M+NH_4]^+$	141	172.2/144.3	0.72	109/129	10/8
Verrucarin A	13.6	520.2 $[M+NH_4]^+$	51	249.1/457.1	0.38	25/19	14/14
Verrucarol	9.7	267.0 $[M+NH_4]^+$	56	249.1/219.0	0.64	11/15	8/14
Verruculogen	14.8	512.3 $[M+H]^+$	16	352.2/494.2	1.06	23/13	10/14
Viomellein	14.7	561.3 $[M+H]^+$	91	530.3/511.3	0.52	41/45	14/14
Viridicatin	14.0	238.1 $[M+H]^+$	81	192.2/165.2	0.74	35/49	12/14
Wortmannin	12.1	429.1 $[M+H]^+$	46	355.2/295.2	0.18	15/35	10/18
α-Zearalenol	14.4	319.2 $[M-H]^-$	−85	160.0/130.0	0.96	−44/−50	−13/−20
α-Zearalenol-4-glucoside	12.7	541.3 $[M+Ac]^-$	−28	319.1/481.1	0.69	−32/−14	−15/−11
β-Zearalenol	13.8	319.2 $[M-H]^-$	−85	160.0/130.0	0.89	−44/−50	−13/−20
β-Zearalenol-4-glucoside	11.7	541.3 $[M+Ac]^-$	−28	319.1/481.1	0.79	−32/−14	−15/−11
Zearalenone	14.5	317.1 $[M-H]^-$	−80	131.1/175.0	0.98	−42/−34	−8/−13
Zearalenone-4-glucoside	12.7	479.2 $[M-H]^-$	−65	317.1/175.0	0.11	−24/−56	−17/−9
Zearalenone-4-sulfate	14.3	397.1 $[M-H]^-$	−75	317.1/175.0	0.17	−32/−48	−15/−13

[a] Declustering potential

[b] Values are given in the order quantifier ion/qualifier ion

[c] Intensity of the qualifier transition / intensity of the quantifier transition

[d] In-source fragment obtained from cleavage of the corresponding peptide bond

(either due to decomposition or due to insufficient solubility) in the multi-analyte standards prepared in the acidified acetonitrile/water mixture.

The large number of analytes (137 substances are scanned in the positive mode) poses a problem concerning the time that is available for data acquisition of each of the 137×2 sMRM transitions, as a minimum of 12 data points per chromatographic peak and an MS/MS dwell time of at least 20 ms is required for a reproducible quantification. In our previous methods, we dealt with that problem by defining fixed retention time periods, each scanning only for the limited number of analytes eluting in the respective time window. However, the applicability of that approach is limited in view of a further increase of the number of analytes, since the chromatogram runs out of points in time that are suitable (which means that no analyte is eluting) for switching from one retention time period to the next. In the sMRM mode, a separate retention time window is defined for each analyte and the dwell time of each sMRM transition is dynamically generated for each point of time by the software from the target scan time (which was set to one second in order to obtain a sufficient amount of data points for a typical chromatographic peak width of approx. 15 s) and the number of sMRM transitions scanned at that time. In this way, the instruments capacity in view of data acquisition is fully exploited.

Extraction efficiency and matrix effects

Dust and indoor materials in general can be considered to be very different matrices in comparison to food. Thus, both extraction efficiency and matrix effects were re-evaluated. The acidified mixture of acetonitrile and water, that was found to be the best compromise for the extraction of the initial set of 39 analytes from wheat [22], was compared to pure methanol and ethyl acetate (that have been applied earlier for extraction of dust and building materials [15, 16] and fungal cultures [20], respectively) in view of the recovery of the extraction of the full set of analytes from settled dust that had previously been collected in offices of our department (see Table 2; note that only a selected list of indoor-relevant analytes is given as well as some food-relevant mycotoxins for comparison purposes). Ethyl acetate clearly performed worst, as recoveries of >70% were obtained only for two out of 36 analytes. Methanol seems to be an acceptable choice (the recoveries of 22 out of 36 analytes are within the target range of 70–120%), but the acidified acetonitrile/water mixture is probably still preferable, as the recoveries of 29 out of 36 analytes are within the target range (in addition, the recoveries of highly indoor-relevant metabolites such as meleagrin and sterigmatocystin—see the section dealing with method application—is clearly better compared to methanol).

Table 2 Recoveries of the extraction step of selected fungal and bacterial metabolites from the collected dust

Analyte	Ethyl acetate	Methanol	ACN/H_2O/HAc (79:20:1, *v*/*v*/*v*)
Sterigmatocystin	40	76	109
Meleagrin	2	71	115
Emodin	25	72	90
Enniatin B	71	76	67
Stachybotrylactam	2	72	117
Roquefortine C	2	59	94
Valinomycin	65	92	110
Chaetoglobosin A	2	145	179
Monactin	0.9	86	106
Beauvericin	54	87	77
Viridicatin	41	106	76
Nonactin	0	83	104
Cytochalasin D	42	80	105
Brefeldin A	40	161	38
Alamethicin F30	0	75	117
Chanoclavine	0	70	97
Fumigaclavine	12	69	104
Alternariol	0	81	86
Alternariolmethylether	40	78	106
Aflatoxin G1	1.2	11	50
Aflatoxin B1	8	34	97
Kojic acid	84	32	69
Viomellein	0	23	90
Penicillic acid	0	79	97
Staurosporin	2	25	73
Chaetomin	15	100	77
Cytochalasin B	39	71	112
T-2 Toxin	59	71	115
Ochratoxin A	0.4	49	108
Fumonisin B1	0	2.1	31
Fumonisin B2	0	4.4	42
Deoxynivalenol	0	n.d.	113
Nivalenol	0	75	103
Zearalenon	n.d.	76	105
Diacetoxyscirpenol	33	87	116
HT-2 Toxin	20	136	116

n.d. not determined due to the occurrence of large interfering peaks eluting close to the retention time of the respective analytes

For investigation of matrix effects, blank extracts of four indoor-relevant matrices were spiked: mortar, carton-gypsum board, coarse-soil-containing small pieces of wood (sampled from a damp cellar) and settled dust. As can be seen in Table 3, the first three matrices do not seem to be critical considering matrix effects, as the analytical signal was altered by more than 20% only in case of 4, 7, and 5 out of 36 analytes. Such a result is not unexpected in case

Table 3 Signal suppression/enhancement (%) in four different indoor materials

Analyte	Mortar	Carton-gypsum board	Soil–wood mixture	Collected dust
Sterigmatocystin	109	105	105	38
Meleagrin	110	94	102	31
Emodin	136	108	74	91
Enniatin B	108	109	135	1,680[a]
Stachybotrylactam	108	109	105	106
Roquefortine C	121	82	95	14
Valinomycin	111	109	103	76
Chaetoglobosin A	109	106	116	85
Monactin	112	113	108	50
Beauvericin	97	88	90	252[a]
Viridicatin	113	111	115	46
Nonactin	107	104	100	73
Cytochalasin D	n.d.	99	97	67
Brefeldin A	79	n.d.	95	n.d.
Alamethicin F30	104	104	112	43
Chanoclavine	109	111	107	46
Fumigaclavine	108	110	108	49
Alternariol	115	98	95	86
Alternariolmethylether	155	119	89	38
Aflatoxin G1	110	96	108	n.d.
Aflatoxin B1	112	97	100	32
Kojic acid	107	120	119	59
Viomellein	97	106	100	40
Penicillic acid	107	110	109	9
Staurosporin	101	71	68	14
Chaetomin	99	111	109	82
Cytochalasin B	107	96	99	49
Diacetoxyscirpenol	109	113	107	58
HT-2 Toxin	98	101	95	89
T-2 Toxin	109	124	105	70
Ochratoxin A	96	125	n.d.	58
Fumonisin B1	112	114	121	92
Fumonisin B2	126	116	122	73
Deoxynivalenol	138	148	113	96
Nivalenol	125	112	102	82
Zearalenon	114	116	95	82

n.d. Not determined due to increased baseline or due to interfering peak eluting closely to the retention time of the respective analyte

[a] Blank matrix was contaminated with enniatins and beauvericin

of mortar and carton-gypsum board (as these matrices can be considered to be rather clean and purely inorganic in case of mortar), but it is rather surprising in case of the soil/wood mixture, which we had previously assumed to be a complex sample. However, it must be expected that in case of moldy real-world samples of these types of materials, matrix effects may be increased due to the co-extracted constituents of the microbial biomass. In contrast to the other investigated materials, settled dust caused severe matrix effects: The analytical signal of 22 analytes was suppressed by more than 20%, in the case of 13 of these metabolites, the related peak area even dropped by more than 50%. These severe matrix effects (that affected the analytical signal of several analytes throughout the chromatogram) probably result from the complex composition of house dust that usually contains cell fragments from microbes as well as many organic compounds that tend to accumulate on particulate matter.

Method performance parameters

In order to investigate whether a quantitative analysis of such a wide range of compounds is feasible even in a challenging matrix such a house dust, the reference material SRM 2583 was chosen as model-sample and it was spiked at ten levels in triplicate. The individual concentration levels as well as the results are summarized in Table 4 (satratoxin G and H, citreoviridin, paspalin, paspalinin, and paspalitrem A and B are not included as the amount of standard available was insufficient).

The analysis of the blank matrix, which was intended to prove the selectivity of the method, revealed that the dust was obviously contaminated with griseofulvin (210 µg/kg, value is corrected for the apparent recovery) which was earlier found to be produced by *M. echinata* on a plaster board [2]. Its occurrence has been verified by product ion scans of the parent mass (m/z=353) of this compound using the Q3 linear ion-trap function of the MS instrument (the so-called "Enhanced Product Ion" (EPI) scan). As can be seen in the upper part of Fig. 1, a lot of interferences exhibiting m/z of 353 were observed in the dust sample. The product ion spectrum that has been acquired at the retention time of griseofulvin includes all three major fragments of griseofulvin (m/z=165, 215, and 285, respectively; note that the intensity ratio of these fragments agrees very well with the respective ratio observed in the standard) but also several other fragments that derive from co-eluting matrix constituents. The extracted ion chromatograms (XICs) exhibit distinct peaks at the retention time of griseofulvin for the above-mentioned major fragment ions (see lower part of Fig. 1), whereas the XICs of other fragments exhibit very broad peaks or an increased baseline. In this way, 5.5 identification points according to [29] have been obtained for the unambiguous identification of griseofulvin in the reference dust, since 1 point is awarded for the parent ion and three times 1.5 points are awarded for three product ions. In addition, the retention time matches to that of an authentic standard (the slight shift in retention time in comparison to Table 1 was due to the use of a new chromatographic column).

Table 4 Method performance characteristics determined in dust: recoveries of the extraction step (R_E), signal suppression/enhancement (SSE), apparent recoveries (R_A), relative standard deviation at the lowest and highest evaluated concentration levels of the spiked samples (RSD), coefficient of variation of the external calibration and the overall procedure (CV_c/CV_P) and limits of detection (LOD)

Analyte	Conc. range (µg kg^{-1})	n[a]	R_E (%)±CL[b]	SSE(%)±CL[b]	R_A (%)±CL[b]	RSD (%) low/high	CV_c/CV_P (%)	LOD (µg kg^{-1})[c]
AAL TA Toxin	1,100–4,400	3	4.9±1	87±3	4.3±3	5.8/19.4	0.9/1.3	22/440
3-Acetyldeoxynivalenol	31–3,100	7	102±32	62±8	63±4	59/3.9	1.3/1.4	15/31
15-Acetyldeoxynivalenol	290–5,800	5	92±36	60±22	55±3	13/4.3	0.6/0.5	29/290
Actinomycin D	15–625	6	75±13	53±6	40±5	45/4.1	3.0/5.3	0.4/1
Aflatoxin B1	30–600	5	29±2	51±4	15±2	17/2.5	1.3/2.3	6/15
Aflatoxin B2	38–160	3	23±4	56±9	13±9	4.3/6.6	2.6/2.9	3/7
Aflatoxin G1	63–630	4	21±6	54±4	11±3	3.7/6.2	1.7/2.3	6/15
Aflatoxin G2	n.d	n.d	n.d	40±3	n.d	n.d	2.1/n.d	15/n.d
Aflatoxin M1	70–1,400	5	28±9	46±4	13±2	9.4/10.7	0.9/1.6	7/35
Aflatoxin M2	76–760	4	33±9	48±4	16±2	17.2/3.4	3.1/2.7	19/76
Agroclavine	6.2–620	7	70±1	34±10	24±2	8.4/3.9	2.0/1.2	1/3
Alamethicin F30	104–4,200	6	76±3	36±2	27±2	4.7/2.3	0.4/3.4	20/40
Altenuene	390–1,600	3	127±47	61±27	77±8	6.7/2.3	3.1/0.8	160/390
Altenusin	n.d	n.d	n.d	147±155	n.d	n.d	1.7/ n.d	400/ n.d
Alternariol	3.1–310	10	85±9	26±4	22±4	47.9/11.8	1.8/3.0	3/3
Alternariolmethylether	3–1,500	9	80±5	41±5	33±5	39.9/4.2	2.8/1.9	1/1
Altersolaniol A	1,800–7,200	3	5.2±6	64±6	3.3±4	6/7.8	1.2/1.8	7/720
Altertoxin-I	44–8,800	9	88±6	78±8	69±7	20.1/2.8	1.1/1.2	17/44
2-Amino-14,16-dimethyloctadecan-3-ol	n.d	n.d	n.d	51±12	n.d	n.d	0.7/ n.d	43/ n.d
Anisomycin	6–600	7	60±2	28±2	17±2	5.1/2.4	1.3/1.6	3/3
Apicidin	1.1–220	8	70±9	72±5	50±7	22.7/19.8	7.4/13.7	0.1/0.1
Ascomycin	34–340	4	89±5	47±3	42±2	16.2/5.9	4.4/1.4	8/34
Asperlactone	n.d	n.d	n.d	53±36	n.d	n.d	0.5/ n.d	22/ n.d
Asperloxin A	88–3,600	6	91±2	57±1	52±2	6.4/2.9	4.5/4.6	36/36
Aspinonene	35–3,600	7	114±3	49±1	56±3	23.9/1.4	3.9/3.9	36/36
Aspyrone	n.d	n.d	n.d	31±6	n.d	n.d	2.7/ n.d	43/ n.d
Asterric acid	4–4,000	10	123±13	127±16	157±9	5.3/10.9	2.4/1.5	4/4
Atpenin A5	11–2,224	8	88±7	84±5	74±6	49.9/10.4	2.0/2.4	2/2
Aurofusarin	220–880	3	140±38	55±22	77±13	18.7/4	4.2/1.2	22/88
Austdiol	n.d	n.d	n.d	44±136	n.d	n.d	1.2/ n.d	2,200/ n.d
Austocystin A	44–4,400	7	90±19	58±7	52±4	4.2/5.5	1.0/0.4	44/44
Avenacein Y	55–2,200	6	705±106	498±408	3,522±387	46.6/5.5	0.98/2.6	550/55
Bafilomycin A1	100–400	3	13±6	15±0.8	1.95±0.29	12/0.9	3.5/2.6	0.3/100
Beauvericin	1.6–88	6	78±14	22±3	17±2	11/6.2	3.2/4.0	0.8/1
Brefeldin A	1,100–4,400	3	63±11	171±17	108±10	12/4.9	0.4/0.4	440/1,100
Calphostin C	55–550	4	65±12	39±7	25±7	10.9/10.9	5.1/2.6	14/55
Cephalosporin C	n.d	n.d	n.d	78±15	n.d	n.d	0.6/ n.d	360/ n.d
Cerulenin	22–2,200	7	38±32	47±18	18±10	7.9/8.7	4.4/5.3	5/22
Chaetocin	44–4,400	7	70±7	71±6	50±5	4.5/3.2	0.4/1.4	22/44
Chaetoglobosin A	96–3,800	6	144±8	96±5	140±8	7.5/7.4	1.5/1.1	19/19
Chanoclavine	3–600	8	77±8	30±3	23±2	12.2/5.3	2.5/1.7	0.6/1
Chetomin	58–11,000	8	31±4	90±3	28±4	22.5/7.7	1.0/2.1	11/11
Chloramphenicol	3.6–3,600	10	115±8	75±6	87±4	20.3/7.3	1.4/1.2	4/4
Chromomycin A3	20–4,000	8	101±18	100±17	102±7	86.5/7.5	1.4/1.7	4/20
Citrinin	46–4,600	7	99±22	102±16	102±21	75.6/22.8	2.6/4.2	23/46
Curvularin	18–3,600	8	95±5	79±5	76±4	7.8/4.4	1.4/0.6	9/18
Cycloaspeptide A	18–3,600	8	91±2	60±21	55±2	29.7/2.6	0.5/0.5	18/18
Cycloheximide	36–3,600	7	98±3	61±2	60±2	9.4/3.7	0.8/0.5	9/36

Table 4 (continued)

Analyte	Conc. range (µg kg^{-1})	n^a	R_E (%)±CL^b	SSE(%)±CL^b	R_A (%)±CL^b	RSD (%) low/high	CV_c/CV_P (%)	LOD (µg kg^{-1})^c
Cyclosporin A	9–9,000	10	37±9	91±9	34±5	50.1/16.5	1.2/3.3	9/20
Cyclosporin C	35–7,000	8	71±10	27±4	20±3	45/2.1	0.7/0.9	18/35
Cyclosporin D	35–7,000	8	83±4	47±2	39±25	16.2/5.1	0.6/0.7	7/35
Cyclosporin H	18–7,000	9	82±6	33±2	27±3	25.7/3.6	0.8/0.4	18/18
Cytochalasin A	n.d	n.d	n.d	78±7	n.d	n.d	0.7/ n.d	44/ n.d
Cytochalasin B	44–4,400	7	93±4	64±3	59±2	40.3/1.5	0.4/0.5	44/44
Cytochalasin C	44–4,400	7	80±12	77±4	60±9	50.1/32.1	0.7/3.4	44/44
Cytochalasin D	44–4,400	7	99±5	77±5	77±4	25.2/3.5	0.8/0.4	11/11
Cytochalasin E	11–1,100	7	52±8	71±7	37±12	25.7/9.1	1.8/2.6	11/11
Cytochalasin H	220–4,400	5	106±4	58±5	62±5	29.4/1.4	0.8/0.6	110/220
Cytochalasin J	110–4,400	6	95±5	63±6	95±5	6.1/1.7	0.4/0.3	44/110
Decarestrictin	35–3,500	7	158±47	48±21	76±13	12.3/9.9	2.4/3.7	18/35
Deepoxydeoxynivalenol	14–2,800	8	110±12	70±7	77±9	44.6/1.6	2.0/3.1	7/14
Deoxynivalenol	15–3,000	8	126±14	73±10	92±9	53/8.7	2.2/2.3	15/15
Deoxynivalenol-3-glucoside	9.5–1,900	8	73±10	41±6	30±7	13.2/7.1	2.5/3.7	4/9
Diacetoxyscirpenol	15–3,000	8	91±3	64±3	58±3	21.6/2	0.8/0.6	15/15
Dihydroergosine	3.9–39	4	77±3	18±5	14±4	3.5/12.1	10.6/9.2	0.3/3
Dihydroergotamine	1–200	7	72±11	29±6	21±5	52.8/3.6	8.8/3.3	0.1/1
Dihydrolysergol	0.48–480	10	62±8	39±3	24±2	39.6/8.5	1.9/2.4	0.5/0.5
Elymoclavine	0.48–480	10	60±8	36±4	22±3	11.1/7	3.1/3.1	0.5/0.5
Elymoclavine fructoside	0.64–640	10	42±5	35±4	15±3	9.6/10.1	3.3/4.9	0.6/0.6
Emodin	7.5–1,500	8	82±8	49±1	40±4	4/2.1	1.3/2.7	1/3
Enniatin A	0.25–5	5	71±4	47±3	34±3	4.1/4.5	10/18.3	0.005/0.2
Enniatin A1	3.3–33	4	106±6	32±1	34±2	5.8/2.1	5.7/6.1	0.03/0.8
Enniatin B	0.35–35	7	87±2	60±1	52±1	4.8/2.8	5.3/5.6	0.03/0.1
Enniatin B1	0.8–80	7	75±4	46±2	35±2	6.6/2.5	3.9/3.5	0.2/0.4
Enniatin B2	2–44	5	80±4	55±3	44±3	13.7/8.2	7.7/7.9	1.1/2
Enniatin B3	0.32–88	8	95±2	64±1	62±1	12.4/4.1	4.6/3.7	0.08/0.08
Equisetin	22–2,200	7	49±9	218±11	108±19	6.8/29.6	2.4/5.4	2/2
Ergine	0.96–190	8	102±3	39±5	39±4	12.7/10.4	5.3/4.4	0.9/0.9
Ergocornine	4–200	6	84±6	30±3	25±3	35.1/2.3	3.7/3.9	1/4
Ergocorninine	1.3–130	7	87±5	71±2	62±2	3.4/3.7	2.9/5.6	0.1/1
Ergocristine	4–200	6	75±5	33±2	25±3	17.7/12	5.2/5.2	0.4/4
Ergocristinine	4–125	5	80±4	66±2	53±3	8.9/6.3	3.9/4.3	3.1/4
Ergocryptine	4–196	6	83±5	25±2	21±2	15.5/14	3.3/5.3	0.9/4
Ergocryptinine	3–125	6	83±5	72±2	60±3	33.1/10.4	3.6/5.1	0.6/3
Ergometrine	0.3–300	10	63±9	40±4	25±2	38.1/8	2.6/4.3	0.3/0.3
Ergometrinine	3–75	5	139±14	71±8	99±7	7.4/9.7	4.3/6.2	0.7/0.7
Ergosine	19–190	4	69±20	29±7	19±5	18.4/13	6.7/6.5	0.9/19
Ergosinine	4–200	6	79±7	29±2	23±2	27/5.3	3.4/4.8	0.1/4
Ergotamine	4–200	6	72±4	48±4	34±4	16.9/6.5	7.1/4.9	1/4
Ergotaminine	0.62–125	8	117±17	16±6	19±6	13/16.4	10.4/8.6	0.6/0.6
Ergovaline	19–190	4	63±10	22±3	14±3	7.3/3.7	2.8/4.9	0.04/0.9
Festuclavine	0.62–620	10	79±3	27±2	21±2	39.2/10.5	2.5/3.2	0.6/0.6
FK 506	28–560	5	83±9	58±6	48±7	25.5/14.2	2.9/3.6	14/28
Fumagillin	200–2,000	4	87±14	57±8	50±6	22.5/9.7	2.6/1.1	50/200
Fumigaclavin	4–4,000	10	78±6	41±3	32±2	41.8/3.3	0.6/0.8	4/4
Fumitremorgin C	33–660	5	99±22	61±14	60±4	17.4/2.9	3.3/1.8	0.6/16
Fumonisin B1	270–11,000	6	0.85±7	85±7	0.73±3	11.4/9.7	0.7/2.0	10/108

Table 4 (continued)

Analyte	Conc. range (μg kg^{-1})	n[a]	R_E (%)±CL[b]	SSE(%)±CL[b]	R_A (%)±CL[b]	RSD (%) low/high	CV_c/CV_P (%)	LOD (μg kg^{-1})[c]
Fumonisin B2	110–11,000	7	2.2±3	100±5	2.16±3	43.9/7.6	0.8/2.0	11/27
Fumonisin B3	n.d	n.d	n.d	84±7	n.d	n.d	2.1/ n.d	2/ n.d
Fusarenon-X	31–3,100	7	138±13	63±6	88±8	46.4/5.4	1.5/2.0	7/31
Geldanamycin	12–610	6	45±9	78±6	35±6	107/14.7	3.3/7.3	2/5
Gibberellic acid	44–8,800	8	65±11	85±8.8	56±4	44.7/2.2	0.7/1.2	8/44
Gliotoxin	4.4–4,400	10	44±7	92±6.9	41±3	16.3/9.5	1.1/2.0	4/4
Griseofulvin	550–2,200	3	94±11	74±5	70±7	93/5.4	0.8/0.6	11/11
HC-toxin	22–4,400	8	83±10	33±5	27±5	28.7/11.9	1.3/2.4	22/22
HT-2 toxin	3.2–3,200	10	87±8	78±6.8	68±4	16.5/3.6	1.4/1.3	3/3
Hydrolysed Fumonisin B1	5.1–5,100	10	57±2	55±1	31±1	30.2/2.3	0.4/1.2	5/5
Ionomycin	220–4,400	5	21±12	97±12	21±9	4.9/9.5	2.3/4.0	4/222
K252a	4.3–430	7	85±10	83±8	71±4	31.3/1.7	3.3/2.8	10/4
K252b	3.9–390	7	75±15	97±15	73±6	56.2/5.2	2.5/4.0	19/3
Kojic acid	870–8,700	4	47±10	51±4	24±6	9.6/5.4	0.4/1.4	175/872
Lysergol	6.2–620	7	59±12	34±4	20±2	5/11.4	2.2/2.5	0.4/6
Macrosporin	7–7,000	10	74±7	39±4	29±4	40/2.4	0.6/2.5	7/7
Meleagrin	38–3,800	7	77±6	25±3	19±3	8.7/5.5	0.9/0.9	3/9
Methysergide	0.48–120	8	68±5	28±2	19±1	13.8/7	4.0/4.1	0.3/0.4
Mevinolin	11–4,400	9	80±7	69±6	55±5	42.6/3.5	1.6/1.5	4/11
Mithramycin	42–420	4	95±24	98±22	93±13	53.1/10	4.3/3.7	10/42
Monactin	1.5–75	6	103±8	79±7	81±5	13.9/5.6	8.7/4.9	0.3/1
Moniliformin	n.d	n.d	n.d	69±5	0±0	0.0/0.0	0.9/ n.d	19/ n.d
Monoacetoxyscirpenol	22–2,200	7	107±2	71±3	77±15	25.4/0.9	0.8/2.4	22/22
Mycophenolic acid	4.3–4,300	10	111±8	78±7	87±3	27.7/2.1	0.8/0.9	4/4
Myriocin	37–370	4	45±8	70±5	32±4	48.6/2.2	1.8/3.2	1/9
Neosolaniol	58–5,800	7	91±6	41±3	37±3	11.4/7.4	0.8/0.7	5/28
Nigericin	5.4–540	7	58±4	37±2	21±2	16/5.4	1.7/3.0	5/5
3-Nitropropionic acid	n.d	n.d	n.d	79±6	n.d	n.d	1.5/ n.d	9/ n.d
Nivalenol	15–3,000	8	121±9	71±10	86±11	85.6/6.2	2.9/2.8	15/15
Nonactin	5–250	6	103±7	89±6	89±4	0.4/5.3	2.5/3.2	0.2/5
Ochratoxin A	11–2,200	8	74±4	63±3	47±3	49.7/6.3	1.2/1.0	2/11
Ochratoxin B	2.4–480	8	107±6	66±4	71±3	45.8/3.5	2.4/2.5	2/2
Ochratoxin α	23–2,300	7	105±23	105±21	111±14	3.7/6.5	2.1/3.8	2/23
Oligomycin A	77–770	4	65±13	74±7	48±9	38.3/25.8	3.1/11.2	4/38
Oligomycin B	180–700	3	70±13	82±9	58±9	59.1/6.3	4.5/9.5	6/35
Oxaspirodion	n.d	n.d	n.d	28±6	n.d	n.d	0.8/ n.d	17/ n.d
oxidized Elymoclavine	15–620	6	79±10	14±4	11±4	21.3/14.9	0.8/3.4	15/15
oxidized Luol	3–600	8	84±4	61±4	52±3	4.7/5.2	1.8/2.4	0.6/0.6
Patulin	66–3,300	6	38±13	76±12	26±10	17.9/15	1.7/5.9	6/66
Paxilline	22–4,400	8	106±16	53±8	56±6	29.5/7.1	1.1/2.5	22/22
Penicillic acid	n.d	n.d	n.d	67±4	n.d	n.d	0.8/0.0	38/0
Penicillin V	n.d	n.d	n.d	67±4	n.d	n.d	1.3/0.0	11/0
Penitrem A	11–2,200	8	84±6	161±12	136±9	13.6/4.1	2.7/1.5	2/5
Pentoxyfylline	5.5–2,200	9	84±3	45±2	38±1	20.2/6.3	0.6/1.0	2/2
Physcion	39–7,800	8	66±12	56.3±6	37±6	24/3.78	1.0/2.9	7/39
Pseurotin A	20–4,000	8	105±21	135±27	142±14	24.7/5.3	2.2/2.5	10/20
Puromycin	2.2–440	8	50±3	37±2	19±2	12.3/6.2	1.5/2.9	0.9/2
Pyripyropene A	11–2,200	8	94±6	61±4	58±3	40.7/6.6	0.9/1.1	11/11
Radicicol	4.6–920	8	116±24	89±24	103±14	42.6/7.2	1.9/4.4	0.8/4

Table 4 (continued)

Analyte	Conc. range (µg kg^{-1})	n[a]	R_E (%)±CL[b]	SSE(%)±CL[b]	R_A (%)±CL[b]	RSD (%) low/high	CV_c/CV_P (%)	LOD (µg kg^{-1})[c]
Rapamycin	36–360	4	113±29	61±19	69±13	43.3/7.2	4.9/12.5	18/18
Roquefortine C	44–4,400	7	94±4	45±4	42±4	11.3/4.6	1.0/0.7	11/22
Roridin A	12–2,400	8	90±3	70±3	64±3	12/1.6	1.3/0.6	2/6
Rubellin D	11–2,200	8	93±11	74±6	69±9	37.9/8.96	2.4/4.4	2/5
Secalonic acid D	77–15,400	8	112±6	113±8	126±6	17/3.6	0.7/0.3	38/38
Stachybotrylactam	5.5 –2,220	9	102±3	73±3	75±3	11.4/5.4	1.4/1.1	2/5
Staurosporine	7–280	6	38±11	39±5	15±2	34.9/4.8	2.86/3.8	0.6/2
Sterigmatocystin	3.1–1,550	9	79±4	56±2	45±2	49.6/5.2	0.7/1.6	3/3
Sulochrin	11–2,200	8	111±10	73±8	82±4	16.1/5	2.2/0.8	2/11
T-2 Tetraol	23–2,300	7	81±6	84±8	68±8	5.1/11.2	2.7/2.4	23/23
T-2 Toxin	31–3,100	7	100±5	68±2	68±3	47.4/1.4	0.6/1.2	8/31
T-2 Triol	25–2,500	7	87±49	66±37	58±21	69.4/3.2	2.4/4.4	62/62
Tentoxin	3–300	7	106±20	137±23	145±17	17.6/6.5	4.9/8.0	0.4/3
Territrem B	88–3,500	6	75±7	60±4	45±4	3.9/13.1	1.0/1.4	3/8
Trichostatin A	7–300	6	81±7	93±8	75±7	40/7.5	5.2/3.3	3/7
Valinomycin	0.32–160	9	97±8	79±7	77±4	28/7.5	3.0/3.4	0.3/0.9
Verrucarin A	9.5–1,900	8	103±9	80±7	82±4	28.6/4.1	1.1/1.5	9/9
Verrucarol	380–3,800	4	174±108	16±18	29±10	4.9/3.5	2.2/2.7	190/390
Verruculogen	38–7,600	8	78±13	77±10	60±5	47.5/17.4	0.8/1.5	7/38
Viomellein	22–4,400	8	95±15	70±10	67±7	18.2/12.2	1.6/2.4	4/22
Viridicatin	11–4,400	9	93±4	97±5	91±3	19.1/2	1.1/0.6	4/11
Wortmannin	248–2,480	5	9.7±8	64±7	6.2±6	18.9/4.3	2.1/5.1	12/62
α-Zearalenol	2.3–2,300	10	82±8	42±8	34±8	11/5.7	3.7/3.4	2/2
α-Zearalenol-4-glucoside	4.6–4,600	10	112±10	74±6	83±6	32.7/9.91	1.1/2.2	4/4
β-Zearalenol	2.3–2,300	10	81±3	47±6	39±5	36/3.21	2.3/2.2	2/2
β-Zearalenol-4-glucoside	23–4,600	8	112±10	58±4	65±6	29.4/10	1.2/2.0	4/23
Zearalenone	3.2–3,200	10	80±6	52±4	42±4	7.2/5.1	1.2/2.7	3/3
Zearalenone-4-glucoside	26–5,200	8	124±12	84±7	105±9	22.1/12.8	1.4/2.0	5/26
Zearalenone-4-sulfate	0.3–60	8	135±16	67±9	91±11	17.7/19.6	19.2/22.2	0.1/0.3

[a] Number of evaluated concentration levels

[b] Confidence limits (α=0.05)

[c] Values are given in the order external standards diluted in solvent/spiked samples

Apart from the identification of griseofulvin in the reference dust, interfering peaks eluting closely to the retention time of the respective analyte were observed for gibberellic acid and fumitremorgin C. Thus, with respect to these three analytes, data evaluation of spiked samples had to be restricted to the highest concentration levels. For all other analytes, linear calibration functions covering a concentration range of up to three orders of magnitudes have been obtained for the liquid standards as well as for the spiked extracts as has been confirmed through Mandel test. This shows that the application of the sMRM mode enables to analyze more than 100 analytes in a single chromatographic run without the need to deal with negative influences on the repeatability of the detector signal due to large MS cycle times or low MS dwell times.

For a couple of analytes, the data obtained for the spiked samples could not be evaluated. Altenusin, asperlactone, aspyrone, austdiol, cephalosporin C, cytochalasin A, oxaspirodion, penicillic acid, and penicillin V decomposed during evaporation of the spiking solvent and/or extraction, as no peaks were visible in the related chromatograms. For moniliformin, 3-nitropropionic acid, and 2-amino-14,16-dimethyloctadecan-3-ol, inconsistent values for the apparent recoveries have been observed. While in case of the two former low molecular weight compounds this may be explained by a partial evaporation of the analyte, no explanation can be given for the behavior of the latter compound. In case of aflatoxin G2 and fumonisin B3, the investigated concentration range was below the limit of detection that has been significantly increased due to a low

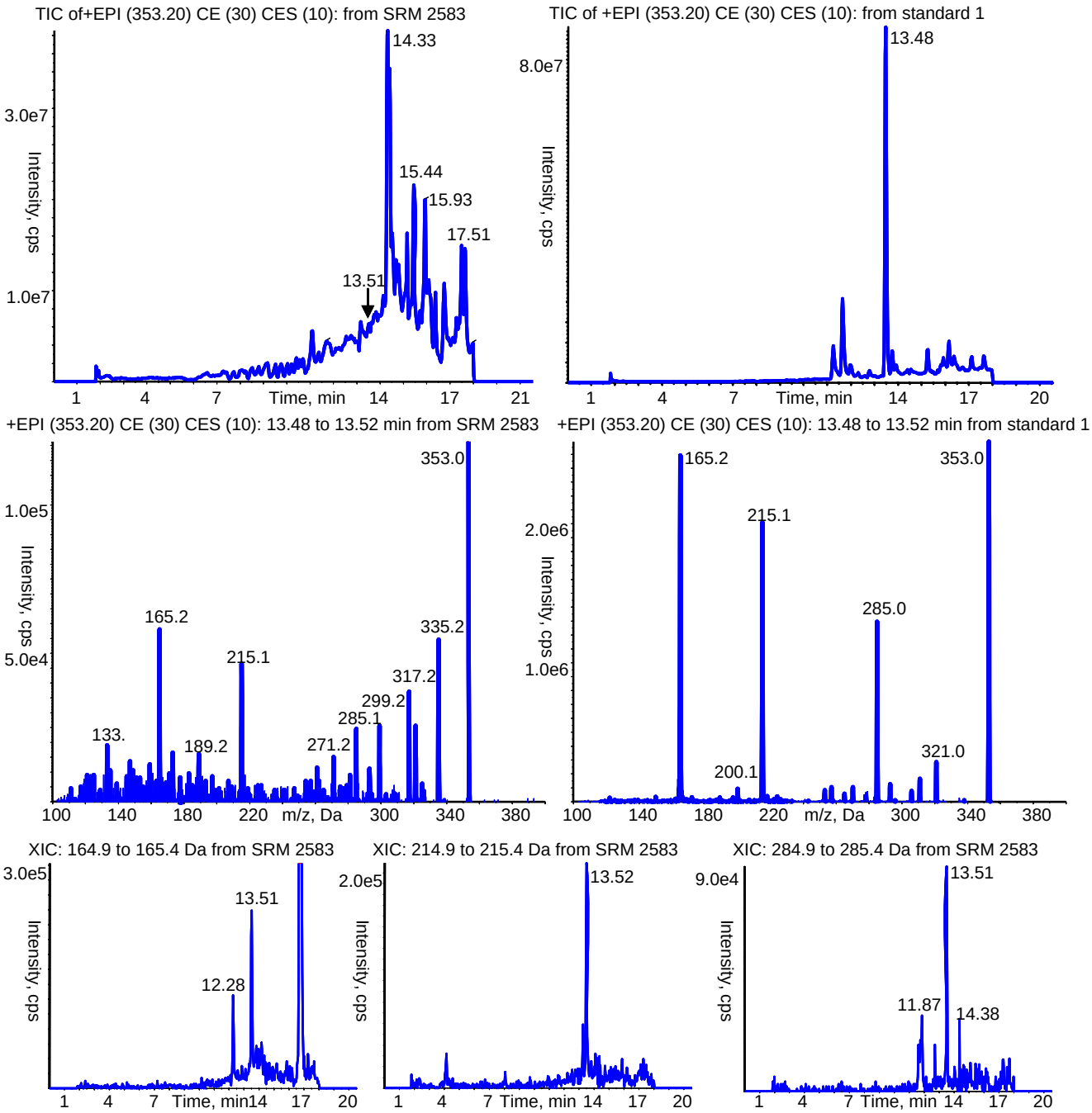

Fig. 1 Verification of the occurrence of griseofulvin in the reference dust SRM 2583; *top* total ion current of the "Enhanced Product Ion" (EPI) scan of SRM 2583 (*left*) and a reference standard (*right*); *middle* related product ion spectra acquired at the expected retention time of griseofulvin; *bottom* extracted ion chromatograms (XIC) of the three major fragment ions of griseofulvin

extraction efficiency and signal suppression due to matrix effects.

For the remaining 160 metabolites the recovery of the extraction step was within the target range of 70–120% for 109 substances. The value of 120% was exceeded in case of 13 analytes (although the respective 95% confidence interval did not overlap with that value only for avenacein Y, chaetoglobosin A, ergometrinine, fusarenon-X, and zearalenone-sulfate) whereas for 38 metabolites the extraction efficiency was lower than 70%. In contrast to our previous work on food and feed analysis [23], incomplete extraction was not restricted to polar and acidic analytes. On the one hand, compounds such as chetomin and nigericin exhibiting strong retention in reversed phase LC were severely affected, whereas polar and acidic analytes such as T2-tetraol and citrinin, respectively, were

almost quantitatively extracted. In comparison to the dust used for the initial experiments, the extraction efficiency of several analytes (most notably fumonisins and aflatoxins) was significantly reduced in SRM 2583. Such deviations were already observed by others [21] and probably result from the heterogeneous composition of house dust. Of course, the trueness of an analytical method is negatively influenced by such effects and it is therefore worth trying to further improve extraction efficiency, e.g., by applying ultrasonic-assisted extraction [15].

As concerns matrix effects, the signals of 60 metabolites were reduced by more than a factor of two. SSE was in the range of 50–70% for 47 analytes and in the range of 70–120% for 63 metabolites, whereas a significant signal enhancement by more than 20% was observed in eight cases. These effects were not restricted to a specific region in the chromatogram as early eluting analytes such as oxidized elymoclavine or chanoclavine were severely affected as well as later eluting metabolites such as beauvericin and cyclosporine C. Similar to the extraction efficiency, the related values obtained for SSE in case of the collected dust and SRM 2583, respectively, varied significantly in case of some analytes. However, such differences between individual samples of an extremely heterogeneous matrix had been previously expected in view of the results we had obtained for different varieties of the same food matrix [31]. The application of matrix-matched calibration must therefore be considered to be insufficient to completely compensate for matrix effects in case of dust and the use of isotopically labeled internal standards would be clearly preferable.

Due to pronounced matrix effects and incomplete extraction, the apparent recovery was below 50% for 79 out of 161 analytes. This findings lie in agreement with the recovery of 33% that has been reported for sterigmatocystin in dust [18] or the range of 7–92% reported for a multi-analyte method [13]. All these losses/effects were reproducible at all concentration levels as indicated by the relatively low values obtained for the coefficient of variation of the whole process, which exceeded 10% only in case of apicidin, enniatin A, oligomycin A, rapamycin, and zearalenone-4-sulfate.

The limits of detection were generally in the low microgram per kilogram range and exceeded 50 μg kg^{-1} only for those compounds exhibiting a low apparent recovery (such as aflatoxin M1, altersolaniol, fumonisin B1, ionomycin, or verrucarol) or a general low MS/MS sensitivity (such as brefeldin A, fumagillin, or T2-Triol). The value of 3 μg kg^{-1} obtained for sterigmatocystin is comparable to the values of 1 and 19 μg kg^{-1}, respectively, reported by other authors for the LOD of this metabolite [16, 17]. A comparison of the LODs (after their conversion to the absolute amount of analyte in 0.05 g dust) to the values obtained by another multi-analyte method [13] (which, however, seems to include some pre-concentration step without stating the respective volumes), reveals that our method is more sensitive with the exception of T-2 toxin, HT-2 toxin, and macrocyclic trichothecenes.

Application of the method to indoor samples from damp buildings

In the framework of a collaborative study, the present method has been applied to the analysis of a large number of dust samples. Results of this work will be published elsewhere (Peitzsch et al., manuscript in preparation). In addition, 14 indoor samples were investigated in the course of the present study using the described method, including scrapings from walls, mixtures of coarse soil and wood pieces sampled from a damp cellar and the paper cover of the underneath of a carpet. As can be seen in Table 5, 20 different metabolites have been identified exhibiting concentrations from the sub-microgram per kilogram range up to 130 mg/kg (note that the concentrations have not been corrected for apparent recoveries; in addition the common way of expressing the concentration as nanogram per square centimeter surface was not applicable as we received the samples as bulk material). Most of these substances have not been detected before in samples of indoor materials from damp buildings, although they were stated to be produced by fungal species that are reported to occur in the indoor environment [32], e.g., *P. chrysogenum* (produces meleagrin and roquefortine C), *C. globosum* (produces chaetoglobosin A and chaetomin), *A. tenuissima* (produces alternariol and alternariol methylether), *S. chartarum* (produces stachybotrylactam), *Penicillium polonicum* (produces viricatin), and *Trichoderma* species (produce emodin and alamethicin).

The most prevalent analytes were meleagrin, sterigmatocystin, and roquefortine C (which is supported by the frequent identification of the related fungi in the investigated samples) as well as enniatins. The low concentrations of the latter compounds (which are produced by *Fusarium* species) probably derived from contaminated particulate matter such as grain dust entering from the outdoor environment. In contrast to that, *Stachybotrys* metabolites were identified only in one sample, although they are probably the most intensively investigated substances in context with indoor molds in damp buildings (which is especially true for the satratoxins). This emphasizes the need to apply the proposed multi-analyte approach in order to get a more authentic picture on the pattern of microbial metabolites that may occur in damp indoor environments.

Table 5 Concentrations of mycotoxins found in the investigated indoor materials

Number	Sample	Identified toxin/estimated concentrations (μg kg^{-1})	Identified fungi
1	Wall scrapings	Meleagrin (62000); Sterigmatocystin (2000); Cytochalasin D (1900); Roquefortine C (1000); Enniatin B1 (69); Enniatin B1 (65); Enniatin A1 (20); Enniatin A (2.7); Emodin (0.75); Beauvericin (0.45)	*Penicillium glabrum* *Aspergillus versicolor*
2	Wall scrapings	Meleagrin (140); Sterigmatocystin (12); Emodin (22); Cytochalasin D (5.4); Roquefortine C (4.2); Enniatin B (0.88); Enniatin B1 (0.22);	*Cladosporium sphaerospermum* *Aspergillus versicolor* *Penicillium chrysogenum* *Penicillium brevicompactum* *Penicillium cf. bilaiae*
3	Wall scrapings	Meleagrin (12000); Sterigmatocystin (3900); Roquefortine C (130); Emodin (11); Enniatin B1 (8.2); Enniatin B (4.3); Enniatin A1 (4.1); Beauvericin (3.7); Enniatin A (2.0);	*Penicillium chrysogenum* *Cladosporium sphaerospermum* *Aspergillus versicolor*
4	Wall scrapings	Meleagrin (3.5); Cytochalasin D (3.3); Alamethicin F30 (2.5); Sterigmatocystine (1.8);	*Cladosporium sphaerospermum* *Penicillium chrysogenum* *Penicillium brevicompactum* *Aspergillus versicolor*
5	Wall scrapings	Alternariol (38); Sterigmatocystin (27); Alternariolmethylether (7.6)	*Cladosporium sphaerospermum* *Aspergillus versicolor* *Penicillium chrysogenum*
6	Wall scrapings	Emodin (180); Enniatin B (0.11); Enniatin B1 (0.16); Enninatin A1 (0.08); Enniatin A (0.012);	*Cladosporium sphaerospermum* *Acremonium sp.* *Acremonium murorum* *Aspergillus versicolor* *Fusarium solani* *Penicillium glabrum* *Penicillium solitum* *Engyodontium album* *Penicillium commune*
7	Wall scrapings	Enniatin B (5.70); Enniatin B1 (0.25); Enniatin A1 (0.32); Enniatin A (0.02);	*Cladosporium sphaerospermum*
8	Wall scrapings	Meleagrin (37); Enniatin B (14); Enniatin B1 (1.2); Emodin (0.97); Enniatin A1 (0.06); Enniatin A (0.01);	*Cladosporium sphaerospermum* *Aspergillus versicolor* *Penicillium chrysogenum* *Aspergillus flavus*
9	Soil/wood	Enniatin B (41); Enniatin B1 (41); Enniatin A1 (34); Beauvericin (18); Meleagrin (7.5); Enniatin A (6.9); Sterigmatocystin (4.7); Cytochalasin D (2.9) Emodin (0.24); Alamethicin F50 (0.11);	Not determined
10	Soil/wood	Alamethicin F30 (10); Sterigmatocystin (2.2); Emodin (0.72);	Not determined
11	Wooden wall scrapings	Chaetoglobosin A (640); Meleagrin (120); Emodin (82); Equesetin (71); Citrinin (47);	Not determined
12	Wooden wall scrapings	Meleagrin (1700); Chaetoglobosin A (55); Chaetomin (23); Roquefortine C (21); Sterigmatocystin (3.1);	Not determined
13	Wooden wall scrapings	Chaetoglobosin A (130000); Meleagrin (110); Emodin (12); Cytochalasin B (9.4); Sterigmatocystin (8.2);	Not determined
14	Carpet cover	Viridicatin (2600); Stachybotrylactam (2000); Sterigmatocytin (340); Meleagrin (260); Roquefortine C (86);	Not determined

Conclusion

The previously published LC-MS/MS multi-mycotoxin method dedicated to food and feed analysis has been extended by 99 analytes and has been successfully applied to the analysis of indoor-relevant matrices. The sMRM mode enables the acquisition of more than 250 fragmentation reactions in a single chromatographic run without the need to make compromises concerning the MS/MS dwell time or the number of data points per chromatographic peak. Due to the minimal sample preparation that had been reduced to a single extraction step during development of our initial method, no major changes in the sample-pretreatment protocol were required upon the analysis of building materials and house dust. Materials such as mortar, carton-gypsum board, and coarse-soil-containing splints did not pose severe problems as considers matrix effects. In contrast to that, settled house dust obviously is an extremely challenging matrix, causing severe matrix effects and incomplete extraction, which is in good agreement with the low recoveries that are often reported in the literature. Whereas the recovery of the extraction step may still be improved, the observed matrix effects pose a fundamental problem concerning the accuracy of the method, as there was a significant difference in the extent of signal suppression between the two investigated dust samples (which seems to be a reasonable result in view of the complex and varying composition of this matrix). Therefore, the application of matrix-matched calibration is probably insufficient to completely compensate for signal suppression in this matrix, which instead would require isotopically labeled internal standards. Despite this limitation, the method in its present form is a valuable tool for obtaining a comprehensive picture of the range of potentially toxic metabolites produced by various fungal and bacterial genera occurring in damp indoor environments, as demonstrated in case of the 20 different analytes identified in the investigated real-world samples. The on-site determination of these compounds will facilitate a sound assessment of their contribution to the symptoms that are frequently reported by inhabitants suffering from the exposure to harmful microorganisms in damp buildings.

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Co-occurrence of toxic bacterial and fungal secondary metabolites in moisture-damaged indoor environments

Abstract Toxic microbial secondary metabolites have been proposed to be related to adverse health effects observed in moisture-damaged buildings. Initial steps in assessing the actual risk include the characterization of the exposure. In our study, we applied a multi-analyte tandem mass spectrometry-based methodology on sample materials of severely moisture-damaged homes, aiming to qualitatively and quantitatively describe the variety of microbial metabolites occurring in building materials and different dust sample types. From 69 indoor samples, all were positive for at least one of the 186 analytes targeted and as many as 33 different microbial metabolites were found. For the first time, the presence of toxic bacterial metabolites and their co-occurrence with mycotoxins were shown for indoor samples. The bacterial compounds monactin, nonactin, staurosporin and valinomycin were exclusively detected in building materials from moist structures, while chloramphenicol was particularly prevalent in house dusts, including settled airborne dust. These bacterial metabolites are highly bioactive compounds produced by *Streptomyces* spp., a group of microbes that is considered a moisture damage indicator in indoor environments. We show that toxic bacterial metabolites need to be considered as being part of very complex and diverse microbial exposures in 'moldy' buildings.

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Key words: Secondary metabolites; Bacterial metabolites; Mycotoxins; House dust; Building materials; Mass spectrometry.

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Practical Implications

Bacterial toxins co-occur with mycotoxins in moisture-damaged indoor environments. These compounds are measurable also in settled airborne dust, indicating that inhalation exposure takes place. In attempts to characterize exposures to microbial metabolites not only mycotoxins but also bacterial metabolites have to be targeted by the analytical methods applied. We recommend including analysis of samples of outdoor air in the course of future indoor assessments, in an effort to better understand the outdoor contribution to the indoor presence of microbial toxins. There is a need for a sound risk assessment concerning the exposure to indoor microbial toxins at concentrations detectable in moisture-damaged indoor environments.

Introduction

Indoor environmental conditions characterized by dampness, moisture damage and mold are recognized risk factors for a number of short-term and long-term health effects (Institute of Medicine, 2004; WHO Regional Office for Europe, 2009). Irritation symptoms are most commonly reported, in particular upper respiratory symptoms such as cough, rhinitis, hoarseness and wheezing. Dampness is also a risk factor for exacerbation and new onset of asthma (Institute of Medicine, 2004; Pekkanen et al., 2007). Recurrent respiratory infections, less specific, neurological or general symptoms and rare health outcomes, such as allergic alveolitis, sarcoidosis and rheumatic diseases, have been linked with moisture damage in indoor

environments (Dales et al., 1991; Myllykangas-Luosujärvi et al., 2002; Park et al., 2006).

The nature of causative agents and mechanisms underlying the adverse health outcomes observed in occupants of moisture-damaged buildings remain yet obscure. The research is complicated by the fact that along with chemical exposures, a whole variety of microbial compounds originating from different indoor molds and bacteria may be involved in generating adverse health effects, such as allergenic proteins, structural elements with inflammatory potential and volatile organic compounds (MVOCs). There is a good body of documentation which suggests that nonvolatile microbial toxins – produced as secondary metabolites during microbial growth – may add another critical constituent to the multiple exposures in damp buildings. Fungal and bacterial strains that produce toxic secondary metabolites are present in moisture-damaged indoor environments (Andersson et al., 1998; Engelhart et al., 2002; Fogle et al., 2007). The production of mycotoxins on building materials is well documented (Nielsen, 2003). Microbial spores and cell fragments get airborne and have been shown to contain microbial toxins (Brasel et al., 2005a); subsequently, occupants of contaminated buildings may be exposed through inhalation of indoor air (Brasel et al., 2005b; Gottschalk et al., 2008; Polizzi et al., 2009). Synergistic interactions in modulation of cellular responses upon simultaneous exposure to bacterial and fungal spores, their metabolites and structural compounds have been shown (Huttunen et al., 2004; Islam et al., 2007; Penttinen et al., 2005; Zhou et al., 1999).

Current data on the natural occurrence of toxic microbial metabolites in indoor environments are limited. Most of the analytical methods applied so far were developed to specifically assess the presence of a restricted set of mycotoxins of primary toxicological interest (Bloom et al., 2007; Engelhart et al., 2002; Gottschalk et al., 2008). Recent work from Polizzi et al. (2009) indicated the presence of a multitude of mycotoxins in indoor sample materials. Toxic bacterial metabolites, referred to here as bacterial toxins, have so far been insufficiently addressed in analytical indoor assessments, even though toxigenic bacterial strains are known to be present in indoor environments (Andersson et al., 1998; Mikkola et al., 2007).

In this exploratory study, we analyzed building material and dust samples from moisture-damaged indoor environments with a multi-analyte liquid chromatography/mass spectrometry (LC-MS)-based method recently published by Vishwanath et al. (2009), allowing a screening for 159 fungal and 27 bacterial metabolites. We report here the simultaneous detection of multiple microbial toxins from indoor sample materials, including – for the first time in naturally infested sample materials – also bacterial toxins.

Methods

Building material and dust samples

Samples of building materials and various dust sample types were primarily derived from single family homes with severe moisture damage that were investigated in the context of the HoTeS study ('Mold-exposure and health survey'). This is an ongoing study that aims to produce novel information on the occurrence of microbes and microbial metabolites in moisture-damaged indoor environments and their association with health effects in exposed residents. Being conducted in an intervention design, this study investigates the effect of building renovations on exposure and health outcomes. Through collaboration with the Finnish Society for Pulmonary Disabled (Heli), HoTeS recruits families across Finland living in homes affected by indoor conditions of severe moisture damage and dampness that require major repair actions. Self-reported health complaints of the residents linked to spending time in the damaged home are an additional inclusion criterion for the study. Initial building inspections by trained civil engineers are conducted to establish the damage status of the building. Subsequently, renovations are planned and carried out. The families and homes are followed up in the course of this intervention, including both health and microbial exposure measurements before and after the renovations. The herein described sample material includes building materials and different dust sample types from the first nine homes that were recruited to the HoTeS study, collected before renovations. Floor dust was typically sampled in the living room, using a regular vacuum cleaner device and nylon dust sampling socks, as described by Hyvärinen et al. (2006). Settled airborne dust samples were collected in the same way, but from surfaces above floor level, such as bookshelves and similar. The dust bags from the vacuum cleaners used by the families in their homes were additionally collected for analyses. Vacuumed floor dust and dust bag dust samples were size homogenized by sieving through a sterile strainer (pore size approx. 1 mm) to remove the coarse fraction. Vacuumed floor dust and settled airborne dust samples were dried in an exsiccator prior to aliquoting and stored at −20°C. In addition to the sample materials of the HoTeS study, five material samples derived from moisture-damaged public buildings in Sweden – day care centres/kindergartens, a cinema and a university building – were included.

Analyses of microbial metabolites

Building materials and dust samples were analyzed with liquid chromatography/tandem mass spectrometry (LC-MS/MS) using the methodology recently published by Vishwanath et al. (2009). The basic list

of target metabolites in this multi-analyte method includes 159 fungal and 27 bacterial metabolites. In brief, the sample materials were extracted in acetonitrile/water/acetic acid (79:20:1, *v*/*v*/*v*), raw extracts were diluted and subsequently analyzed without further clean-up. Detection and quantification was done with a QTrap 4000 LC-MS/MS (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization source and a 1100 Series HPLC System (Agilent, Waldbron, Germany). Sample preparation, instrumental parameters, method performance characteristics (coefficients of variation, limits of detection, recoveries) and standards used in this multi-target method have been described in detail by Vishwanath et al. (2009). A subset of building material samples was included in an interlaboratory method comparison and was additionally analyzed using another LC-MS/MS-based method previously described by Bloom et al. (2007, 2009) targeting selected *Aspergillus* spp. and *Stachybotrys* spp. toxins.

Cultivation and microbial identification

Cultivation and microbial identification was performed on 37 building material samples derived from the HoTeS study. Samples were processed as described previously by Hyvärinen et al. (2002) and plated on 2% malt extract agar and dichlorane glycerol agar (DG18) for fungi, and on tryptone yeast extract glucose agar and Gauze agar for bacteria. Morphological identification and enumeration of fungi and of 'dry' actinobacteria-type colonies were done microscopically.

Results and discussion

Table 1 summarizes the results of the analyses of various indoor sample materials derived from moisture-damaged buildings with a multi-analyte HPLC-MS/MS-based method. In total, 33 different microbial metabolites were detected, with all of the 69 samples analyzed being positive for at least one of the targeted analytes. Between 3 and 13 different microbial metabolites were present in the investigated residential homes. Up to 16 metabolites were found in single gypsum board samples of public buildings. Along with well-known indoor mycotoxins, such as sterigmatocystin and satratoxins, we detected in our study metabolites that are novel in the context of naturally infested indoor sample materials. This list includes several fungal metabolites, such as mycophenolic acid or physcion, as well as the bacterial metabolites chloramphenicol, monactin, nonactin, staurosporin and valinomycin (Table 1).

The fungal compounds emodin, enniatin B, beauvericin and the bacterial toxin chloramphenicol were

Table 1 Summary of the microbial metabolite findings in the sample materials collected from moisture-damaged indoor environments

Sampling location	Type and number of sample materials (N)	Microbial secondary metabolites present in the sample materials	
		Fungal	Bacterial
Home 1	BM (9)	Chaetoglobosin A, Emodin, Meleagrin, Roquefortin C, Stachybotrylactam, Sterigmatocystin	Monactin, Valinomycin
Home 2	BM (4), DBD (1), FD (2), SD (1)	Alternariol, Alternariolmethylether, Apicidn, Beauvericin, Emodin, Enniatin A, Enniatin B, Equisetin, Festuclavin, Mycophenolic acid	Chloramphenicol
Home 3	BM (5), DBD (1), FD (2), SD (1)	Beauvericin, Emodin, Enniatin B, Equisetin, Mycophenolic acid, Ochratoxin A, Physcion, Viridicatin	Chloramphenicol, Valinomycin
Home 4	FD (1), SD (1)	Emodin, Enniatin B	Chloramphenicol
Home 5	DBD (1), FD (1), SD (1)	Beauvericin, Emodin, Enniatin B, Equisetin, Mycophenolic acid, Sterigmatocystin	Chloramphenicol
Home 6	BM (5), DBD (1), FD (2), SD (1)	Beauvericin, Emodin, Enniatin B, Mycophenolic acid, Ochratoxin A, Sterigmatocystin	Chloramphenicol
Home 7	DBD (1), FD (1), SD (1)	Beauvericin, Emodin, Enniatin B	Chloramphenicol
Home 8	DBD (1), FD (2), SD (1)	Alternariol, Alternariolmethylether, Beauvericin, Emodin, Equisetin, Enniatin A1, Enniatin B, Physcion	n.d.
Home 9	BM (14), DBD (1), FD (2)	Altenuene, Alternariol, Alternariolmethylether, Beauvericin, Chaetoglobosin A, Emodin, Enniatin A1, Enniatin B, Equisetin, (Hydrolysed Fumonisin B1[b]), Kojic acid, Macrosporin, Physcion, Sterigmatocystin	n.d.
Public buildings 1[a]	BM (2)	Beauvericin, Brefeldin A, Emodin, Enniatin B, Meleagrin, Physcion, Satratoxins G+H, Stachybotrylactam, Sterigmatocystin	Monactin, Nonactin, Valinomycin, Staurosporin
Public buildings 2[a]	BM (3)	Alamethicin, Beauvericin, Brefeldin A, Chaetoglobosin, Chanoclavin, Emodin, Enniatin B, Fumigaclacvine, Meleagrin, Physcion, Roquefortin C, Satratoxins G+H, Stachybotrylactam, Sterigmatocystin, Viridicatin	Monactin, Nonactin, Valinomycin
Total	BM (42), DBD (7), FD (13), SD (7)	28 different fungal metabolites	5 different bacterial metabolites

n.d., not detected; BM, building materials; DBD, vacuum cleaner dust bag dust; FD, vacuumed floor dust; SD, settled airborne dust.

[a]Public buildings 1 include a cinema and a university building; Public buildings 2 include day care centres and kindergartens.

[b]A signal for hydrolysed fumonisin B1 was detected on both MRM (multiple reaction monitoring) transitions, therefore formally complying the criteria for positive identification according to Commission Decision 2002/657; however, because no fumonisin B1 was detected, this finding is highly improbable and can therefore be regarded as interference.

overall most prevalent and were detected in 62%, 57%, 38% and 38% of all samples, respectively (Table 2). The biggest variety of fungal and bacterial metabolites was found in wood-based and gypsum-based building materials, both of which are known to provide a good substrate for fungal and bacterial growth (Hyvärinen et al., 2002). Among house dust samples, floor dust and dust bag dust seemed to equally well capture the variety of microbial metabolites present in the study homes (detection of 12 and 11 different target compounds, respectively; Table 2). Floor dust samples are easily collected in a standardized way by field workers or residents themselves and may therefore be a good sample material for an initial assessment of the indoor presence of microbial metabolites. The amount of toxin(s) detected in a defined floor area may also be useful in calculating a worst case exposure scenario, considering for example an infant re-suspending and inhaling as well as ingesting floor dust while playing on a carpet floor. Naturally, such approach is far from attempting an accurate inhalation exposure assessment.

Six different fungal – emodin, enniatins A1 and B, beauvericin, equisetin, physcion – and the bacterial metabolite chloramphenicol were detected in settled airborne dust (SD) samples. These metabolites are an addition to the list of previously reported, potentially toxic microbial compounds that may occur in indoor

Table 2 Fungal and bacterial metabolites detected in building materials and dust samples collected in moisture-damaged buildings

	Prevalence (%) of microbial metabolites in building materials					Prevalence (%) of microbial metabolites in dust			Overall prevalence (%) $N = 69$
	Wood based $N = 14$	Mineral fibres[a] $N = 10$	Paper based $N = 6$	Gypsum based $N = 5$	Other $N = 7$	Dust bag dust $N = 7$	Floor dust $N = 13$	Settled airborne dust $N = 7$	
Fungal metabolites									
Emodin	71.4	20.0	33.3	60.0	71.4	100.0	92.3	28.6	62.3
Enniatin B	28.6		16.7	80.0	42.9	100.0	100.0	100.0	56.5
Beauvericin	7.1	10.0	16.7	80.0	14.3	71.4	61.5	71.4	37.7
Equisetin	21.4	70.0	16.7		42.9	57.1	38.5	14.3	34.8
Physcion	14.3	60.0		60.0	28.6	42.9	15.4	14.3	27.5
Sterigmatocystin	50.0	10.0	16.7	80.0	14.3	14.3	7.7		23.2
Meleagrin	35.7		50.0	100.0	14.3				20.3
Chaetoglobosin A	28.6	10.0	50.0	40.0			15.4		17.4
Enniatin A1		30.0			14.3	28.6	30.8	14.3	15.9
Stachybotrylactam	7.1		33.3	100.0	14.3				13.0
Alternariolmethylether	7.1				42.9	14.3	7.7		8.7
Alternariol	7.1				28.6	14.3	7.7		7.2
Roquefortine C			33.3	60.0					7.2
Brefeldin A				60.0	14.3				5.8
Kojic acid	7.1	10.0			28.6				5.8
Mycophenolic acid	14.3		16.7			14.3			5.8
Satratoxin G				60.0	14.3				5.8
Satratoxin H				60.0	14.3				5.8
Altenuene		10.0					15.4		4.3
Enniatin A	7.1				28.6				4.3
Ochratoxin A	7.1	20.0							4.3
Viridicatin				20.0	14.3				2.9
Fumiclavine				40.0					2.9
Chanoclavine				40.0					2.9
Alamethicin				20.0					1.4
Apicidin					14.3				1.4
Festuclavin	7.1								1.4
Macrosporin	7.1								1.4
Total no. of different fungal metabolites	17	10	10	16	18	10	11	6	28
Bacterial metabolites									
Chloramphenicol	7.1	20.0	33.3		14.3	71.4	69.2	85.7	37.7
Valinomycin	42.9		33.3	60.0	14.3				17.4
Monactin	7.1		33.3	40.0	14.3				8.7
Nonactin				40.0	14.3				4.3
Staurosporin					14.3				1.4
Total no. of different bacterial metabolites	3	1	3	3	5	1	1	1	5

[a]Man-made mineral fibres (MMF), including mineral wool, stone and glass wool.

air in moisture damage conditions (Brasel et al., 2005b; Gottschalk et al., 2008; Polizzi et al., 2009). The concentrations of metabolites we found in dust and building materials were mostly in the low to mid nanogram per gram range, reaching low microgram per gram levels in a few cases (Table 3). These levels are well comparable to earlier reports on microbial toxin content of indoor dust and building materials (Bloom et al., 2007; Engelhart et al., 2002).

The detection of a multitude of 28 different fungal metabolites in our sample materials confirms earlier reports that have indicated the presence of multiple mycotoxins in moisture-damaged indoor environments (Polizzi et al., 2009; Vishwanath et al., 2009). The results of our exploratory study underline the relevance of using a multi-analyte screening method, which allows to conclude on the spectrum of microbial toxins present indoors. Larsson (2008) has stressed the possible relevance of not only fungal, but also bacterial secondary metabolites as toxic indoor contaminants. While toxic metabolite producing bacterial strains have been isolated from indoor samples (Andersson et al., 1998; Mikkola et al., 2007), actual measurement data on the natural indoor occurrence of these metabolites have so far not been provided. The multi-analyte method applied in our study targeted 27 bacterial metabolites, most of them produced by *Streptomyces* species. Five such compounds were found, with monactin, nonactin, staurosporin, and valinomycin – unlike chloramphenicol – being exclusively detected in building materials, but not house dusts (Table 2).

Streptomyces is a genus of Gram-positive bacteria that commonly occur in soil, but are also known to be present in indoor environments (Hyvärinen et al., 2002; Nevalainen et al., 1991), where they can indicate moisture damage and dampness (Rintala et al., 2004). *Streptomyces* species are also highly potent producers of a large variety of bioactive metabolites (Demain, 1999), such as antibiotics, immunosuppressive agents, enzyme inhibitors, and other pharmacologically active compounds. It is striking to find a toxic metabolite-producing group of bacteria being associated with moisture damage conditions in indoor environments, with some of these metabolites being readily detectable in damaged indoor sample materials.

In this context, it is important to mention that cultivation of building materials did not always predict the presence of the bacterial metabolites in the samples (*data not shown*). The detection of 'dry' actinobacteria-type colonies – a morphology typical for *Streptomyces* and related genera – in culture was not always accompanied by the detection of *Streptomyces* metabolites in the same original material sample and *vice versa*. However, there are several possible explanations for a lack of co-detection of metabolite and producing microbe, including most importantly a still very limited set of bacterial metabolites included in the list of analytes, limitation of cultivation technique to only

Table 3 Levels of microbial metabolites in four moisture-damaged homes: concentrations of microbial metabolites in building materials (BM) and traceability into the respective dust samples

	Microbial metabolites in building materials and dust samples of the same home (maximum concentrations in ng/g building material or dust)														
	Home 2				Home 3				Home 6				Home 9		
	BM	DBD	FD	SD	BM	DBD	FD	SD	BM	DBD	FD	SD	BM	DBD	FD
Altenuene													33		1200
Alternariol	400												13		
Alternariolmethylether	55												1.2		1.3
Apicidin	1300														
Beauvericin			1.4	0.67	0.60	0.32			70		0.75	1.6	0.11	0.05	
Chaetoglobosin A													83		3100
Chloramphenicol		4.8	14	21	14	5.9	17	11	19	5.0	22	42			
Emodin	140	2.4	0.24		78	39	15	84	45	10	5.0		47	12	6.9
Enniatin A	0.83														
Enniatin A1													0.86	2.7	1.0
Enniatin B	0.65	0.75	4.9	0.35	0.78	1.0	4.1	1.1	0.44	0.63	2.4	1.1		9.4	1.6
Equisetin			24			6.4							26	6.1	3.4
Festuclavin	3.3														
Kojic acid													2000		
Macrosporin													120		
Mycophenolic acid	91				88				67						
Ochratoxin A					8.3				32						
Physcion						460							420	46	
Sterigmatocystin									21				110		1.3
Valinomycin					37										
Viridicatin					5.1										

DBD, vacuum cleaner dust bag dust; FD, vacuumed floor dust; SD, settled airborne dust.

viable and culturable microbes and a longer persistence of the microbial metabolites compared to the viability of the producing bacterial cells. In any case, in each home where one or more of the bacterial metabolites were detected and cultivation of building materials was performed, we found also actinobacteria-type colonies in culture, establishing the biological plausibility for the presence of bacterial metabolites in these homes.

The bacterial metabolites present in the sample materials are potent, bioactive substances. Valinomycin, monactin and nonactin are known ionophors that disrupt transmembrane ion gradients. Monactin and nonactin are members of the macrotetrolid antibiotics family and have been shown to modulate cytokine production and T-cell proliferation (Mori et al., 2000; Umland et al., 1999). Research on the biological activity of valinomycin proposed mitochondrial swelling, reduced natural killer cell activity and at higher doses apoptosis in peripheral blood lymphocytes (Paananen et al., 2000). Staurosporin gathers a whole range of biological activities, including anticancer activity by inducing apoptosis in mammalian cells (Stepczynska et al., 2001). Chloramphenicol is an efficient broad-range antibiotic, which because of resistance and safety concerns has been banned for use in food-producing animals and is restricted in human applications. Rare, but severe adverse side effects of chloramphenicol treatment have been reported, such as aplastic anaemia and leukaemia (Rich et al., 1950; Shu et al., 1987).

In addition to bacterial compounds, the multitude of fungal metabolites detected in our study (Table 2) represents an even longer list of different biological activities, which cannot be discussed in detail here. However, it becomes evident that a sound risk assessment on the health implications of indoor microbial toxins is needed. When attempting to assess the health relevance of chronic, low level exposure to microbial metabolites in indoor environments, their variety and co-occurrence as well as the possibility of synergistic effects of different metabolites and other microbial compounds need to be considered. Previous research has shown that the indoor microbes *Stachybotrys chartarum* and *Streptomyces californicus* produce synergistic inflammatory responses in mouse macrophages (Huttunen et al., 2004; Penttinen et al., 2005). Stimulation of the production of toxic secondary metabolites during cocultivation was postulated to be one possible mechanism (Penttinen et al., 2006). Endotoxin – the lipopolysaccharide (LPS) of Gram-negative bacteria – and other microbial compounds are present in all living environments, but are found in elevated levels in moisture damage situations (Garrett et al., 1998; Gorny et al., 2002; Park et al., 2006). The documentation on mycotoxins and LPS acting synergistically in modulating inflammatory processes and causing adverse effects on organ systems is well established (Islam et al., 2007; Kankkunen et al., 2009; Pestka and Zhou, 2006; Zhou et al., 1999).

Emodin, enniatin B, beauvericin and the bacterial toxin chloramphenicol were the most prevalent compounds found in this study and were particularly frequently detected in house dust samples. Preferential detection of certain microbial metabolites in house dusts compared to building material samples could suggest sources other than indoor microbial growth. Plant material and soil, mold-contaminated grain dusts and even insects (Berendsen et al., 2010; Molnár et al., 2010) transferred from outdoors to the indoor environment represent potential sources for low indoor levels of microbial toxins. A recent study from Berendsen et al. (2010) supports the theory that chloramphenicol occurs naturally in soil, produced by common soil bacteria, such as *Streptomyces venezuela*, and accumulates in herbs and grasses. Thus, low indoor levels of chloramphenicol may result from transfer of plant material or dust from out- to indoors. Table 3 summarizes data on the 'traceability' of microbial metabolites in four homes, where both building materials and dust samples were available for analyses. The detection of a given metabolite in damaged building materials along with dust is an indication that microbial growth associated with the moisture damage may be the source of the metabolite. In two of three homes, in which chloramphenicol was found, the analyte was detected not only in dust but also in building material samples. Similarly, the fungal metabolites occurring most frequently in house dust (i.e. beauvericin, emodin, enniatin B) were present also in material samples in the majority of the respective homes, with the exception of equisetin. Nevertheless, uncertainty concerning the sources of microbial toxins detectable indoors suggests conducting sampling and analyses of outdoor air in parallel with indoor assessments in the future.

Limitations of this exploratory study include the relatively small number of samples, which prompted us to present our novel findings on co-occurrence of bacterial and fungal metabolites in a rather descriptive manner. Statistical analyses using microbial toxin data and other exposure and health parameters will be conducted in a next step, once a more complete dataset derived from the HoTeS study will become available. We furthermore aim at concluding on a short-list of the most relevant microbial toxins associated with damage conditions, which will require larger datasets including both sample materials derived from moisture damage and from non-damaged indoor environments.

Sampling and analyses of outdoor air have not been considered in the initial sampling strategy, as this will require more laborious sampling of large air volumes. Based on the study findings, however, we are certainly planning on conducting a systematic study to investi-

gate the outdoor contribution to the presence of microbial metabolites indoors.

Performance characteristics, analytical standards used and other details on the applied multi-analyte HPLC-MS/MS-based methodology have been described previously in a dedicated paper (Vishwanath et al., 2009). As this method was developed as a screening tool for the detection of more than 180 different microbial compounds, there are certain restrictions when it comes to exact quantification. The screening method was shown to be quantitative in case of building materials, however, signal suppression because of matrix effects and non-quantitative extraction impede accurate quantification in dust (Vishwanath et al., 2009). Limits of detection are not consistent for the multitude of target compounds, which may somewhat bias the frequency of detection of certain metabolites. For example, enniatin B and beauvericin show a very high response in the analytical LC-MS/MS methodology, meaning that they are detectable also at very low levels, which could contribute to their relatively high prevalence in the indoor sample materials.

Nevertheless, the applied multi-analyte screening method proved to be an excellent tool to fulfil the objective of assessing the variety of microbial metabolites present in moisture-damaged indoor environments and at creating knowledge on indicative concentrations of the prevalent compounds. The reliability of the multi-analyte HPLC-MS/MS screening method was further confirmed in an interlaboratory method comparison including five building material samples derived from public buildings (Table 1). In addition to the analyses with the multi-analyte screening method, these samples were subjected to analyses with more dedicated methodology targeting a small set of *Stachybotrys* and *Aspergillus* spp. metabolites (Bloom et al., 2007, 2009). The presence of satratoxins G and H and sterigmatocystin in the sample materials – targeted by both methods – was confirmed using the two different approaches.

Conclusions

A whole variety of microbial metabolites is present in sample materials of moisture-damaged indoor environments. Up to 13 different microbial metabolites were found to co-occur in the same home. With the detection of five different metabolites produced by *Streptomyces* species, including chloramphenicol, we provide here for the first time direct proof for the indoor presence of bacterial toxins and their co-occurrence with mycotoxins. Analytical methodology aiming at assessing microbial toxins in indoor samples needs to consider both bacterial and fungal metabolites. Floor dust may be a good sample for an initial assessment of the presence of microbial toxins, as it seems to capture a wide range of indoor metabolites in detectable levels and moreover, is easy to collect.

Several microbial toxins were detectable in settled, previously airborne dust, which indicates that inhalation exposure to these compounds may take place. It remains to be determined whether the detected concentrations of the microbial metabolites are of toxicological relevance or implicate adverse health outcomes. Links to health effects identified among occupants as well as mechanistic, toxicological data on inhalation exposure will help to assess the risk related to indoor exposure to microbial toxins.

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Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC–MS/MS and GC–MS methods

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ABSTRACT

This study reports on detection of a large number of biological and anthropogenic pollutants using LC–MS/MS and GC–MS technologies in settled floor dust (SFD). The latter technique was applied to obtain a general picture on the presence of microbial as well as non-microbial volatile organic compounds, whereas the targeted LC–MS/MS analysis focused on identification of species specific secondary metabolites. In the absence of moisture monitoring data the relevance of finding of stachybotrylactam and other metabolites of tertiary colonizers are confined only to accidental direct exposure to SFD. To the best of our knowledge 30 of the 71 identified volatile organic compounds (VOCs) are newly reported in SFD matrix. Coordinated application of "AMDIS and Spectconnect" was found beneficial for the evaluation and identification of prime volatile pollutants in complex environmental samples. Principal component analysis (PCA) of peak areas of 18 microbial volatile organic compounds (MVOCs) resulted in identification of nonanal as potential MVOC marker. Two more volatiles toluene and 1-tetradecanol though had discriminative influence, are not regarded as MVOC markers, considering their probable alternate origin from paints and cosmetics, respectively.

1. Introduction

The advantages of various methods and matrices for the purpose of indoor evaluation are well documented in earlier reports [1–9]. The suitability of settled floor dust (SFD) for the above mentioned purpose can be best explained on the basis of mode of its formation. SFD is typically formed by the deposition of indoor aerosols, outdoor particles (due to ventilation) and also particles brought by foot trafficking. Moreover criticality and relevance of chosen SFD matrix for the mass spectrometric evaluation are also justified by the reports of Rosas et al. [1] for the detection of antibiotic multi resistance *E. coli* serotypes and reports of SFD surface adsorption of polycyclic aromatic hydrocarbons and other volatile and semi-volatile organic compounds [10,11]. Since the process of degradation indoors is typically slow, settled floor dust is also considered as a well preserved fossil evidence for indoor evaluation [12].

The new findings since reports of sick building syndrome (SBS), indoor pollutants are classified either as biogenic or anthropogenic pollutants. Biogenic pollutants include aerosols of viruses, bacteria, fungal spores and mycelial fragments containing toxic metabolites (e.g.: *Stachybotrys* containing Stachybotrylactam and Satra toxins), pollen, animal dander, dust mite residues and other particles of biological origin [13]. Anthropogenic pollutants are hazardous chemicals which arise as a consequence of their wide spread use in day to day consumer products (phthalate in PVC products). A variety of adverse health effects following human exposure to bioaerosols have been well documented [14]. Some of them are allergy, hypersensitivity, respiratory and toxicological problems, and infectious diseases [15]. Microbial role in indoor pollution and health implications are well known. For instance, fungi as well as bacteria (antigens, structural components, bioactives compounds as endotoxins) in humidifiers have been implicated in "humidifier fever", a disease with both toxic and allergic manifestations [16]. Bacteria in indoor air of houses or offices have also been reported to be associated with extrinsic allergic alveolitis among occupants [17].

Under non obvious moisture complications types and levels of fungal spores in the indoor samples are generally lower to those

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detected in the outdoor samples. Fungal flora of damp buildings comprises species addition to outdoor fungi that can utilize the nutrients present in building materials and adaptable to particular level of water activity. Based on water activity molds grown on building materials can be divided into primary, secondary and tertiary colonizers. Many of the toxigenic species *Stachybotrys*, *Chaetomium*, *Memnoniella*, *Aspergillus* and *Trichoderma* belong to the class of tertiary colonizers. Indoor isolates of *A. versicolor* from building materials are reported to produce sterigmatocystin, a class 2B carcinogen and biosynthetic precursor of aflatoxin B_1. Prevalence of airborne spores of *Stachybotrys chartarum* in houses with water incursion [18] and satratoxin G–albumin adducts (in vivo) upon human and animal exposures to *S. chartarum* [19] are some evidences for probable health threats due to mycotoxins in water damaged indoor environments. Animal exposure studies of low molecular weight compounds from fungi from the built environment have shown implications on health, such as inflammatory processes [20]. Microbial volatile organic compounds (MVOCs) are an addition to the list of biogenic indoor pollutants and have drawn attention as potential contributors for adverse health effects observed in residents of moisture damaged buildings [21]. In vitro studies of histamine release by bronchoalveolar cells exposed to MVOCs of *Trichoderma viride* [22], is a good example of the experimental evidences of MVOCs connections to clinical aspects.

Studies on house dust have shown for the presence of banned chemicals, and chemicals with endocrine disrupting, carcinogenic, neurotoxic potentials at levels that are considered to be of concern for human health [23,24]. Traditionally, consumption of food has been considered a primary route of exposure to contaminants mentioned above. However, it is becoming clear that exposure through ingestion and/or inhalation of indoor dust may be comparable to corresponding food consumption especially for younger children [24]. Independent studies monitoring for occupational exposure to volatile organic compounds (VOCs) toluene, o-xylene and n-butyl acetate and correlation of these substances to clinical symptoms upon long-term exposure such as deficits in concentration and memory, and an increase in the reaction time are proofs for ill effects of some of the VOCs in adults too [25,26].

State-of-the-art technology GC–MS may be useful for detecting hidden mold [27] and proved to be useful to differentiate between fungal strains [28]. Extremely low MVOC concentrations and the existence of many disturbing concomitants indoor complicate the analysis of microbial VOC in moldy houses leading to false positives [29]. Secondary metabolites being inconsistent in distribution throughout the fungal kingdom are unique as markers for speciation and chemotaxonomy purposes [30]. This was also reported true for fungal species found indoors [31,32]. Hence application of complementary technologies GC–MS and LC–MS/MS for evaluation of complex indoor matrix as SFD can be highly advantageous for comprehensive indoor evaluation and to monitor, e.g., ongoing remediation processes.

Enumeration studies based on microbial viability [1,2], detection of microbial volatiles [3–6] and non volatiles [7–9] in indoor matrices including settled dust are known. To our knowledge this is the first report of comparison of dust matrix from indoors used for different purposes using microbial volatiles and secondary metabolites. Dust samples from vastly differing indoor environments such as waste management and recycling units (WMU), houses with and without any water damage and mold infestation are screened for microbial effectors and subsidiary chemicals. Additionally, we wanted to test feasibility of source recognition/apportionment based on (M)VOC pattern among various indoor dust samples using principal component analysis (PCA) and hierarchical cluster analysis (HCA). Some of the methodological challenges in GC–MS such as sample volume, extraction time, and temperature for the optimal extraction of volatile substances in dust matrix are addressed. Appertain to data evaluation we demonstrated usability of AMDIS deconvoluted chromatograms in combination with Spectconnect [33] for additional verification of sampling and method performance. Practical relevance of the study can be best explained in relation to safety of toddlers in homes with crawling and hand to mouth behavior and workers of WMUs. The extraction temperature condition (max 90 °C) of our GC–MS method is similar considering the working temperature of common home appliances (e.g. surface of a electric bulb (110–160 °C)). This aforementioned fact makes volatile pattern generated under our experimental conditions realistic and comparable to real world scenario. This is the first comparative study of individual SFD samples derived from relatively differing indoor environments in their purposes using both LC–MS/MS and GC–MS methods.

2. Materials and methods

2.1. Study sites and sampling

2.1.1. Waste management facilities

Settled floor dust samples were collected using vacuum cleaner from different waste management units (WMU) in Germany dealing with municipal waste or paper recycling. Samples MWD 1–7 (Municipal waste dust, Group A) were collected from waste handling facilities treating municipal waste with "biological and mechanical" or "biological" methods. Samples PWD 1–8 (Paper waste dust, Group B) were from enclosures used for paper recycling activities such as sorting, storage, or mechanical pressing (Table 1).

2.2. Residential indoors

Settled floor dust samples were collected from houses inhabited by small group of people, generally less than 5. Samples AHD 1–5 (Affected house dust, Group D) were vacuum cleaner dust bag dust samples derived from single family houses located in Eastern and southern Finland. These houses had severe moisture damage/dampness problems that were confirmed by trained engineers upon building inspection. Residents of these buildings typically complained about building related symptoms. Samples CHD 1–2 (Control house dust, Group C) were samples, respectively, from United States of America (SRM 2583) and India (CHD-2). SRM 2583 is a certified reference material (CRM) for 5 elements viz., Arsenic, Cadmium, Chromium, Lead and Mercury. CHD-2 is a self collected house dust where no clinical symptoms of ill health or visible mold growth were observed or reported.

3. Methods for analysis of non volatile and volatile substances

Volatile organic compounds (microbial and anthropogenic) were evaluated using GC–MS technology. Non volatile microbial organic compounds were monitored using LC–MS/MS technology.

3.1. GC–MS screening for volatile and microbial volatile organic compounds

3.1.1. Analytical reagents and supplies

The alkane mixture C_5–C_{10} was mixed in-house. C_8–C_{20} and C_{21}–C_{40} straight chain alkanes of 40 mg L^{-1} concentration in hexane and toluene, respectively, were purchased from Fluka (Buchs, Switzerland). All pure GC–MS standards (substances in Table 2 and Supplementary Table 1 highlighted with "*") used in this study were purchased from Sigma–Aldrich, Vienna, Austria. To avoid artifact originating from GC-column bleeding, SPME fiber coating or from laboratory air, head space vials were left open for 24 h in laboratory

Table 1
Sample description. MW: municipal waste management units, PW: Paper waste recycling units, TA: treatment area, ST: storage, NA: not applicable.

Sample	Nature of sample	Sampling site	Mode of waste treatment at the sampling site	Ventilation of sampling site	Processing load/turn over of waste handling units (kilotons/year)
MWD-1	MW	TA	Biological and Mechanical	Mechanical	62
MWD-2	MW	TA	Biological and Mechanical	Mechanical	62
MWD-3	MW	TA	Biological and Mechanical	Mechanical	62
MWD-4	MW	TA	Biological and Mechanical	Mechanical	62
MWD-5	MW	TA	Biological and Mechanical	NA	62
MWD-6	MW	TA	Mechanical	Natural	400
MWD-7	MW	TA	Mechanical	Natural	200
PWD-1	PW	ST	Paper-Storage	Natural	7.3
PWD-2	PW	TA	Mechanical	Natural	100
PWD-3	PW	TA	Sorting-Mechanical & Manual	Natural	40
PWD-4	PW	TA	Sorting-Mechanical	Natural	20
PWD-5	PW	TA	Sorting-Mechanical	Natural	60
PWD-6	PW	TA	Sorting & Pressing-Mechanical	Natural	10
PWD-7	PW	TA	Sorting & Pressing-Mechanical	Natural	10
PWD-8	PW	TA	Sorting & Pressing-Mechanical	Natural	10

and subsequently analysed. Substances identified this way were excluded during final compilation of data.

3.1.2. GC–MS

Automated sample extraction, chromatographic separation and MS detection was done with an Agilent 6890 GC (Waldbronn, Germany) instrument, coupled to a 5975B MSD detector for recording the mass spectra. The following pair of GC–MS columns were used one at a time during the entire study:

(A) HP-5MS 30 m × 0.25 mm, 0.25 μm f.th. (Agilent, Waldbronn, Germany),
(B) Optima® Wax 30 m × 0.25 mm, 0.25 μm f.th. (Agilent, Waldbronn, Germany).

For both columns Helium (5.0, Messer, Austria, Gumpoldskirchen) was used as carrier gas at a constant flow rate of 1 mL/min. Oven program: HP-5MS: (apolar) 35 °C (hold 2 min), 5 °C/min to 230 °C, 40 °C/min to 260 °C (hold 5 min). Optima® Wax: 35 °C (hold 2 min), 5 °C/min to 250 °C (hold 10 min). The inlet was equipped with a headspace inlet glass liner, 1.5 mm i.d. (Supelco, Bellefonte, USA) and set to 250 °C in splitless mode during desorption (2 min) of analytes from the fiber. The transfer line to MS was at 270 °C. MSD parameters: electron impact ionisation (EI) at 70 eV, source temperature 230 °C, quadrupole temperature 150 °C, full scan mode, mass range 35–500 amu.

3.1.3. HS-SPME

Sampling was done by headspace volatiles extraction procedure fully automated by an auto sampler (MPS 2 XL, Gerstel, Mülheim a.d. Ruhr, Germany).

Fiber selection and extraction optimization were done on a 30 m HP-5MS column using real world settled dust ($n = 5$) and SRM 2583 (certified indoor reference dust) with an empirical sample amount of 0.05 g which was found to be well suited upon validation in due course of the study. The following SPME-fibers with different polarities were tested: polydimethylsiloxane (PDMS), 100 μm; polydimethylsiloxane/divinylbenzene (PDMS/DVB), 65 μm; carboxene/polydimethylsiloxane (CAR/PDMS), 85 μm; polyacrylate (PA), 85 μm and divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS), 50/30 μm (Supelco, Bellefonte, USA). Selection of fiber was on the basis of number of conserved component upon extraction of representative sample of each group using above mentioned 5 fibers. All fibers tested and used were preconditioned according to the manufacturer's guidelines. Dust samples were weighed into 20 mL head space glass vials covered with Teflon capping and heated to 90 °C for 30′ to release dust bound volatiles. The conditioned fiber was then inserted 21 mm into the head space vial and incubated for 60′ unagitated at constant temperature of 90 °C for adsorption of volatiles. For desorption, the fiber was inserted for 2 min in the split less injector (250 °C, fiber penetration depth 57 mm).

The following SPME parameters were tested: equilibration time (0 and 30 min), extraction time (30 and 60 min), and equilibration and extraction temperature (30, 60 and 90 °C). For all subsequent experiments of parameter evaluation the best found SPME fiber CAR/PDMS, 85 μm was employed (fiber selection elaborated in Section 4).

3.1.4. Method evaluation: reproducibility, representative sampling and source recognition/apportionment studies

For all the above purposes systematic conserved component identification was done, using open source software http://spectconnect.mit.edu. The working principle, algorithm, data extraction procedures followed by Spectconnect are described elsewhere in detail [33]. This is the first report of application of Spectconnect for evaluation of both analytical method and comparability of samples without compound identification. Criterions for picking conserved components were kept stringent and are as follows: elution threshold of 0.5 min (high), support threshold occurrence in all samples (high) and similarity threshold with minimum spectral similarity of 90% (high). Statistical software Unscrambler® [34] and R package (R 2.12.0) [35] were used for multivariate statistics Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), respectively. The data was leverage corrected and centered before subjecting to PCA and for the hierarchical clustering "Euclidean" distance and "Ward" linkage methods (between groups) were used. The effect of several well established clustering methods as single linkage, complete linkage and centroid methods were tested before finalizing with ward linkage. The results from PCA and HCA were used in concurrence for drawing final conclusion.

3.1.5. Volatile data evaluation: identification and confirmation of components

The data acquisition software MSD Chemstation G1701EA E.01.00.237 (Agilent, Waldbronn, Germany) was used to compare mass spectra of chromatographic peaks found in combined Nist and Wiley 2008 databases/spectral libraries. For peak picking all chromatograms were queried to an empty/blank msl library prior querying against combined Nist and Wiley 2008 spectral library. This method was found beneficial avoiding omission of peaks. Addition to this in-house sub libraries were built deriving mass spectra from Nist 05a and Wiley 7n for all those volatiles with AMDIS mass

Table 2
List of identified volatile organic compounds originating from settled floor dust samples from Groups A, B, C and D.

CAS No.	Name	*m/z* (precursor ion)	RT-HP5 (min)	RI-HP5 literature	RI-HP5 experimental	RT-Optima® Wax (min)	RI-Optima® Wax literature	RI-Optima® Wax experimental	References
123-73-9	2-Butenal, (E)-	70.08	6.29	644.0	657.3	9.95	1046.0	1046.4	
79-09-4	Propanoic acid	74.07	8.02	740.0	732.7	23.37	1528.0	1560.1	
71-36-3	1-Butanol	74.12	6.52	668.9*	669.3	13.05	1140.0	1162.6	[45,47]
110-62-3	Pentanal	86.13	7.22	698.0	704.3	8.35	983.0	981.0	[45,47]
107-92-6	Butanoic acid	88.10	10.81	831.0	827.4	25.57	1637.0	1654.7	
71-41-0	1-Pentanol	88.14	9.13	764.0	771.8	15.89	1261.0	1266.6	[47]
108-88-3	Toluene	92.13	9.10	771.2*	770.9	9.78	1037.9*	1040.0	
108-95-2	Phenol	94.11	16.01	983.9*	985.6	33.32	2023.4*	2026.0	[48]
109-08-0	2-Methylpyrazine	94.11	10.99	832.0	832.7	16.45	1312.0	1287.0	
6728-26-3	2-Hexenal, (E)-	98.14	11.81	859.7*	857.9	14.90	1228.8*	1230.1	
66-25-1	Hexanal	100.15	10.07	804.8*	804.6	10.97	1086.3*	1086.0	[45,47]
109-52-4	Valeric acid	102.13	13.15	921.0	899.0	28.10	1744.0	1770.0	[47]
503-74-2	Isovaleric acid	102.13	11.74	876.0	855.8	26.55	1674.0	1698.0	[47]
111-27-3	1-Hexanol	102.17	12.28	873.3*	872.3	18.61	1370.2*	1369.0	[47]
100-42-5	Styrene	104.14	13.09	880.0	897.3	15.73	1260.0	1260.6	[45]
100-41-4	Ethylbenzene	106.16	12.35	869.6*	874.4	12.12	1128.1*	1128.9	
106-42-3	p-Xylene	106.16	12.36	875.0*	874.6	12.31	1119.0	1135.9	
108-38-3	1,3-Dimethyl-benzene	106.16	12.35	864.4	874.6	12.31	1140.0	1136.0	
95-47-6	o-Xylene	106.16	12.36	879.0*	874.8	13.69	1189.2*	1185.7	
100-51-6	Benzyl alcohol	108.13	17.83	1042.0*	1041.3	30.86	1905.2*	1902.15	
4313-03-5′	2,4-Heptadienal, (E,E)-	110.15	17.03	1015.2*	1016.7	22.28	1515.8*	1514.7	
57266-86-1	2-Heptenal, (Z)-	112.16	15.22	958.0	961.5	17.83	1331.0	1339.1	
111-71-7	Heptanal	114.18	13.36	904.4*	905.4	13.79	1186.25*	1189.4	[45,47]
53535-33-4	1-Heptanol	116.20	15.58	974.0	972.6	21.24	1458.0	1472.2	
104-87-0	4-Methyl-benzaldehyde	120.14	19.45	1079.0	1091.0	25.44	1653.0	1649.0	
108-67-8	1,3,5-Trimethyl-benzene	120.19	16.48	1006.0	999.6	14.70	1221.0	1222.8	
526-73-8	1,2,3-Trimethyl-benzene	120.19	16.48	1019.9	999.7	18.00	1344.0	1345.5	[45]
611-14-3	o-Ethylmethylbenzene	120.19	15.95	975.0	983.7	15.36	1248.0	1247.2	
620-14-4	m-Ethylmethylbenzene	120.19	15.37	958.5	966.2	14.80	1231.0	1226.4	
622-96-8	p-Ethylmethylbenzene	120.19	15.50	960.0	970.0	14.15	1181.0	1202.5	
95-63-6	ψ-Cumene	120.19	16.46	989.0	999.2	15.36	1252.0	1247.2	
589-18-4	4-Methyl-benzenemethanol	122.16	20.96	1135.0	1141.2	32.58	1977.0	1989.0	
2363-89-5	2-Octenal	126.19	18.48	1060.0	1061.2	20.61	1436.0	1447.1	
124-13-0	Octanal	128.21	16.69	1005.6*	1006.3	16.70	1294.33*	1296.4	[45,47]
3391-86-4	1-Octen-3-ol	128.21	15.92	981.9*	982.7	21.00	1462.6*	1462.7	
104-76-7	2-Ethyl-1-hexanol	130.22	17.46	1031.4*	1030.0	22.03	1504.0*	1503.8	
111-87-5	1-Octanol	130.22	18.81	1073.1*	1071.2	23.73	1574.67*	1575.0	[47]
1195-32-0	Dehydro-p-cymene	132.20	19.54	1087.0	1093.7	20.25	1415.0	1433.0	
874-41-9	1-Ethyl-2,4-dimethyl-benzene	134.21	19.06	1078.0	1079.0	18.27	1348.0	1356.0	
99-87-6	p-Cimene	134.21	17.46	1040.0*	1029.9	16.08	1250.0	1273.3	[11]
138-86-3	α-Limonene	136.23	17.61	1041.9*	1034.3	13.92	1190.6*	1194.1	
5989-54-8	L-Limonene	136.23	17.61	1031.0	1034.3	13.92	1199.0	1194.1	[45]
80-56-8	α-Pinene	136.23	14.47	939.0	939.0	9.28	1026.0	1020.3	[46]
3777-69-3	2-Pentylfuran	138.20	16.26	994.0*	992.9	14.84	1222.8*	1228.2	[45,47]
18829-56-6	2-Nonenal, (E)-	140.22	21.59	1164.0	1162.3	23.26	1524.0	1555.5	
90-12-0	1-Methyl-naphthalene	142.19	25.77	1306.8	1310.6	30.46	1875.0	1882.4	
124-19-6	Nonanal	142.23	19.90	1107.0*	1105.4	19.52	1403.0*	1403.6	[47]
4180-23-8	Anethole	148.20	25.31	1283.0	1293.7	29.76	1818.0	1848.4	
25152-84-5	2,4-Decadienal, (E,E)-	152.23	22.90	1314.0	1207	29.56	1800.0	1838.6	
3913-81-3	2-Decenal, (E)-	154.24	24.53	1261.0	1265.5	25.78	1630.0	1664.3	
112-31-2	Decanal	156.26	22.90	1207.7*	1207.1	22.185	1510.33*	1510.3	
112-05-0	Nonanoic acid	158.23	24.70	1273.7*	1271.8	36.51	2233.5*	2196.0	

Table 2 (*Continued*)

CAS No.	Name	*m/z* (precursor ion)	RT-HP5 (min)	RI-HP5 literature	RI-HP5 experimental	RT-Optima® Wax (min)	RI-Optima® Wax literature	RI-Optima® Wax experimental	References
644-08-6	p-Phenyltoluene	168.23	30.58	1492.0	1498.9	35.11	2117.0	2117.1	
2463-77-6	2-Undecenal	168.27	27.28	1350.0	1368.2	28.26	1755.0	1777.0	
334-48-5	n-Decanoic acid	172.26	27.72	1380.0	1384.9	38.35	2258.0	2303.4	[47]
112-53-8	1-Dodecanol	186.33	30.82	1472.0	1509.0	32.57	1920.0	1988.4	
143-07-7	Dodecanoic acid	200.31	32.55	1565.0*	1582.8	41.97	2564.0*	2526.5	[48]
112-70-9	n-Tridecan-1-ol	200.36	32.46	1577.0*	1578.7	34.47	2076.0	2083.5	[48]
96-76-4	2,4-Bis(1,1-dimethylethyl)-phenol	206.32	30.82	1519.0	1509.1	38.71	2323.3*	2324.75	
120-51-4	Benzyl benzoate	212.24	36.98	1765.0	1783.9	44.27	2613.0	2658.6	
128-37-0	Butylated hydroxytoluene	220.35	30.80	1513.6*	1508.3	31.28	1920.6*	1923.1	
629-80-1	Palmitaldehyde	240.42	37.72	1811.0	1819.7	35.76	2124.0	2153.5	
36653-82-4	Cethyl alcohol	242.44	38.99	1876.0	1882.8	39.97	2363.0	2400.6	
57-10-3	Palmitic acid	256.42	40.63	2010.0	1966.7	49.04	2865.0	2903.0	[48]
502-69-2	Hexahydrofarnesyl acetone	268.47	38.18	1846.0*	1842.6	35.55	2134.0	2141.7	
1921-70-6	2,6,10,14-Tetramethyl-pentadecane	268.52	35.29	1703.0	1704.6	25.93	1669.0	1670.7	
110-27-0	Isopropyl myristate	270.45	37.78	1824.0	1822.7	33.77	2023.0	2048.3	
112-39-0	Hexadecanoic acid methyl ester	270.45	39.81	1926.7*	1924.3	37.15	2229.5*	2233.0	
112-92-5	1-Octadecanol	270.49	42.87	2081.0	2084.4	43.26	2569.0	2607.0	
84-69-5	Isobutyl-o-phthalate	278.34	38.66	1868.0	1866.2	42.78	2526.0	2578.1	
84-74-2	Dibutyl phthalate	278.34	40.51	1969.0	1960.3	45.18	2726.0	2705.3	

* Retention index (RI) values determined by us with pure reference standards. Listed in the order of increasing precursor ion mass.

spectral match factor ≥90. This was done to minimize data evaluation time in addition to convenient automation. Moreover, the in-house sub libraries dedicated for individual column specifications were supplemented with linear temperature programmed retention indices (LTPRI, Van Den Dool and Kratz index values) corresponding to the stationary phases of the GC used in the study. The added LTPRI values were either experimentally determined by us using authentic standards or literature values retrieved from NIST Chemistry Web Book (2009). In case a putatively identified substance was reported with more than one LTPRI, the value most frequently stated was taken into account. Automated data evaluation was done by AMDIS software (automated mass spectral deconvolution and identification system, version 2.64) [36]. The optimization of AMDIS parameters for deconvolution and identification were done as described earlier by Meyer et al. [37]. The following parameters were found optimal and used for deconvolution and identification during the study; width, 20; adjacent peak subtraction, 1; sensitivity, high; resolution, high; shape requirement, high. Mixture of alkane standards (C_5–C_{10}), (C_8–C_{20}) and (C_{21}–C_{40}) were analysed separately and LTPRI values were determined [38]. Data presented in Table 2 are designated as "identified" when LTPRI value of a volatile compound was within relative deviation of ±2% from literature or from experimentally determined value (using pure standard) in addition to mass spectral match factor greater or equal to 90 on both columns of inverse polarities in triplicates. In cases where detection was on one of the columns meeting the other three criterions for identification, compounds were designated "annotated" (Supplementary Table 1). The set criterions in this study are based on our previous investigation [39] of fungal and other complex matrices and other reports for impact of matrix composition on RI [40,41].

3.2. Liquid chromatography/tandem mass spectrometry – non volatile microbial metabolites

3.2.1. Analytical reagents and supplies

Methanol, acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands), ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma–Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France). Individual fungal and bacterial metabolites were from the same sources as mentioned in our earlier publication [7].

3.2.2. LC–MS/MS

Detection and quantification was done with a QTrap 4000 LC–MS/MS System (Applied Biosystems, Foster City, CA) equipped with a TurboIonSpray electrospray ionization (ESI) source and an 1100 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Gemini® C18-column, 150 mm × 4.6 mm i.d., 5 μm particle size, equipped with a C18 security guard cartridge, 4 mm × 3 mm i.d. (all from Phenomenex, Torrance, CA, USA). Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10:89:1 (v/v/v; eluent A) and 97:2:1 (v/v/v; eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% within 12 min, followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1000 μL min^{-1}.

ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and in negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte. The sMRM detection window of each analyte was set to the respective retention time ±24 s and the target scan time was set to 1 s. The settings of the ESI source

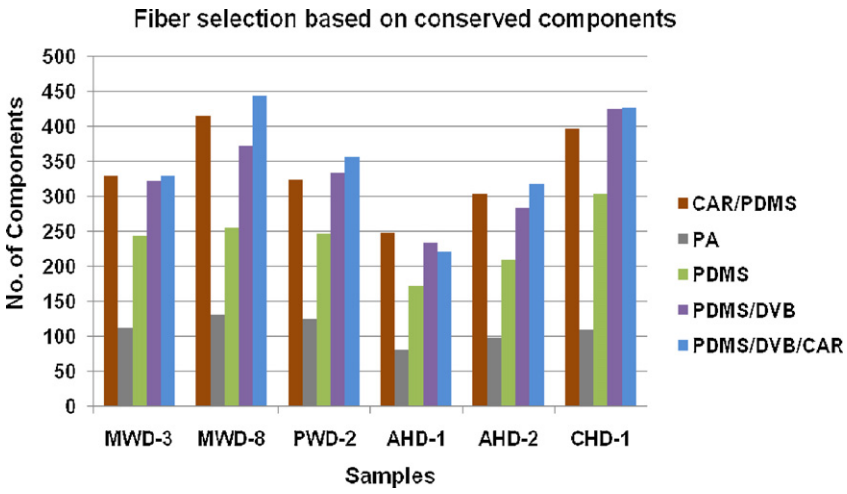

Fig. 1. Comparison of different fiber coatings and number of adsorbed components.

were as follows: source temperature 550 °C, curtain gas 10 psi (69 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 50 psi (345 kPa of nitrogen), ion source gas 2 (drying gas) 50 psi (345 kPa of nitrogen), ion-spray voltage −4000 V and +4000 V, respectively, collision gas (nitrogen) high. Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte, which yields 4.0 identification points according to commission decision 2002/657/EC [42]. In addition, the LC retention time and the intensity ratio of the two sMRM transitions have to agree with values of corresponding standards.

3.2.3. Secondary metabolite data evaluation

LC–MS/MS data evaluation was done using the Analyst® 1.5 (AB SCIEX 2008). Identification of positive target analytes in samples was confirmed by comparing retention time (RT) and ratios of qualifier to quantifier to authentic standards, measured before and after a particular sample batch of 30 samples.

4. Results and discussion

4.1. SPME and GC–MS

The sensitivity of the HS-SPME-GC–MS technique depends mainly on the distribution constant of analytes partitioned between sample and stationary phase of fiber (K_{fs}) [43]. To generate comprehensive profile of volatiles with variable volatility, selection of optimal fiber becomes a crucial factor in qualitative but mainly in quantitative analysis, where limits of detection are related to amount of adsorbed compound on phase covering the fiber. Among the tested fibers the most polar fiber coatings of polyacrylate (PA) as well as non polar polydimethylosiloxane (PDMS) were found not suitable for our profiling study. Fiber performances of CAR/PDMS, PDMS/DVB and PDMS/DVB/CAR were similar concerning the number of conserved components derived from Spectconnect (Figs. 1 and 2a–e). Hence annotation was considered as an additional criterion for the selection. Among the three semipolar fibers CAR.PDMS was found superior for the purpose with 30% more identification or annotation (Fig. 3). CAR.PDMS is often a fiber of choice in food industries for sensory aroma evaluation [44]. Better suitability of this fiber for profiling purpose is also reported for fungal volatile profiling [6]. Comparison of different extraction temperatures showed that use of 90 °C was suitable for evaluation of substances with wide range of volatilities. Choice of extraction temperature becomes critical, since use of higher temperatures though promoting detection of low volatile substance can be inappropriate as causing premature desorption of other more volatile analytes from the fiber coating which is not suitable for profiling studies like ours [43]. Other factors that need to be considered selecting extraction temperature are nature of matrix and stationary phase. Sample volume and pre-incubation time and temperature were also evaluated in order not to overlook low abundant or moderately volatile substances. Optimization for these parameters resulted in values of 0.05 g sample mass/vial and extraction at 90 °C for 30 min, respectively (Supplementary Tables 2 and 3). Increasing the sample volume did not show any significant increase in number of conserved components indicating either fiber saturation or limitation of the fiber in terms of its potential to adsorb different compounds. The reduction of empirical sample amount (0.025 g) reduced number of components by 25% possibly due to inadequate ion current/intensity of low abundant substances. In our method pre incubation and extraction temperatures are kept constant to minimize temperature ramping. We assume that this in addition to reducing ramping time is also beneficial achieving equilibrium of low and semi volatile compounds in the mixture. Application of a pair of columns with inverse polarities for identification was compared to earlier reports based on single column [6,11,45–47], found advantageous in case of stereo isomers o- and p-xylenes and constitutional isomers ψ-cumene, mesitylene, hemimellitene. The compounds which were barely resolved on apolar column were well resolved on polar Optima® Wax with RT and RI of 13.69 min and 12.31 min and 1186 and 1136, respectively, for o and p-xylenes. Similarly mesitylene, ψ-cumene, hemimellitene had RT of 14.7, 15.4 and 18.0 min and LTPRI 1223, 1247, 1346, respectively.

Total of 71 volatile organic compounds were detected (Table 2) on both columns of different polarity. 20 of the compounds have been reported as microbial volatile organic compounds produced by either individual or mixtures of microbes under laboratory conditions on different matrices [5,6,45,48–51]. Additionally 85 substances designated as “annotated” (Supplementary table 1) were detected on only one of the two columns HP5(89%)/Optima® Wax (11%) with exact LTPRI match or LTPRI within relative deviation of ±2% from literature or from a value determined by measuring a pure standard. Spectral match factor for both identified and annotated compounds were above or equal to 90. Assigning the origin of a compound might not be straight forward as many of the detected volatiles are produced by microbes and plants, as well as they were known to be integral part of many solvents that are commonly used indoors. The best observed examples for

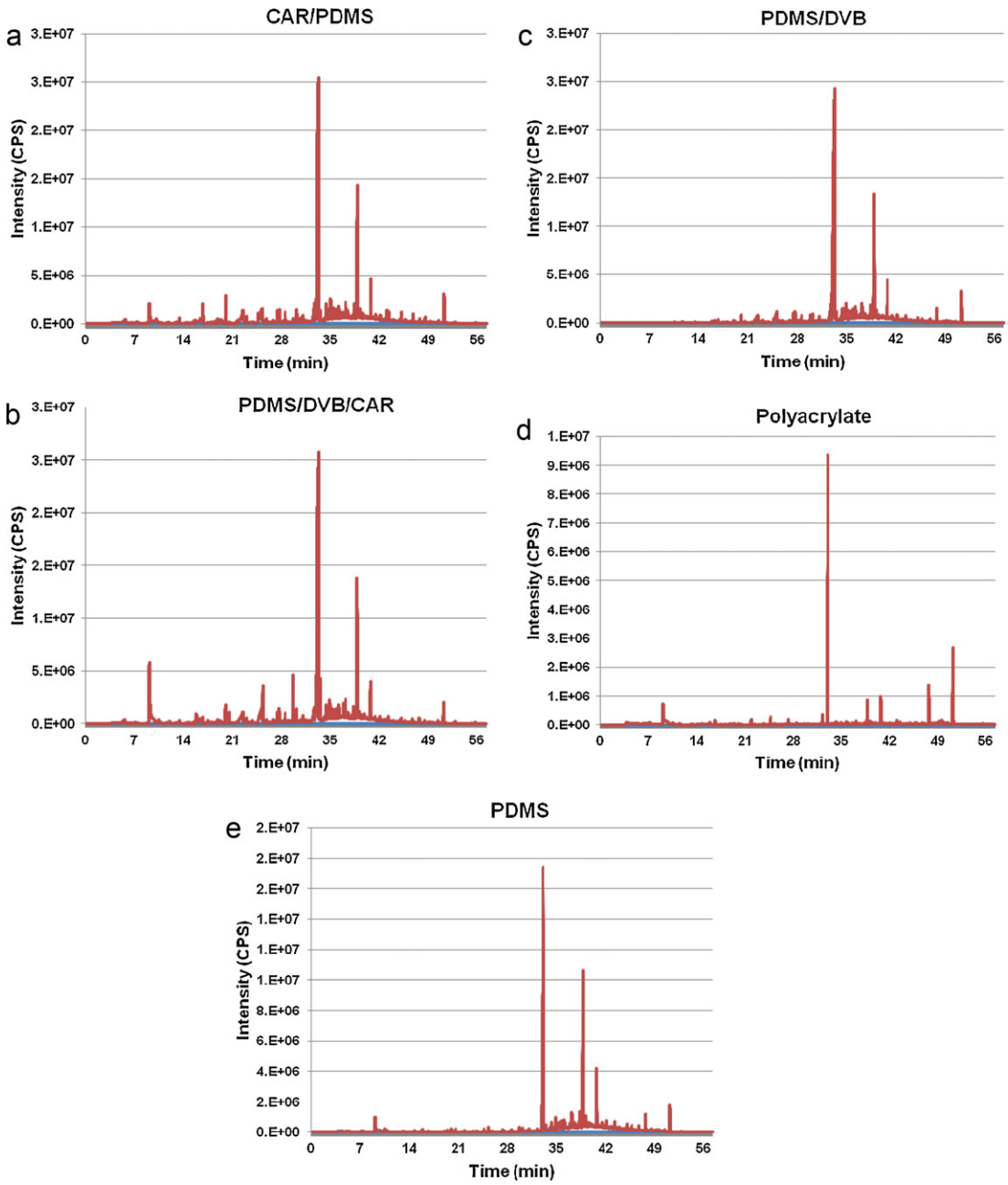

Fig. 2. Fiber selection-chromatographic profile of extracted volatile from sample AHD-1 on different fiber coatings.

this class of compounds were toluene, phenol, ethylbenzene, 1-butanol, limonene, styrene and α-pinene [51–53]. The compounds nonanal, toluene, butanoic acid, benzyl alcohol, phenol, 1-octanal, phthalic acid, and dibutyl phthalate were uniform in their occurrence across all four groups of samples. 1-Octanol and nonanal without any ambiguity could be related to microbial origin [51]. All other frequently found substances with exception of toluene and phenol could be traced back to either combustion by product of gasoline, adhesive or plasticizers [53]. Limonene is a constituent of many household consumer products such as deodorizers, polishes, fabric softeners, cigarettes and food beverages [52,54]. Limonene occurrence in samples derived exclusively from municipal waste management units and not in other can be an argument for its insignificant synthesis and release by microbes compared to non biological sources.

4.1.1. Statistical evaluation: GC–MS method performance and source identity recognition/apportionment based on volatile profile of samples

Multivariate statistics PCA and HCA were employed to identify probably existing differences and similarities in volatile profiles of different indoor environments. Our assumption for this was discrepancies in volatile profiles of different indoor environments also exist in dust samples (due to surface adsorption and particle accumulation), and arises as a result of dissimilar indoor purposes, climate and geographical location. For this purpose ISmatrix generated by Spectconnect was used. ISmatrix is a result output Microsoft Excel CSV file, consisting of complement peaks areas consistently detected in multiple chromatograms across sample groups or sample replicates. ISmatrix of the order 66*1993 representing conserved peak areas across 22 samples in replicates of

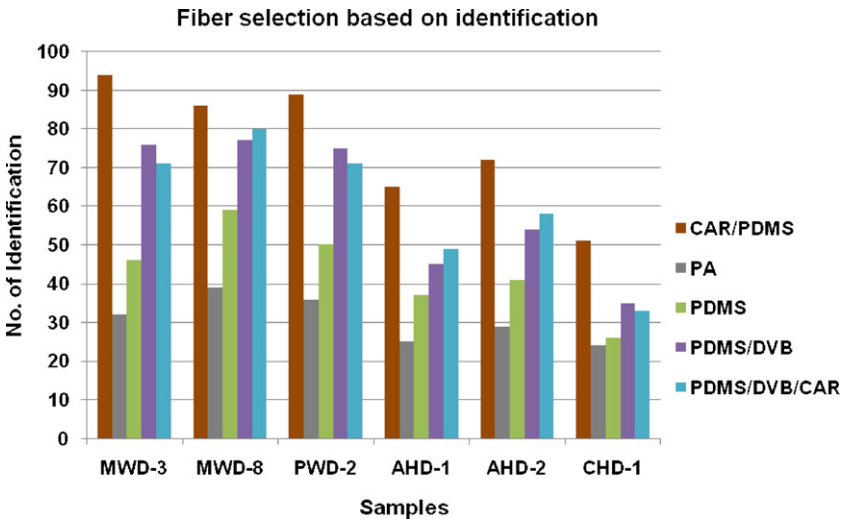

Fig. 3. Comparison of different fiber coatings and number of identified components.

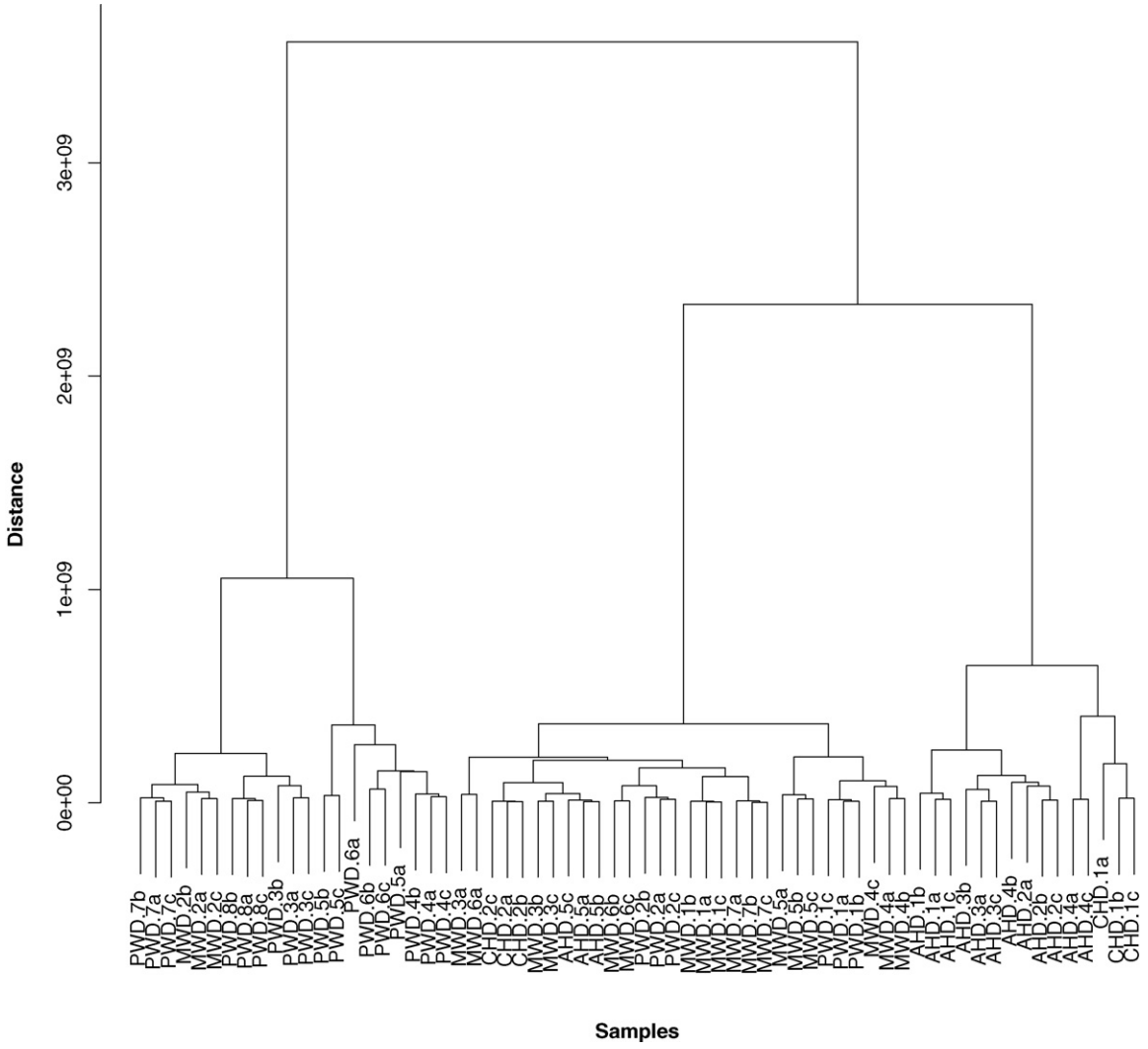

Fig. 4. Hierarchical clustering considering selected volatile components with loading score >0.1 with resultant matrix (66*7). Data clusters 1: PWD, Data clusters 2: AHD, CHD, MWD, PWD, Data clusters 3: AHD.

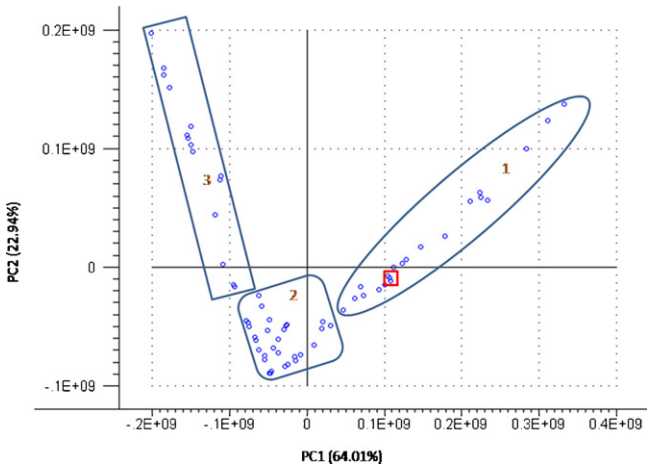

Fig. 5. PCA of selected volatile components with loading score >0.1 with resultant matrix (66*7). Data clusters 1: PWD, Data clusters 2: AHD, CHD, MWD, PWD, Data clusters 3: AHD.

three or at least conserved in triplicates of a single sample was used for hierarchical clustering to verify analytical aspects (sample homogeneity and component distribution) in addition to other mentioned purposes. Concerning homogeneity and component distribution 17 of the 22 samples showed good clustering among replicates. This resulted in two super-clusters with 2 sub clusters each (Supplementary Fig. S1). Replicates of individual house dust samples from distant geographical origins clustered along with the samples from municipal waste handling units forming one of the four major clusters consisting of 11 elements (Supplementary Fig. S1) with the exception one sample MWD-2. Four of the five Finnish house dust samples from moisture damaged houses (AHD 1–5) were equidistant and clustered together and were different to other groups in their volatile pattern. Contrary to anticipation samples from paper recycling units (exception of PWD 1–2) split into two sub clusters of four and three elements each, representing occurrence of probable intrinsic differences. This minor separation could be explained by the different waste handling methods and its influence on aerosols or deposited particles and ultimately leading to formation of dust with compositional irregularities. One or more sample replicates clustering sparsely or distant samples merging into a close knit cluster may not be amenable for an easy explanation (example Fig. 5, clustering of MWD-2 along with PWD samples highlighted with red square). Nevertheless clustering among the majority of sample replicates is good evidence for validity and applicability of our method and instrumental set up for indoor evaluation studies. The recommended procedure for PCA for differentiation of samples is to analyse all variables at the same time. But in cases where numbers of variable are higher than the number of cases this may not be feasible. The observation of loading scores from the matrix 66*1993 suggests that the majority of variables had typically low in the magnitude of <0.1 indicating insignificant influence on separation. Hence a cut off loading score of 0.1 was fixed for variable selection. The resulting new matrix (66*7) comprising both volatiles and reported microbial volatiles (two) accounted for 86.95% of the total variance in the data on PCA. The first principal component (PC1) explained

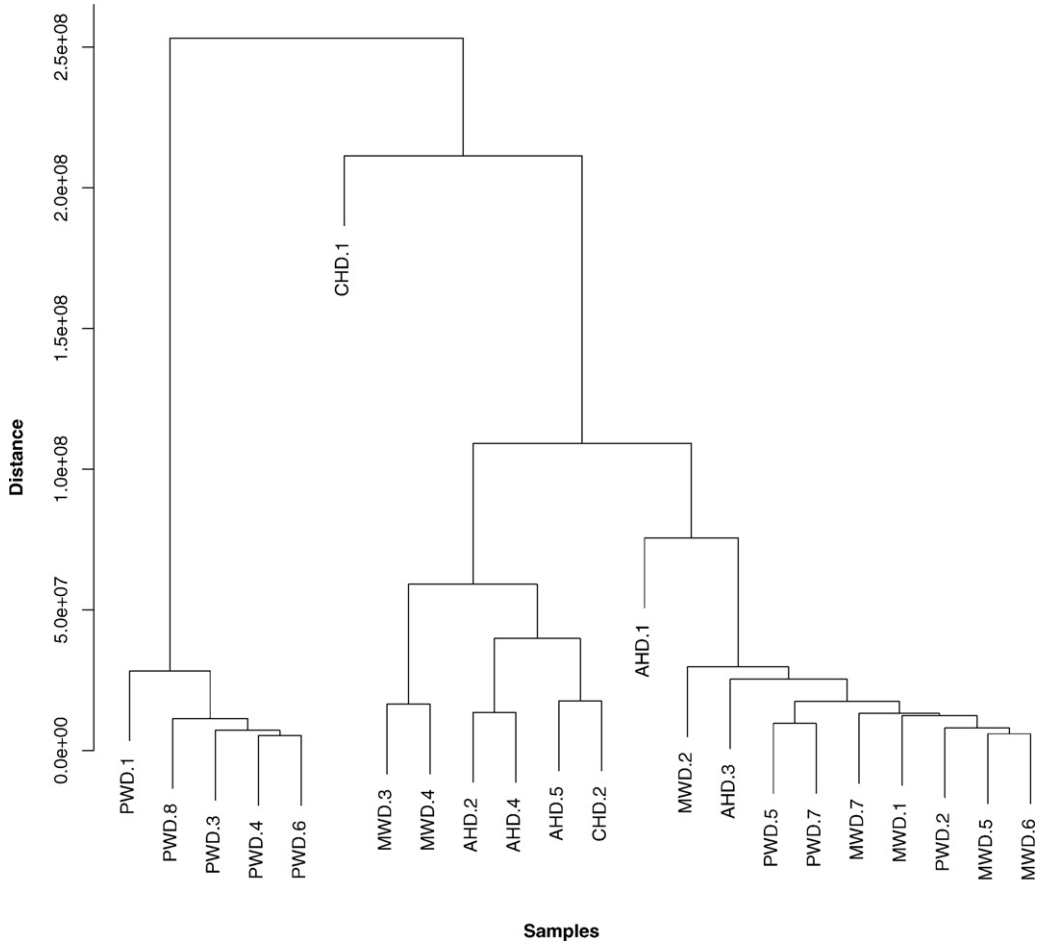

Fig. 6. Hierarchical clustering considering literature reported microbial volatile components with resultant matrix (22*18). Data cluster: 1: CHD1, Data cluster: 2: AHD, MWD, PWD, Data cluster: 3: AHD, MWD, CHD, Data cluster: 4: PWD.

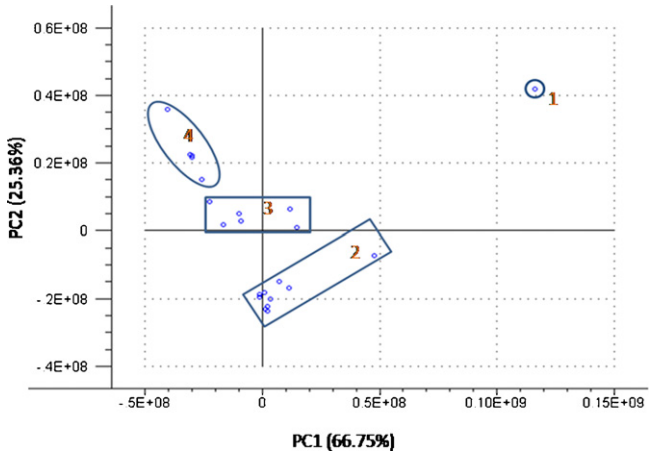

Fig. 7. PCA of selected microbial volatile components with loading score >0.1 with resultant matrix (22*18). Data cluster: 1: CHD1, Data cluster: 2: AHD, MWD, PWD, Data cluster: 3: AHD, MWD, CHD, Data cluster: 4: PWD.

64.01% of the variance separating samples and second principal component (PC2) with 22.94% of variance. The PC1 identified as linear combination of 2,6-diisopropylnaphtalene and 1-tetradecanol. The PC2 was mainly characterized by variables diethyl phthalate and minor influence by 1-butyl 2-isobutyl phthalate and Toluene. Scattered plot corresponding to this discrimination is illustrated in Fig. 5.The conclusion that can be drawn from the illustrated classification study is that 71% of affected house dust samples and 75% of waste management units dealing with paper were clearly separable on first principal component. Considering samples from a distinct cluster on negative PC1 and PC2 it can be concluded that overlap in volatile and semi volatile profiles are consistent with the statistical reports of the Parliament office of science and technology, England (statistics discussed at later part) for household contribution for municipal waste formation. Hierarchical clustering of the same matrix resulted in two major clusters each having two minor clusters (Figs. 4 and 5). Comparative evaluation of dendrograms showed grouping in Fig. 4 is similar to Supplementary Fig. S1, thus confirming the independent influence of chosen variables in separation for the group of samples. In parallel averaged replicates peak areas of identified MVOCs that are well documented in the scientific literature were considered for PCA. This was done to verify their probable role in separation of samples and differentiation of enclosures (22*18). The PCA of MVOCs indicated the extraction of two principal components representing total variance of 92.10% of the data set. PC1 represented 66.75% of variance and was strongly characterized by toluene, PC2 on the other hand accounted for 25.36% of variance due to nonanal. Due to ambiguity concerning the origin of toluene this compound may not be an ideal volatile marker for separation of samples and sources, based on microbial volatile profile. In this view nonanal underlying PC2 and explaining a variance of 25.36% could be an important microbial marker for separation of samples in the indoor environments. HCA of the same matrix (22*18) resulted in three super-clusters. Extreme right cluster (Fig. 6), where samples belonging to paper recycling units can be seen grouped together (PWD 1, 3, 4, 6, 8). This may explain similar purposes of indoor or occurrence of common abundant cellulose matrix promoting climate favorable for particular set microbes, in addition to influencing their physiology and volatile pattern. Similarly another main cluster consisting of house dust samples (AHD 2, 4, 5 and CHD 2) cluster at similar height which could be best reasoned as a consequence of comparable thermal and humidity comfort pattern practiced in homes leading to comparable/general micro climates in these indoor environments (Figs. 6 and 7).

4.2. Evidence for natural occurrence of microbial secondary metabolites in settled dust

The analysis of non volatile/secondary microbial metabolites in settled dust matrix was done using a validated method described earlier [7]. In cases of samples from waste management units containing paper and other matrices with absorptive consistency a larger sample to solvent ratio of 1:8 or 1:12 was used to ensure complete submersion of samples and optimum extraction. Dust samples were source of 38 different microbial metabolites to variable quantities. The concentrations of the investigated toxins in the positive samples are listed in Table 3. Their relative standard deviation between replicates was generally below 20% (e.g. 50% of samples), which we consider to be a reasonable value in view of the heterogeneity of the matrix (thus confirming the accuracy of the method). The microbial metabolite spectrum detected included following microbial taxa, *Penicillum* ($n=12$), *Aspergillus* ($n=5$), *Fusarium* ($n=7$), *Beauveria* ($n=1$),*Trichoderma* ($n=1$), *Claviceps* ($n=1$), *Alternaria* ($n=2$), *Stachybotrys* ($n=1$), *Metarrhizium* ($n=1$), *Chaetomium* ($n=1$) and Bacterial ($n=6$) [55]. A few of the metabolites could be attributed to more than one genus of indoor fungi and bacteria.

Samples from municipal and paper recycling waste management units were similar in terms of microbe-metabolite pattern and quantities. A post note released by the Parliament office of science and technology, England (http://www.parliament.uk/documents/post/postpn252.pdf) provides some insight concerning organic content of municipal waste and offers an additional indirect explanation for high prevalence of saprophytic/parasitic microbes in dust samples procured from municipal waste dealing units. The above mentioned report states that, the bulk of municipal waste generation is contributed by the households and consists of biodegradable material (41%, kitchen, garden, soil), biodegradable & recyclable material (18%, paper, cardboard), recyclable waste (17%, glass, plastic, metal) and other materials (20%, wood, non-combustibles, textiles). Municipal waste with complex organic content thus could be a potential matrix nurturing diverse set of fungi and bacteria. Each group of enclosures was unique having set of metabolites not found in the other sampling sites. The samples of WMUs dealing with municipal waste were distinct from samples of WMUs dealing with paper by the presence of griseofulvin, dechlorogriseofulvin, chlamydeosporal, malformin C, myriocin, patulin and puromycin. Presence of griseofulvin and its halogenated derivative, dechlorogirsofulvin in one and the same municipal waste management sample is confirmation for mutual occurrence addition to proving the greater relevance of our chosen indoor target metabolites in the course of method expansion [56,57]. Patulin, a mycotoxin on decomposing apples and a metabolite of species of *Pencillium* and *Aspergillus* is not frequently found in indoor dust makes its presence interesting. Presence of malformin C could indicate occurrence of the indoor mold. *Aspergillus niger* [58] known for causing skin diseases and ear infection [59]. Similarly samples from paper recycling units were different to those of municipal waste management units in their microbe-metabolite pattern due to the presence of deoxybrevinamide E and cytochalsin D. Cytochalasin D is a metabolite produced by filamentous saprophobic ascomycetes of the genus *Chaetomium* found in soil, air and plant debris [60]. Several species of *Chaetomium* are common in indoor environments such as *C. elatum*, *C. globosum*, *C. murorum* [61]. Similarities in the metabolite patterns of the two waste handling units might be a result of similarities in the fungal spectra present in such work places due to similar micro-climatic conditions, substrates and the hygiene principles applied.

As apprehended settled dust samples from inhabited houses showed lower metabolite diversity ($n=18$) compared to other groups. Metabolites enniatin B2 and alternariol were exclusively

Table 3
Non volatile/secondary microbial metabolites detected in settled floor dust samples from Groups A, B, C and D. Quantification values presented are average of replicates.

Metabolite	Precursor ion	Municipal waste (Gp A) management (μg/kg)	Household paper (Gp B) recycling (μg/kg)	Settled dust from (Gp C) house (μg/kg)	Settled dust from (Gp D) house (μg/kg)
3-Methylviridicatin	281.07$[M+H]^+$	5.8–10.0	8.5–9.0	–	–
Alamethicin F30	775.5 $[y7^d+H]^+$	4.0–35.0	14.0–15.0	–	–
Alternariol	257.0 $[M-H]^-$	–	–	39.7	34.6–41.0
AME	271.1 $[M-H]^-$	11.2–42.0	17.3–37.0	8.0	7.3–10.0
Apicidin	622.4 $[M-H]^-$	1.1–1.4	0.5–0.8	–	–
Beauvericin	801.5 $[M+NH_4]^+$806.5 $[M+Na]^+$	1.6–22.0	0.3–10.4	3.1	0.7–1.8
Chaetoglobosin A	695.0 $[M-H]^-$	193.0–258.0	83.0–242.0	–	–
Chanoclavine	257.1$[M+H]^+$	1.9–2.8	0.6–5.9	–	–
Chlamydosporol	245.2$[M+H]^+$	16.0–59.0	–	–	–
Chloramphenicol	320.9 $[M-H]^-$	39.5–108.3	2.7–22.6	–	3.6–4.6
Cyclopenin	295.1$[M+H]^+$	32.0–188.0	26.0–303.0	–	–
Cyclopeptine	281.07$[M+H]^+$	7.8–50.0	3.0–46.0		–
Cycloaspeptide A	642.3 $[M+H]^+$	9.6–29.0	10.8–11.7	155.4	–
Cytochalasin D	609.3 $[M+2H]^{2+}$	–	51.0–221.0	–	57.1
Dechlorogriseofulvin	319.1$[M+H]^+$	230.5	–	13.0	–
Deoxybrevianamid E	352.2$[M+H]^+$	–	221.3	–	–
Emodin	269.0 $[M-H]^-$	61.0–314.0	26.0–88.0	15.15	4.0–117.0
Enniatin A	699.4 $[M+NH_4]^+$	7.4–17.0	3.0–17.0	–	0.7–223.0
Enniatin A1	685.4 $[M+NH_4]^+$	2.0–30.0	1.3–14	–	1.4–185.0
Enniatin B	657.5 $[M+NH_4]^+$	2.2–23.0	0.2–8.1	–	0.9–10.3
Enniatin B1	671.4 $[M+NH_4]^+$	4.0–49.0	1.3–19.3	–	–
Enniatin B2	643.5 $[M+NH_4]^+$	–	–	1.9	0.6–2.0
Equisetin	372.2 $[M-H]^-$	21.2–422.4	12.5–185.3	–	19.0–20.0
Fumigaclavine	299.3 $[M+H]^+$	13.0–86.0	10.0–23.0	–	–
Griseofulvin	353.2 $[M+H]^+$	87.0–1598.0	–	210.0	–
Malformin C	530.3$[M+H]^+$	8.0–78.0	–	–	–
Meleagrin	434.3 $[M+H]^+$	14.0–52.0	29.0–67.0	–	–
Monactin	768.8 $[M+NH_4]^+$	4.0–101.0	0.4–2.3	–	0.65
Myriocin	402.4 $[M+H]^+$	52.0–1941.0	–	–	–
Nonactin	754.6 $[M+NH_4]^+$	0.2–50.0	0.5–1.2	0.8	0.2–0.3
Patulin	152.9 $[M-H]^-$	49144.0	–	–	–
Pentoxyfylline	279.2 $[M+H]^+$	11.0–186.0	2.0–101.0	–	–
Physcion	283.0 $[M-H]^-$	409.0–1034.0	231.0–1565.0	–	–
Puromycin	472.4 $[M+H]^+$	40.0–126.0	–	–	–
Roquefortine C	390.2 $[M+H]^+$	83.0–176.4	18.0–350.0	–	–
Stachybotrylactam	386.3 $[M+H]^+$	52.6–104.5	87.0–160.0	–	–
Sterigmatocystine	325.1 $[M+H]^+$	3.0–45.3	6.0–32.0	–	1.6–11.0
Valinomycin	1128.8 $[M+NH_4]^+$	0.05–8.0	0.3–2.0	0.4	0.04–0.6
Viridicatin	238.1 $[M+H]^+$	110.0–920.0	108.0–369.0	–	–

detected in settled floor dust (SFD) samples of control and houses with severe moisture damage (Table 3) along with some other *Fusarium* metabolites. However control and damaged houses had certain metabolite signature pattern. The control houses differed by the presence of metabolites griseofulvin, dechlorogriseofulvin and cyclosapeptide A. The moisture damaged houses were positive for the presence of alternariol monomethyl ether (AME) and monactin which are metabolites of tertiary colonizers *Alternaria alternata* and *Actinomycetes* [14] indicating for water damage. The presence of bacterial metabolites produced by *Streptomyces* and related genera in the damaged houses clearly links to conditions of excess moisture damage and dampness indoors, as this groups of bacteria has been described earlier as being indicative for such indoor conditions [62].

5. Conclusion

The detection and quantification of wider range of metabolites in concentration range of 0.04–49,144.0 μg/kg are evidences for the competency of our developed multi target LC–MS/MS method for the purpose. Occurrence of broader array of metabolites (primary, secondary and tertiary colonizing microbes) in samples of waste handling units (municipal waste (>50%) and paper recycling units (>33%)) is affirmation for microbial succession. Metabolites of *S. chartarum,* which is well known to be indoor specific fungi was found in waste management units in high concentrations (52–160 μg/kg). In the absence of moisture monitoring data of evaluated environments, origin (indoor or outdoor) of stachybotrylactam remains to be an open question. The presence of emodin, griseofulvin and dechlorogriseofulvin along with metabolites of tertiary colonizer are not unexpected findings, considering processes involved in waste handling (collection, transportation, storage) during which moisture accumulation is an obvious possibility. To the best of our knowledge 30 of the 71 identified volatiles are new report in the indoor context, particularly in settled floor dust matrix. Though as many as 20 previously reported MVOCs were found in our study, a direct correlation to secondary metabolite profiles could not be possible due to the known ambiguity in MVOC and the non-availability of authentic environmental control samples and volatile profiles corresponding to them. The applications of AMDIS and Spectconnect for volatile profiling are advantageous identifying major indoor pollutants clouded amidst extremely high number of variables/components. The wide range biogenic and anthropogenic pollutants in dust qualify it as an indicator matrix of indoor status, hence can be valuable for evaluation purposes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.07.043.

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Supplementary data:

CAS.No	Name	m/z(precursor ion)	RT-HP-5MS (min)	RI-HP5-MS Literature	RI-HP5 Experimental
64-19-7	Acetic acid	60.05	6.02	600.0	643.0
3102-33-8	3-Penten-2-one, (E)-	84.11	8.34	735.0	743.9
498-60-2	3-Furaldehyde	96.08	11.16	815.0	838.2
98-01-1	2-Furaldehyde	96.08	11.18	836.0	838.6
98-00-0	2-Furanmethanol	98.09	12.01	853.0	863.9
80-62-6	2-Methyl-2-propenoic acid methyl ester	100.11	7.50	732.0	714.1
100-52-7	Benzaldehyde	106.12	15.51	961.0	970.3
18829-55-5	2-Heptenal, (E)-	112.16	15.22	956.0	961.5
142-62-1	Hexanoic acid	116.15	17.61	981.0	1034.3
111-70-6	1-Heptanol	116.20	15.59	969.0	972.7
122-78-1	Benzeneacetaldehyde	120.14	18.14	1042.0	1050.8
529-20-4	2-methyl- Benzaldehyde	120.14	19.45	1067.0	1091.0
98-86-2	Acetophenone	120.14	18.85	1066.0	1072.5
65-85-0	Benzoic acid	122.12	22.49	1214.4*	1192.7
60-12-8	Benzyl Carbinol	122.16	20.32	1120.3*	1119.4
91-20-3	Naphthalene	128.17	22.62	1183.0	1197.1
111-13-7	2-Octanone	128.21	16.24	992.0	992.3
646-14-0	1-nitro- hexane	131.17	18.04	1046.5	1047.8
122-00-9	p-Acetyltoluene	134.17	22.50	1189.0	1193.1
585-74-0	1-(3-methylphenyl)- ethanone	134.17	22.50	1176.0	1193.1
488-23-3	1,2,3,4-tetramethyl- Benzene	134.21	20.46	1150.0	1124.3
95-93-2	1,2,4,5-tetramethyl- benzene	134.21	20.42	1131.0	1122.8
272-16-2	1,2-Benzisothiazole	135.18	23.81	1221.0	1239.8
95-16-9	Benzothiazole	135.18	23.81	1228.0	1239.9
5989-27-5	D-Limonene	136.23	17.61	1027.0	1034.3
122-99-6	2-Phenoxyethanol	138.16	23.43	1245.3	1226.1
5910-87-2	2,4-Nonadienal, (E,E)-	138.20	23.26	1219.0	1220.1
6750-03-4	2,4-Nonadienal	138.20	23.26	1219.0	1220.1
60784-31-8	2-Nonenal, (Z)-	140.22	21.59	1147.0	1162.3
91-57-6	Naphthalene, 2-methyl-	142.19	25.76	1290.5	1310.6
124-07-2	Octanoic Acid	144.21	21.95	1177.3*	1174.5
149-57-5	2-ethyl- Hexanoic acid	144.21	20.13	1116.7	1113.0
143-08-8	Nonan-1-ol	144.25	21.85	1173.4*	1171.3
106-46-7	p-Dichlorobenzene	147.00	17.24	1013.0	1023.0
541-73-1	1,3-dichloro- Benzene	147.00	17.24	1005.0	1022.9
104-46-1	Anethole	148.20	25.31	1289.3	1293.7
1197-01-9	p-Cymen-α-ol	150.21	22.28	1182.0	1185.8
2363-88-4	2,4-Decadienal	152.23	25.45	1317.0	1298.7
92-52-4	Phenylbenzene	154.20	27.94	1377.3	1393.4
2497-25-8	2-Decenal, (Z)-	154.24	24.53	1250.0	1265.5
571-61-9	1,5-Dimethylnaphthalene	156.22	29.00	1439.8	1435.4
575-37-1	1,7-dimethyl- Naphthalene	156.22	29.00	1418.7	1435.4
575-43-9	1,6-dimethyl- naphthalene	156.22	29.00	1419.6	1435.3
581-42-0	2,6-dimethyl- naphthalene	156.22	28.69	1401.0	1423.1
582-16-1	2,7-dimethyl- naphthalene	156.22	28.69	1402.2	1423.1
112-32-3	n-Octyl formate	158.23	20.56	1117.0	1127.7
112-30-1	1-Decanol	158.28	24.74	1272.0	1273.2
643-58-3	2-Methylbiphenyl	168.23	28.33	1402.3	1408.7
643-93-6	3-Methylbiphenyl	168.23	30.58	1487.8	1499.0

90-43-7	o-Xenol(fungicide)	170.20	31.35	1506.0	1531.6
112-12-9	2-Undecanone	170.29	25.30	1295.1*	1293.3
112-42-5	1-Undecanol	172.30	27.90	1370.0	1391.8
120-12-7	Anthracene	178.22	37.56	1786.4	1811.8
136-60-7	n-Butyl benzoate	178.22	27.63	1377.0	1381.4
85-01-8	Phenanthrene	178.22	37.56	1780.0	1811.7
78-40-0	Triethyl phosphate	182.15	20.32	1137.2	1119.5
119-61-9	Benzophenone	182.21	34.02	1621.0	1647.3
3796-70-1	trans-Geranylacetone	194.31	29.36	1454.6	1450.1
295-17-0	Cyclotetradecane	196.37	34.74	1673.0	1679.7
30021-74-0	1-Isopropyl-7-methyl-4-methylene-1,2,3,4,4a,5,6,8a-octahydronaphthalene	204.35	30.82	1477.0	1509.1
13360-61-7	1-Pentadecene	210.39	30.41	1492.5	1492.2
24157-81-1	2,6-Diisopropylnaphthalene	212.33	35.91	1728.0	1733.7
124-25-4	Tetradecanal	212.37	33.32	1611.0	1616.1
112-72-1	1-Tetradecanol	214.38	34.73	1672.0	1679.5
84-66-2	Diethyl phthalate	222.23	32.80	1597.0	1593.4
295-65-8	Cyclohexadecane	224.42	38.99	1883.0	1882.8
544-63-8	Tetradecanoic acid	228.37	36.62	1780.0	1767.2
84-15-1	o-Terphenyl	230.30	39.37	1903.0	1901.3
64437-47-4	9-hexadecenol (E)	240.42	39.00	1868.0	1883.3
18435-45-5	1-Nonadecene	266.50	39.01	1892.0	1883.5
17851-53-5	1-Butyl 2-isobutyl phthalate	278.34	38.66	1900.0	1866.3
117-82-8	2-Methoxyethyl phthalate	282.28	40.52	1965.0	1961.0
638-36-8	2,6,10,14-tetramethyl-Hexadecane	282.54	37.45	1810.7	1806.6

CAS.No	Name	m/z (precursor ion)	RT-Optima® Wax **(min)**	RI-Optima® Wax Literature	RI-Optima® Wax Experimental
4170-30-3	Crotonaldehyde	70.08	9.95	1408.0	1046.4
123-72-8	Butanal	72.10	6.28	877.0	877.0
534-22-5	2-methyl- Furan	82.10	6.14	871.0	867.3
1003-29-8	α-Pyrrolaldehyde	95.09	33.91	2044.0	2055.5
142-83-6	2,4-Hexadienal, (E,E)-	96.12	20.01	1423.6*	1423.1
3658-80-8	Dimethyl trisulfide	126.26	18.81	1378.0	1376.8
629-33-4	n-Hexyl formate	130.18	18.61	1382.0	1369.0
104-61-0	γ-Nonalactone	156.22	34.19	2028.0	2069.1
112-34-5	O-Butyl diethylene glycol	162.22	29.36	1796.0	1829.0
1454-84-8	n-Nonadecanol-1	284.52	43.28	2637.0	2607.8

Supplementary Table **(1); List of annotated volatile organic compounds found in settled floor dusts from Groups A, B , C and D, ***

Retention index(RI) values determined by us with pure reference standards. Listed in the order of precursor ion mass.

	CAR.PDMS		Polyacrylate		PDMS		PDMS.DVB		PDMS.DVB.CAR	
Samples	**Conserved**	**Identified**	**Conserved**	**Identified**	**Conserved**	**Identified**	**Conserved**	**Identified**	**Conserved**	**Identified**
MWD-3	329	94	112	32	244	46	322	76	329	71
MWD-6	415	86	131	39	255	59	372	77	443	80
PWD-2	323	89	126	36	247	50	334	75	356	71
AHD-1	248	65	81	25	173	37	233	45	221	49
AHD-2	303	72	98	29	210	41	284	54	318	58
CHD-1	396	51	110	24	303	26	425	35	426	33

Supplementary Table **(2); Fiber selection for SPME :All samples used for the purpose are naturally contaminated real world samples•**

AHD-1 sample extraction and chromatography with CAR.PDMS and apolar HP5 column								
Sample amount (grams)			**Extraction/incubation temperature**			**Extraction/incubation time**		
	Spectconnect	**AMDIS**		**Spectconnect**	**AMDIS**		**Spectconnect**	**AMDIS**
0.025	132	34	**30'60'30°C**	40	6	**0'30'90°C**	145	39
0.05	248	65	**30'60'60°C**	76	19	**0'60'90°C**	170	44
0.1	171	49	**30'60'90°C**	248	65	**30'30'90°C**	152	45
						30'60'90°C	248	65

Supplementary Table **(3); HS-SPME: The values shown above are averages of triplicates. Spectconnect (conserved components) and AMDIS (Identification).**

Occurrence of toxic bacterial and fungal metabolites on mold damaged building materials

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SUMMARY
This exploratory study on a small set of mold damaged building material samples was performed based on the hypothesis that various different microbial toxins may be present in indoor environments, following the observed diverse microbial growth in situations of dampness and moisture damage. We detected up to eight different fungal and – for the first time – also bacterial toxins in single building material samples. In the majority of cases, fungal and bacterial genera being potential producers of the detected toxins were identified in the samples. The findings of our study raise attention towards potentially occurring synergistic interactions between different microbial metabolites, spores and cell fragments upon co-exposure.

KEYWORDS
indoor environment; microbial toxins; mycotoxins; mold damage; building materials

INTRODUCTION
Indoor environmental conditions characterized by dampness, moisture damage and mold are recognized risk factors for a number of short-term and long-term health effects. While the association between moisture and microbial growth with adverse effects on human health is well documented (e.g. Bornehag et al. 2001; IOM, 2004), the nature and mechanisms of causative agents remain yet obscure. It is likely that observed adverse health outcomes are a consequence of synergistic interactions of different compounds.
Microbial toxins produced during excessive microbial growth may play a role in the complex picture of multiple exposures in damp buildings. We base this statement on the following documentation: i) fungal and bacterial strains that produce toxic secondary metabolites are present in moisture damaged indoor environments (e.g. Andersson et al. 2009; Engelhart et al. 2002; Fogle et al. 2007); ii) production of fungal toxins on building materials has been shown (e.g. Nielsen et al. 2003); iii) microbial spores and cell-fragments do get airborne and may contain microbial toxins (Brasel et al. 2005a); subsequently, residents are exposed to a variety of microbial compounds through indoor air (Brasel et al. 2005b; Charpin-Kadouch et al. 2006; Gottschalk et al. 2008) iv) synergistic interaction in simultaneous exposure of bacterial and fungal spores and their metabolites has been shown (e.g. Huttunen et al. 2004).
Data on the natural occurrence of toxic microbial metabolites in indoor environments is limited. A small number of mycotoxins have been addressed by dedicated methods based on gas or liquid chromatography coupled mass spectrometry (GC-MS and LC-MS; Bloom et al. 2007a,b). Other LC-MS based methods for fungal metabolite screening have been restricted

to the analysis of isolated fungal or artificially inoculated building materials, due to limitations in selectivity and sensitivity. Improvements in the latest generation of mass spectrometers, however, have made the simultaneous analysis of a large number of target compounds in complex samples feasible.
The hypothesis underlying this exploratory study was that mold-damaged building materials may be contaminated with a number of different microbial metabolites. The objective was to perform GC- and LC-MSMS analysis on set of severely damaged building materials for a large number of fungal and bacterial metabolites. A subset of samples was also subjected to cultivation to identify potential producers of the detected metabolites.

METHODS

Building material samples. Samples of building materials were derived from the kitchen area of a penthouse in Southern Finland. The residents of the home reported severe health complaints and sent nine building material samples to National Institute for Health and Welfare for microbial analyses. The samples included mostly wooden materials (ID 4105, 4106, 4108, 4109, 4113), paper-covered gypsum or building board (4107, 4110, 4111) and one cardboard sample (4112).
Cultivation and microbial identification. Cultivation and microbial identification was performed from 4 of the mold damaged building material samples (4107, 4108, 4110, 4113). Samples were treated as described previously by Hyvärinen et al (2002). Aliquots were plated on 2% malt agar extract (MEA) and dichloran glycerol agar (DG18) for fungi, and on tryptone yeast extract glucose agar (TYG) for bacteria, and colony forming units (CFUs) were calculated per gramme fresh weight. Morphological identification of fungi (mostly on genus level) and of actinobacteria-type colonies was done using an optical microscope.
Analytical methodology. Nine building material samples were extracted and subsequently analyzed without clean-up according to an LC-MSMS method that had been initially developed for analysis of 87 mycotoxins in food (Sulyok et al. 2007) and has been extended by another 90 fungal and bacterial metabolites (external calibration using a liquid multi-analyte standard; positive identification by monitoring two specific fragmentation reactions per analyte). Material samples were additionally analyzed for verrucarol, trichodermol and sterigmatocystin using the methods described by Bloom et al. (2007a,b).

RESULTS

Table 1. Counts of total viable fungi, identified fungal groups and actinobacteria-type colonies in four material samples.

	CFUs/g building material sample			
	4107	**4108**	**4110**	**4113**
Fungi				
Total fungal count	$2{,}6 \times 10^6$	$1{,}9 \times 10^6$	$3{,}3 \times 10^6$	$3{,}7 \times 10^5$
Acremonium	$5{,}0 \times 10^4$	n.d.	n.d.	n.d.
Aspergillus	n.d.	n.d.	$3{,}0 \times 10^5$	$4{,}5 \times 10^3$
A. versicolor	n.d.	$2{,}5 \times 10^5$	n.d.	$1{,}8 \times 10^5$
*Eurotium**	n.d.	$2{,}3 \times 10^4$	$5{,}0 \times 10^4$	n.d.
Penicillium	$1{,}5 \times 10^6$	$1{,}5 \times 10^4$	$4{,}0 \times 10^5$	$4{,}1 \times 10^4$
Stachybotrys	$3{,}5 \times 10^5$	$1{,}3 \times 10^6$	$1{,}6 \times 10^6$	n.d.
Ulocladium	n.d.	n.d.	n.d.	$9{,}1 \times 10^3$
yeasts	n.d.	$5{,}0 \times 10^4$	$1{,}5 \times 10^5$	n.d.
sterile (non-sporulating) CFUs	$7{,}5 \times 10^5$	$2{,}5 \times 10^5$	$8{,}5 \times 10^5$	$1{,}4 \times 10^5$
Bacteria				
actinobacteria-type colonies	$5{,}8 \times 10^5$	$2{,}2 \times 10^5$	$3{,}0 \times 10^5$	$7{,}0 \times 10^4$

CFUs/g of viable fungi were generally determined on MEA; *colony counts of ***Eurotium*** were determined on DG18 agar; CFUs/g of viable bacteria were determined on TYG; n.d., not detected

Table 2. Occurrence of fungal and bacterial metabolites on building materials and their potential producing fungal and bacterial genera.

	Detected microbial metabolites	Potential producers* (genera)	Detection of microbial metabolites in the building material samples								
			4105	4106	4107	4108	4109	4110	4111	4112	4113
Fungal	Chaetoglobosin A	*Chaetomium*	+	+	+	+		+	+	+	+
	Emodin	*Penicillium, Aspergillus*	+	+		+		+			+
	Meleagrin	*Penicillium*	+	+	+	+	+	+	+	+	+
	Roquefortine C	*Penicillium*			+				+	+	
	Stachybotrylactam	*Stachybotrys*			+			+		+	+
	Sterigmatocystin	*Aspergillus*	+	+		+	+			+	+
	Trichodermol	*Stachybotrys*								+	
Bac-terial	Monactin	*Streptomyces, Actinomadura*					+	+		+	
	Valinomycin	*Streptomyces*		+		+	+	+		+	+

*according to AntiBase 2007 (The Natural Compound Identifier software)

DISCUSSION

Our paper describes the parallel detection of up to eight different fungal and bacterial toxins in single building material samples. These results clearly indicate the need to introduce comprehensive analytical approaches – as applied here – capable of simultaneous monitoring of a large array of microbial metabolites produced on-site. Already from a small set of investigated samples derived from one location in one home a whole variety of different microbial toxins was identified (see Table 2).

We are not aware of any previous reports on the detection of bacterial toxins directly from building material samples. Valinomycin and/or monactin were found in six of the nine samples, which indicates that bacterial toxins may frequently co-occur along with other microbial compounds. This finding deserves particular attention, since synergistic interactions in simultaneous exposure to spores and metabolites of fungi and bacteria have been shown in several studies (e.g. Huttunen et al. 2004).

We found good agreement in the majority of cases between the identified microbial toxins and potentially producing organisms. The detection of *Penicillium* spp. and *Aspergillus* spp. matches the findings of emodin, meleagrin and roquefortin C. Sterigmatocystin and its most common producer, *A. versicolor*, were detected in the same samples. Actinobacteria-type colonies were present in all cases and could explain the findings of valinomycin and monactin. Co-detection of stachybotrylactam and the producing *Stachybotrys* spp. was not consistent. *Chaetomium* spp. were not identified even though chaetoglobosin A was present. Limitations in culturing (not all fungal spores present are cultivable) and identification (non-sporulating strains) may explain the lack in agreement of these findings. Fogle et al. (2007) reported recently that *Chaetomium globosum* did not sporulate on ME agar. Hence, the non-sporulating CFUs in our samples may have accounted for *Chaetomium* spp..

CONCLUSIONS

This exploratory study reports the simultaneous detection of up to eight different fungal and - for the first time - also bacterial toxins in material samples from a water damaged home. The plausible presence of several microbial toxins in 'moldy homes' raises attention and urges for more research to explore synergistic interactions between different metabolites, spores and cell fragments. The necessity of applying analytical methods that are capable of simultaneous

detection of a variety of fungal and bacterial toxins directly from indoor samples is evident. Results on the presence of fungal/bacterial strains alone cannot predict the presence of toxins, as their production may vary between distinct growth situations. Both, toxin analyses and information on the present fungal and bacterial strains, are required to present a complete picture of the potential exposures in moisture damaged indoor environments.

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Microbial toxins in surface swab and dust samples from school buildings in three European countries.

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Keywords: microbes, secondary metabolites, school study, exposure

1 Introduction

Indoor environmental conditions characterized by dampness, moisture damage and microbial growth are associated with a number of adverse health outcomes (WHO Regional Office in Europe, 2009). Nature and mechanisms of the causative agents remain yet obscure. Secondary metabolites of microbes, often referred to as microbial toxins, are part of a complex exposure situation in damp buildings and are suggested to contribute to the adverse health outcomes observed in residents. Microbial metabolites exhibit a wide range of biological activities and include numerous pharmacologically active and in some cases highly toxic compounds.

Previous research has led to the conclusion that toxin producing fungi and bacteria are part of the microbial flora in moisture damaged indoor environments. Toxin production has been shown on building materials (Nielsen, 2003). In recent years, several studies have reported the presence of mycotoxins (i.e. fungal toxins) in indoor dust and indoor air, suggesting exposure through inhalation of spores and cellular fragments. However, information on the presence and prevalence of microbial toxins in indoor environments is limited, mostly due to the fact that only few target compounds have been considered so far.

HITEA (Health Effects of Indoor Pollutants: Integrating microbial, toxicological and epidemiological approaches) is an EU funded multicenter study aiming at clarifying the health impacts of indoor exposures on children and adults. One major part of HITEA refers to the school environment. The prevalence of moisture damage and dampness in schools in three European countries is surveyed, and respiratory health in pupils and teachers is investigated in relation to IAQ factors and specific microbial exposures in the schools.

As part of the HITEA project, the aim of the present study was to analyze indoor settled dust and surface swab samples collected in water-damaged and reference school buildings for a wide range of toxic fungal and bacterial metabolites by using mass-spectrometry based analytical methodologies.

2 Materials/Methods

Sample materials were collected from 95 school buildings in Finland, The Netherlands and Spain, representing three climatic regions in Europe. Building investigations were performed by trained field workers following a common protocol and using standardized checklists and surface moisture recorders. School buildings were categorized as either 'index' or 'reference' or 'non-categorized' buildings based on the extent, severity, and location of observations on moisture damage, dampness and visible mold. Dust settled above floor level (SDS) and moldy spot surface swab (MSS) samples were collected from different locations in the school buildings (i.e. classrooms, hallways, etc.). Sample

materials were analyzed for the presence of a wide variety of microbial secondary metabolites (161 fungal and 28 bacterial compounds) using mass-spectrometry based methods recently published by Vishwanath et al. (2009) and Bloom et al. (2007 a,b).

3 Results and Discussion

In total, 675 SDS and 66 MSS samples were collected from different locations in 49 index, 37 reference and 9 non-categorized school buildings situated in Spain, The Netherlands and Finland. 42%, 58% and 44% of all samples collected in Spanish, Dutch and Finnish schools, respectively, were positive for at least one of the metabolites analyzed. The toxin profiles varied between countries. Prevalence of single compounds in the samples was rather low, typically not exceeding 10%. Exceptions here were emodin and enniatin B, which were detected frequently in schools in all three countries, independent of building status. Enniatins are commonly detected in *Fusarium* mold infested grains; thus, transfer of contaminated grain dust from outdoors to indoors may explain the relatively high prevalence of low levels detected in index as well as reference buildings. For emodin and some of the other metabolites detected, however, source allocation is less straight forward, as both production by indoor molds as well as outdoor origin are feasible.

In general, we found a greater variety of different metabolites in the index buildings than in the reference school buildings; this tendency was least pronounced in the Dutch schools and clearest in Finnish schools. On a country level, certain indoor related microbial toxins were detectable only in the index, but not in the reference schools. For example the indoor source related mycotoxins meleagrin and alamethicin were exclusively detected in index school buildings in Spain and Finland. However, this was not the case for Dutch schools, where alamethicin was not detected, while meleagrin was detected in both index and reference schools. Differences in the detection of toxins based on building status were rarely consistent between the three countries and in most cases failed to reach statistical significance, partly due to low prevalence of the single analytes. Differences in the detection of toxins in the three different countries reached statistical significance ($p<0.05$ according to Chi-Square Test) for several potential 'indoor' metabolites - alamethicin more frequently detected in Spain; beauvericin, meleagrin and valinomycin most frequent in Netherlands -, as well as for a number of metabolites more likely to originate from outdoor sources (eg. enniatins, physcion).

4 Conclusions

To the best of our knowledge, this is the most comprehensive study on detection of microbial toxins in schools, with additional information being provided on moisture damage, dampness and other factors contributing to poor IAQ. Microbial metabolites were frequently found in settled dust and surface swab samples collected in index as well as in reference school buildings, with prevalence of single compounds being typically rather low. Our result highlight the need to screen for a wide variety of microbial toxins that are present in indoor environments in order to identify the most relevant compounds in terms of prevalence, concentrations and associations with indoor environmental conditions, such as moisture damage. In a next step, linking such exposure data with health data from the exposed residents will allow to evaluate the risk related to indoor microbial toxins and human health.

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An Intervention Study In Moisture Damaged Homes – preliminary results before renovation

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Keywords: Moisture and mold damage, homes, microbes and microbial toxins, respiratory symptoms, intervention

1 Introduction

Numerous population studies have shown that moisture damage and microbial growth are associated with adverse health effects. Nevertheless, the causative agents behind these symptoms are still obscure. Microbial secondary metabolites are among potential candidates, since they have a wide range of biological activities and may exhibit different toxic effects. Mycotoxins, such as macrocyclic trichothecenes,have been shown to be present in indoor air (Brasel *et al.* 2005, Charpin-Kadouch *et al.* 2006) indicating possibility for inhalation exposure.

An intervention study in severely moisture and mold damaged homes was designed to produce information on microbes and microbial metabolites associated with health effects as well as effects of renovation on exposure and health effects. At present, the first part of the study (before the remediation) is underway.

2 Materials and methods

Through collaboration with the Finnish Society for Pulmonary Disabled (Heli), the HoTeS study (Mold-exposure and health survey) recruits serious cases of 'moldy homes' and their families across Finland. Upon initial building inspections by trained civil engineers, appropriate renovation plans are developed and executed. The homes and occupants are followed up in an intervention design, including both health and microbial exposure measurements before and after the renovations. Microbial exposure is determined using several different dust samples (floor, vacuum cleaner dust bag, airborne settled) and building material samples (only taken before renovation). Building material samples are taken from all damaged locations. Floor dust samples are taken from a living room and the most damaged room if not the living room and airborne settled dust is taken from eg. shelves, door frames and other horizontal surfaces through out the home. Microbial toxins are analyzed from all sample types with liquid chromatography / tandem mass spectrometry (LC-MS/MS) (Vishwanath *et al.* 2009). The basic list of target metabolites in this multi-analyte method includes 159 fungal and 27 bacterial metabolites. Cultivation and microbial identification is performed on building material samples. Health symptoms and other background information are assessed with questionnaires sent to the participating families before and after renovation and in 3-months-intervals in between. All family members are asked to conduct clinical measures twice, before

and after remediation of homes, including measurements on cardiovascular, respiratory and inflammation parameters.

In this paper, preliminary data from the situation before renovation are presented from 21 homes including 38 adults and 40 children (aged 2-71). Based on the median levels of viable fungi in the building materials, homes were classified into two categories: homes with more severe mold growth and homes with less severe mold growth. The associations between mold growth and symptom questionnaire, PEF and eNO-levels are analyzed with logistic regression adjusted for age and gender. In addition, the occurrence of microbial toxins in building material and dust samples is examined.

3 Results

Before renovation, in all homes except two microbial growth (total concentration >10 000 cfu/g (Ministry of Health and Social Affairs 2003)) was observed in one or more building materials (n=124). Fungal concentrations varied between <45 – $36*10^6$ cfu/g. Microbial metabolites were detected in all building material (n=49) and dust samples (n= 47) studied so far. Number of detected microbial toxins in one home varied between 2 to 14. Compounds with known toxic potential such as ochratoxin A, sterigmatocystin and chaetoglobosin A were found, with enniatins, emodin, and beauvericin detected most frequently. Building materials showed the largest diversity of toxins in all except three homes, out of which two had less severe microbial growth. Nevertheless, high concentration of toxin was found in a one of these homes (sterigmatocystin 1145 ng/g building material). Some of the toxins were also found in various house dust types, suggesting that those compounds might have originated from a damaged building material.

In this cross sectional setting before renovation, the occupants with more severe fungal growth in building materials suffered more frequently from eye symptoms, stuffy nose and night cough during the last 12 months than the occupants with less microbial growth in their homes (Figure 1). The findings were very similar when assessing the symptoms during the last three months. No differences were found in levels of FEV_1 and eNO measured during the field visit. Associations between symptoms and microbial toxins will be presented in the conference.

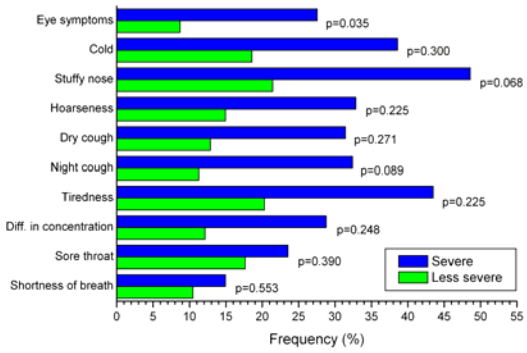

Figure 1. Frequency of symptoms among residents (N=78) in homes with severe and less severe moisture damage

4 Conclusions

Microbial toxins are found frequently in moisture damaged indoor environments. Respiratory symptoms are associated with severe microbial growth. This study produces information on microbes and microbial metabolites and their association to adverse health effects. Such information will be essential for a sound risk assessment of microbes and microbial toxins indoors.

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Determination of moulds and their metabolites in indoor environments

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Summary

Indoor mold growth has recently become a concern with regard to the health of residents. A primary requisite addressing this issue would be to scan various indoor samples and generate a knowledge bank of fungal, bacterial and other microbial species growing indoor. For the same a robust LC-MS/MS based qualitative and quantitative analytical method is developed. The method is an extension of an existing multi metabolite screening method developed for analysis of food samples with 87 analytes. The extended method encompasses a total of 168 fungal and bacterial metabolites with relevance to food, feed and indoor environments. Limit of detection (LOD) and extraction efficiency were analysed for silica rich house dust. Three extraction solvents methanol, ethyl acetate and acidified acetonitril/water were tested for extraction efficiency. Among the three, a mixture of acetonitril/water/acetic acid (79:20:1) proved to be the most efficient. The analysed water damaged building materials and settled house dusts were tested positive for fungal metabolites sterigmatocystin, meleagrin and emodin.

Introduction

The quality of indoor air and architecture of living environment has a profound influence on the health of individuals. The importance of air sanitation and surface sterilisation of walls in houses are documented in ancient Vedic scriptures. The clinical symptoms such as fatigue, irritability, poor concentration and learning disabilities are observed in adults as well as in children with no previous history of neuro-psychiatric complaints. Surprisingly these symptoms are found at a higher frequency in the inhabitants of buildings with reported water damage or houses with inadequate ventilation. Exact reasons for these symptoms are unclear, hence grossly referred as symptoms of sick building syndrome (SBS). Further it may be attributed the symptoms observed in SBS are the result of cumulative influence of volatile and non-volatile metabolites of various micro flora growing indoor. The primary mode of entry of these metabolites are by the inhalation of volatile compounds from air or house dust containing bacterial and fungal spores and fragments of mycelia.

Species of ***Penicillium***, ***Aspergillus, Chaetomium, Fusarium***, ***Stachybotrys*** and ***Trichoderma*** are the most common fungal dwellers of the damp buildings. These species produce metabolites such as meleagrin, sterigmatocystin, stachybotrylactam, chaetoglobosin, emodin, enniatins and histamines capable of influencing human health. Though clinical investigations have proved the biological consequences of some of these metabolites the actual amounts produced by the microbes indoor or on building materials, daily exposure concentrations and tolerable limits are still a matter of debate. As

a matter consequence this became the reason for anxiety and confusions among various strata of society.

The natural disasters in the past like hurricane Katrina over the Bahamas and southern Florida and Asian Tsunami have prompted the speedy formulation of unified guidelines for the assessment of building health. The issue has gained significant public and private sector attention in the aftermath of devastating hurricane Katrina, which caused huge amounts of loss to lives and properties. A pilot study in the affected houses of New Orleans done by US public health departments with universities in the aftermath of Katrina reported a baseline, cultivable mold ranging from 22,000 to 515,000 colony-forming units/ m^3, spore counts ranging from 82,000 to 630,000 spores/m^3, and endotoxin ranging from 17 to 139 endotoxin units/ m^3 (Chew GL et.al 2006).

The need of the hour is a rapid analytical method for assessing the building health and also for monitoring the effectiveness of preventive management methods in practice. Hyphenation of liquid chromatography and tandem mass spectrometry has become the method of choice for most toxicologists due to its precision and sensitivity. In the present study a previously described LC-MS/MS method (Sulyok M et.al 2007) is extended to 168 analytes by the addition of metabolites of indoor relevance. The method involves single step extraction with no additional clean up procedure. The salient features of the method are economised cost and time and high rates of analyte extraction.

Analyte extraction was optimised using spiked settled dust. Three widely used extraction solvents were compared for extraction efficiency. LOD was investigated for the analytes stable under acidic conditions. The overall goal of the study is to generate an accurate and comprehensive compendium of indoor microbiota, substrate specificity of microflora for growth and metabolite concentrations prevailing in different indoor environments.

Materials and methods.

Unless stated all chemicals/reagents used are of highest purity or of analytical grade.
Samples: The samples were collected from houses with visible or reported water damage. Both organic and inorganic samples such as wood peelings, settled dust and wall scrapings were considered for analysis.

Extraction: Both spiked and real samples were extracted on orbiter shaker with 1:4 (w/v) of extraction solvent for 90 minutes at room temperature. Higher sample to solvent ratios were adopted in case of dust due to the highly absorptive nature of this matrix. Three extraction solvents methanol, ethyl acetate and acetonitril/water/acetic acid (79:20:1) were evaluated for extraction efficiency.

LC-MS/MS: Chromatographic separation was performed using an 1100 Series HPLC System (Agilent, Waldbronn, Germany) equipped with a Gemini® C18-column, 150×4.6mm i.d., 5 µm particle size, and a C18 4×3mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA,USA) in gradient elution mode. Detection and quantification of the mycotoxins was performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo V Ion source for electrospray ionisation (ESI).

Microbiology: Subcultivation and screening of mold was done as three-point-inoculations on Czapek Yeast Extract agar (CYA) and Yeast extract agar (YES) which were isolated on Dichloran Rose Bengal Chloramphenicol Agar (DRBC). Subcultures were grown at 25 °C for 10 days and then stored at 4 °C. Identification was done by microscopy.

Results and discussion.

During the method extension special emphasis was given to metabolites related to fungal and bacterial species that are described to be indoor relevant, e.g. ***Stachybotrys*** (satratoxins G and H, stachybotrylactam), ***Aspergillus*** (austocystin A, fumigaclavine A, fumagillin), ***Chaetomium*** (chaetochromin, cochliodinol), ***Cladosporium*** (calphostin C), ***Nocardiopsis*** (k 252a and k 252b) and ***Streptomyces*** (eg. valinomycin). A few of these 84 metabolites that were added to the LC-MS/MS protocol were not compatible with the acidic conditions during extraction and/or are not sufficiently stable in the solvents used for preparing multi-analyte standard solutions. Among the tested solvents acidified acetonitrile water mixture proved to yield best recoveries; extraction efficiency of 13 analytes were outside 100±50% in case of the acetonitrile/water/acetic acid mixture (79:20:1), where as 31 analytes were out of the range in case of methanol. The LODs were in the order of 0.01 to 10 µg/L for the investigated analytes.

An additional, 54 house samples with suspected or reported water damage was subjected to analysis with the extended method. 19, 17 and 15 samples, respectively, were positive for sterigmatocystin, meleagrin (Fig.1) and emodin reflects infection due to spp. of ***Aspergillus***, ***Penicilliu***m, and ***Cladosporium***.

The numbers show a predominance of ***Aspegillus*** and ***Penicillum*** taxa followed by ***Cladosporium*** in the indoor environment. The emodin is a common metabolite produced by all three above mentioned species. Hence determining the source of emodin needs further investigation with molecular diagnostic methods or culture based screening procedures. Other less frequently detected metabolites were stachybotrylactam, cytochalasin D, beauvericin, alternariol-methylether and roquefortine C.

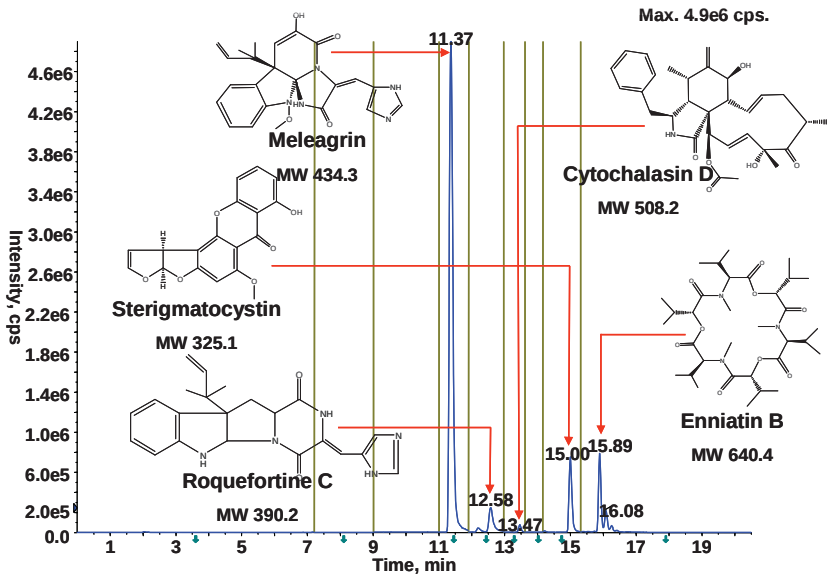

Figure 1: HPLC-ESI(+) MS/MS chromatogram of wall scrapings from a children's room. Identified species were ***Penicillium chrysogenum and Aspergillus versicolor.***

The most prevalent metabolite streigmatocystin is a metabolite of few species of ***Penicillium*** and ***Aspergillus***. From the toxicological perspective sterigmatocystin is a potential hepatocarcinogen and known to form reversible DNA adducts (Essigmann JM et al 1979). Earlier publications reported for the indoor carpet dust occurrence of ***A versicolor*** as a potential source for sterigmatocystin (Engelhart S et al 2002). Selected samples with significant amounts of sterigmatocystin were screened by classical microbiological procedures. Based on experimental evidences and literature we designate ***A. versicolar*** as the source for sterigmatocystin in indoor samples***.***

The metabolite meleagrin was the second most frequently detected metabolite from the damp building samples. Substances such as insulation, gypsum wallboard, manufactured wood, ceiling tiles and textiles upon water damage are known to harbor species of ***Penicillum*** (Miller JD et.al 2007). Species of ***Penicillium chrysogenum*** produce the nitrogenated alkaloid metabolite meleagrin on building materials (de la Campa R et.al 2007). Toxicolological studies with the extract of ***P. atramentosum*** containing rugulovasines, meleagrin and oxaline was reported to induce cytotoxicity in MTT-cell culture assay (Larsen TO et.al. 2002), though this is not a clear evidence for meleagrin induced cytotoxicity. Based on the earlier reports it can be speculated for a synergistic mode action of meleagrin with other mycotoxins in inducing cytotoxicity. Parallel to the findings from isolates of damp buildings, production of meleagrin is also reported by ***Penicillium chrysogenum*** and ***Penicillium expansum*** strain isolated from Mir space station (Kozlovskiĭ AG 2002). This suggests for a probable

co-existence of species of ***Penicillum*** and humans even in the absence water damage. Based on the literature and microbiological examination of building materials positive for meleagrin we conclude ***Penicllium chrysogenum*** to be the source of meleagrin.

Emodin is a widely distributed natural anthraquinoid metabolite found both in fungi and plants. It is a product of species of ***Penicillium, Aspergillus*** and ***Chaetomium*** growing on the damp surfaces. Plant rhizome extracts of ***Rheum palmatum*** containing emodin is shown to inhibit casein kinase II, a regulator of proliferation and differentiation of eukaryotic cells (Yim H et.al 1999). Another independent study comparing uncoupling effects of Emodin and Skyrin in rat liver mitochondria showed a stronger uncoupling influence of Emodin (Kawai K et.al 1984). Since the vital life processes such as cell proliferation and differentiation and energy metabolism can be influenced by emodin, it gains a critical analyte status in the indoor context. As expected earlier, several species belonging to ***Cladosporium, Penicillium*** and ***Aspergillus*** were identified in the culture isolates of damp wall scrapings.

The findings of the study show the applicability of liquid chromatography coupled to tandem mass spectrometry for analysis of building materials and other infected indoor samples. The ability of accurate quantification makes this method a key tool for the assessment of water damage and authentication of building health. The present initiative is expected to include MVOCs (microbial volatile organic compounds) for assessing health and occupational risks with appropriate methods.

Acknowledgement

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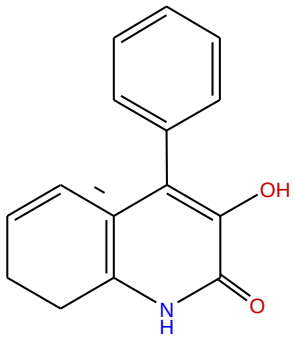
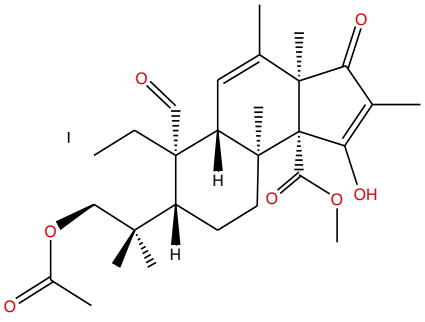
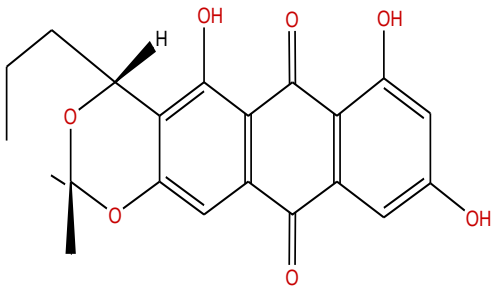
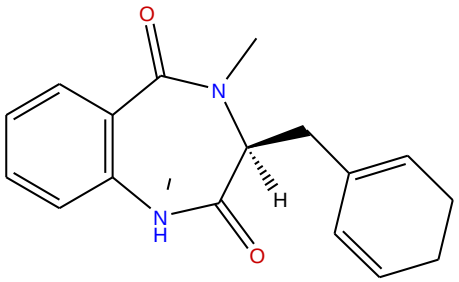
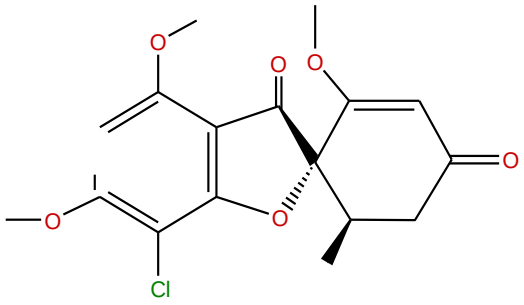
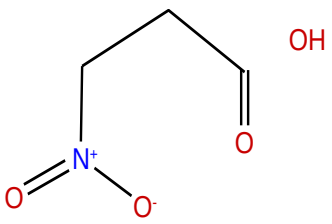
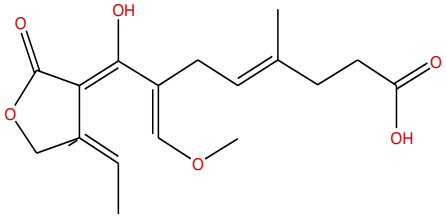
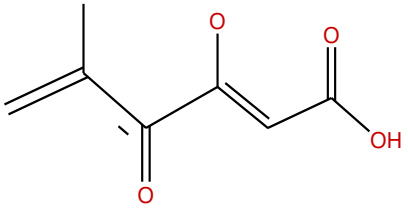
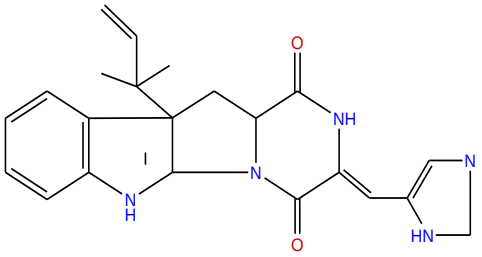
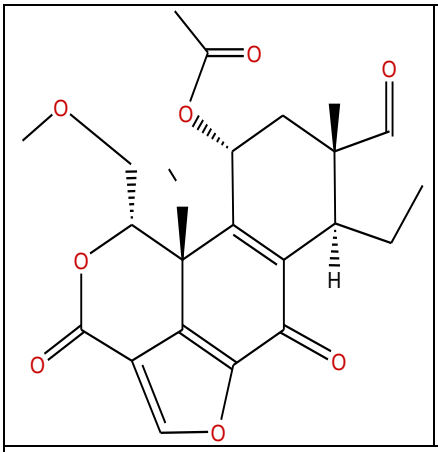
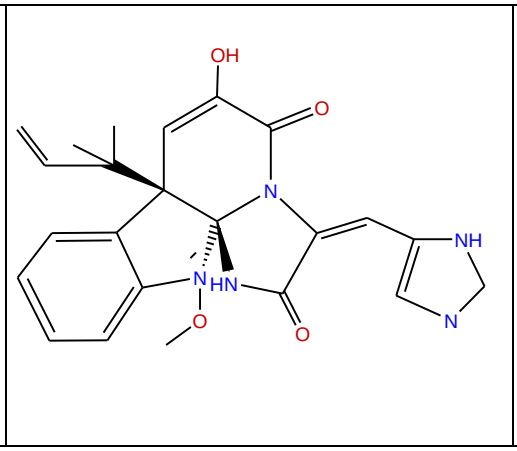
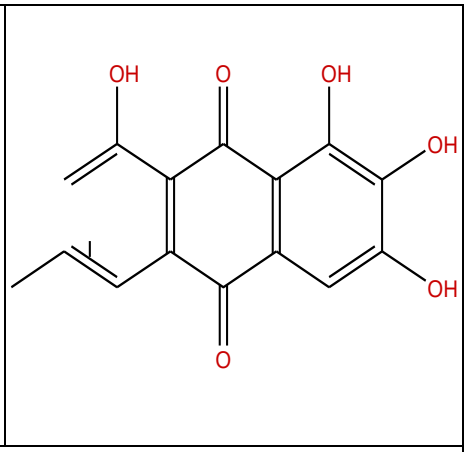
Appendix

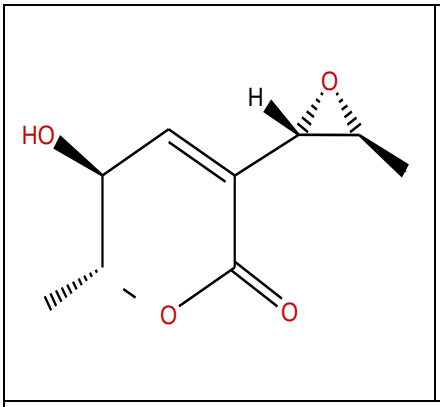
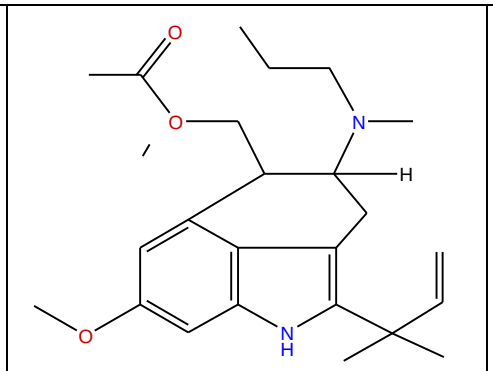
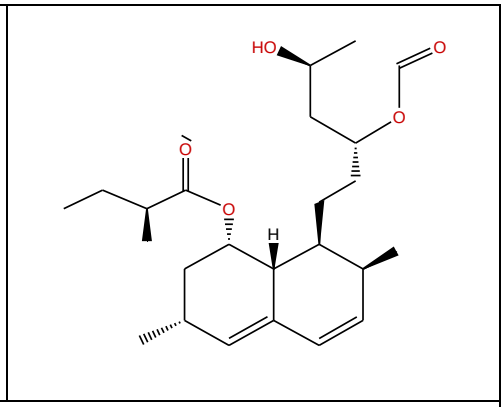
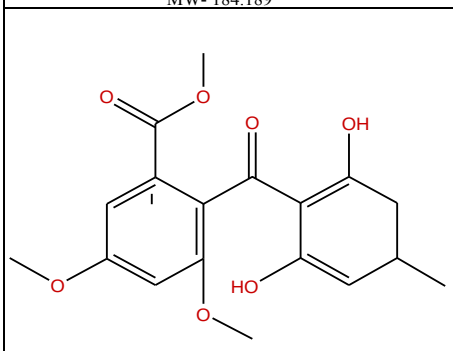
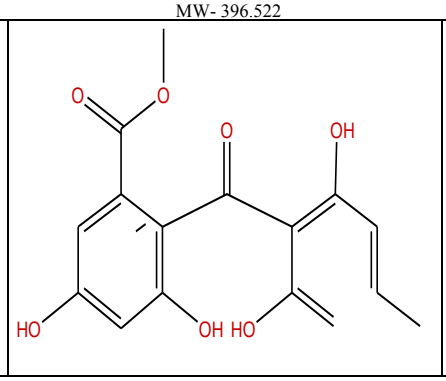
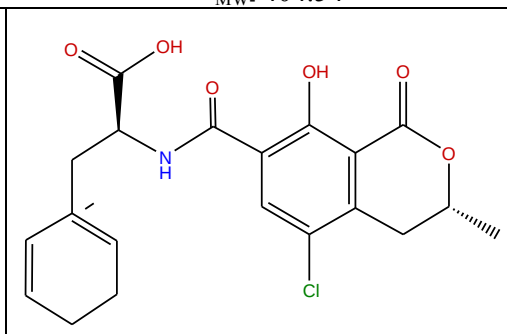
Chemical structures of mycotoxins/ microbial bioactive compounds found in indoor environments

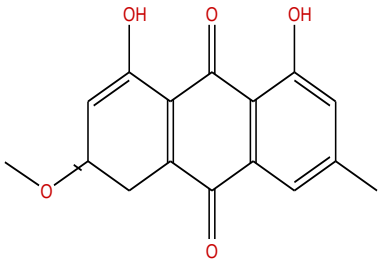
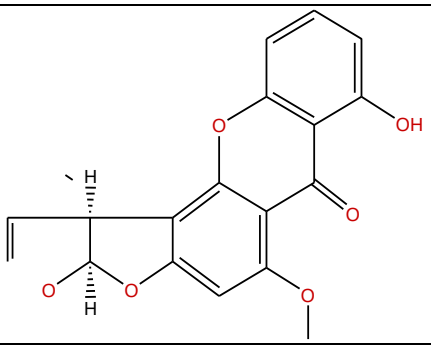
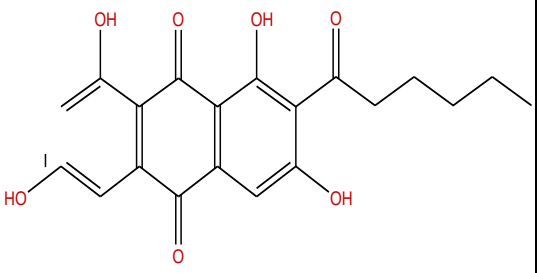
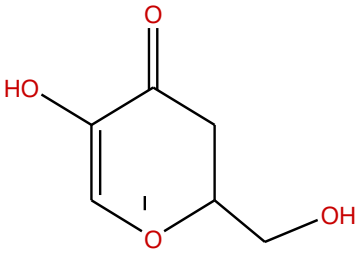
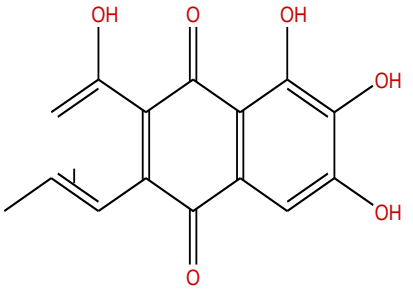
Penicillium **metabolites**

		
Viridicatin $C_{15}H_{11}NO_2$ MW- 237.253	**Andrastin A** $C_{28}H_{38}O_7$ MW- 486.597	**Averufin** $C_{20}H_{16}O_7$ MW- 368.337
		
Cyclopeptine $C_{17}H_{16}N_2O_2$ MW- 280.321	**Griseofulvin** $C_{17}H_{17}ClO_6$ MW- 352.766	**3-nitropropionic acid** $C_3H_5NO_4$ MW- 119.08
		
Mycophenolic acid $C_{17}H_{20}O_6$, MW- 320.337	**Penicillic acid** $C_8H_{10}O_4$ MW- 170.163	**Roquefortine C** $C_{22}H_{23}N_5O_2$ MW- 389.450

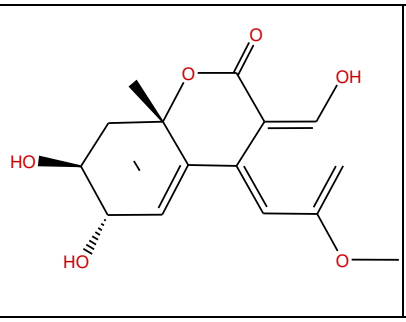
		
Wortmannin $C_{23}H_{24}O_{8}$ MW- 428.432	**Meleagrin** $C_{23}H_{23}N_5O_4$ MW- 433.459	**Emodin** $C_{15}H_{10}O_5$ MW- 270.237

***Aspergillus* metabolites**

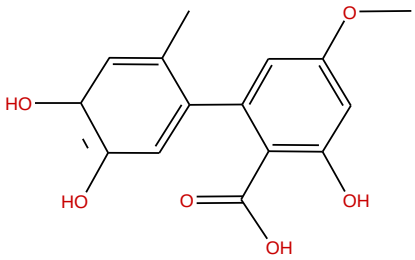
		
Aspyrone $C_{9}H_{12}O_{4}$ MW- 184.189	**Fumigaclavine** $C_{24}H_{32}N_2O_3$ MW- 396.522	**Mevinolin** $C_{24}H_{36}O_5$, MW- 404.54
		
5-O-Methylsulochrin $C_{18}H_{18}O_7$ MW- 346.331	**3-O-Demethylsulochrin** $C_{16}H_{14}O_7$ MW- 318.278	**Ochratoxin-A** $C_{20}H_{18}ClNO_6$, MW- 403.813

		
Physcion $C_{16}H_{12}O_5$ MW- 284.263	**Sterigmatocystin** $C_{18}H_{12}O_6$, MW- 324.284	**Norsolorinic acid** $C_{20}H_{18}O_7$, MW- 370.353
		
Kojic acid $C_6H_6O_4$ MW- 142.109	**Emodin** $C_{15}H_{10}O_5$ MW- 270.237	

Alternaria metabolites



Altenuene
$C_{15}H_{16}O_6$
MW- 292.284



Altenusin
$C_{15}H_{14}O_6$
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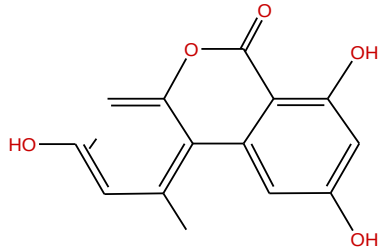
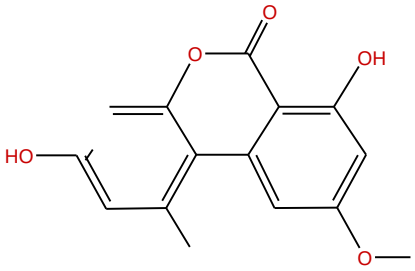
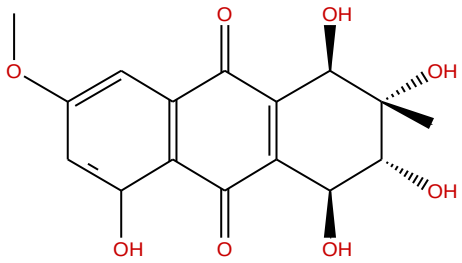


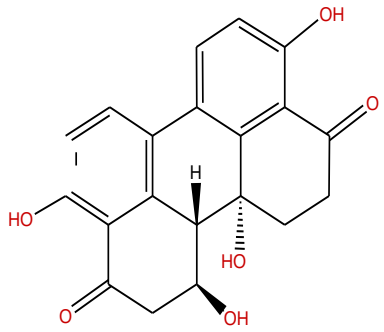
Alternariol
$C_{14}H_{10}O_5$
MW- 258.226



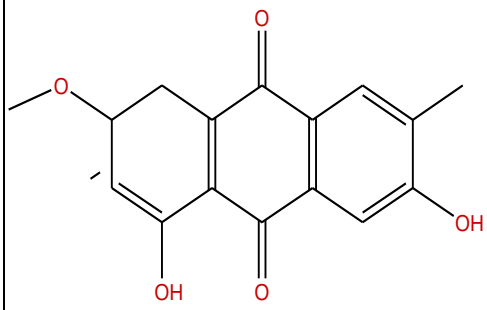
Alternariol monomethylether
$C_{15}H_{12}O_5$
MW- 272.253



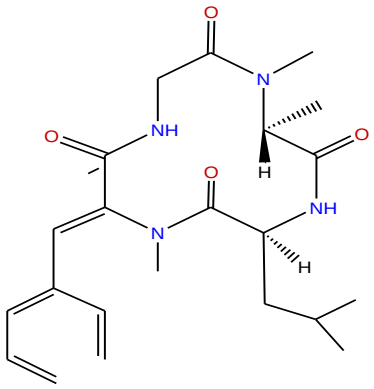
Altersolanol A
$C_{16}H_{16}O_8$
MW- 336.293



Altertoxin I
$C_{20}H_{16}O_6$
MW- 352.337

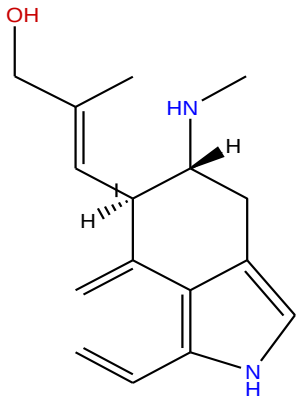


Macrosporin
$C_{16}H_{12}O_5$
MW- 284.263



Tentoxin
$C_{22}H_{30}N_4O_4$
MW- 414.498

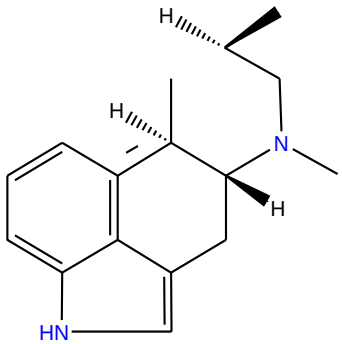
Other indoor relevant fungal metabolites



Chanoclavine-I

$C_{16}H_{20}N_2O$

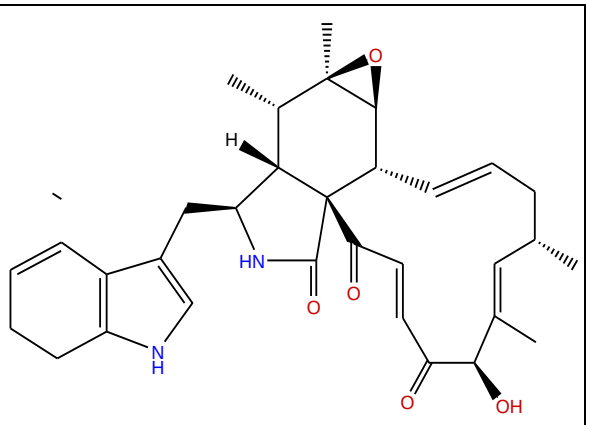
MW- 256.343



Festuclavine,

$C_{16}H_{20}N_2$

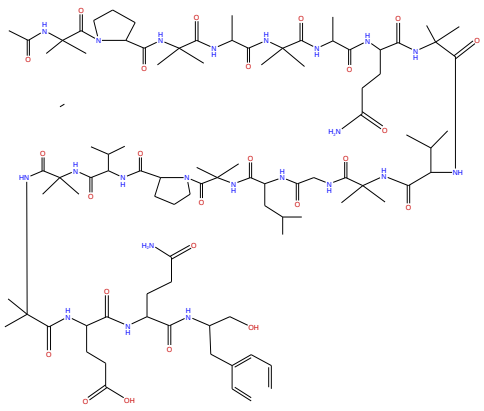
MW- 240.343



Chaetoglobosin-A

$C_{32}H_{36}N_2O_5$

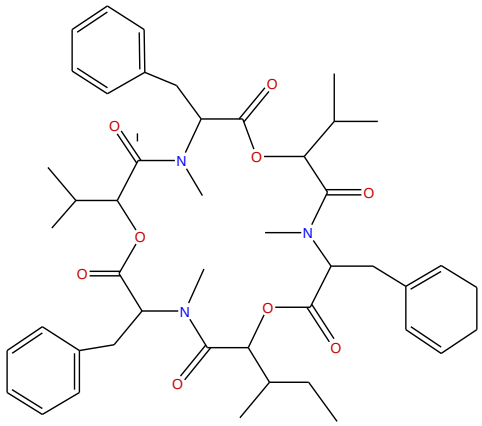
MW- 528.639



Alamethicin F30

$C_{92}H_{150}N_{22}O_{25}$

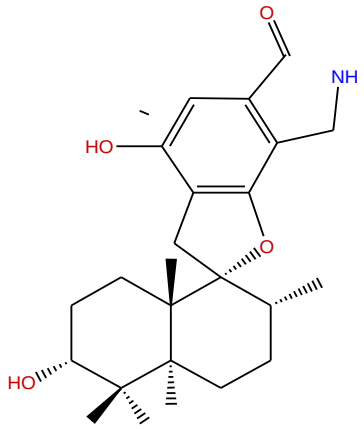
MW- 1964.31



Beauvericin-A

$C_{46}H_{59}N_3O_9$

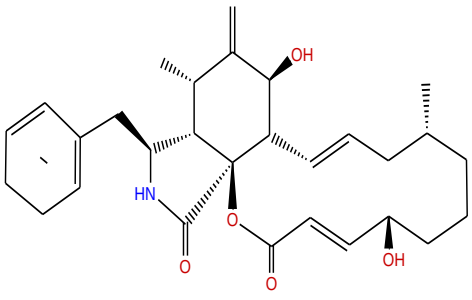
MW- 797.975



Stachybotrylactam

$C_{24}H_{33}NO_4$

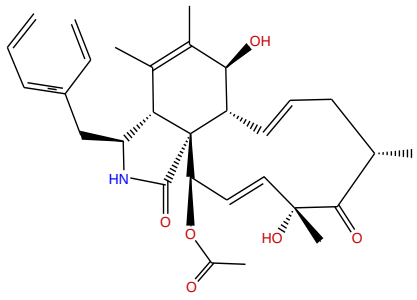
MW- 399.523



Cytochalasin B

$C_{29}H_{37}NO_5$

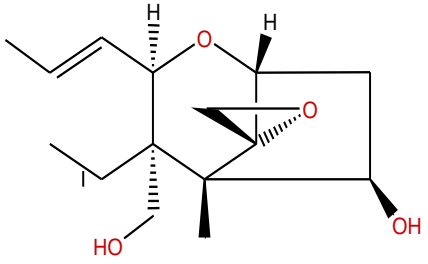
MW- 479.608



Cytochalasin C

$C_{30}H_{37}NO_6$

MW- 507.618

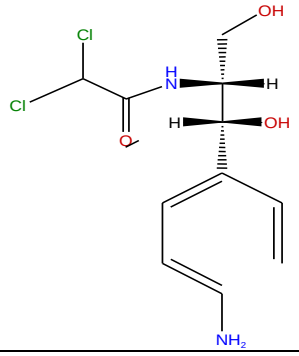


Verrucarol

$C_{15}H_{22}O_4$

MW- 266.333

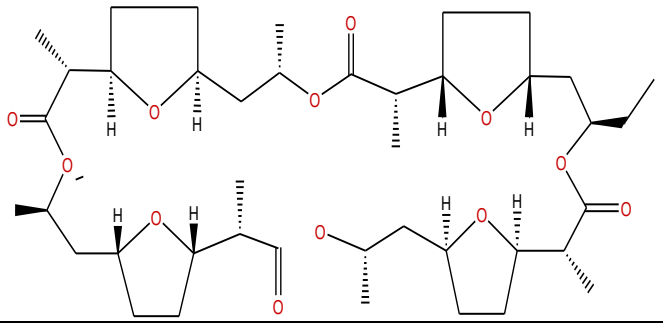
Bacterial metabolites



Chloramphenicol amine

$C_{11}H_{14}Cl_2N_2O_3$

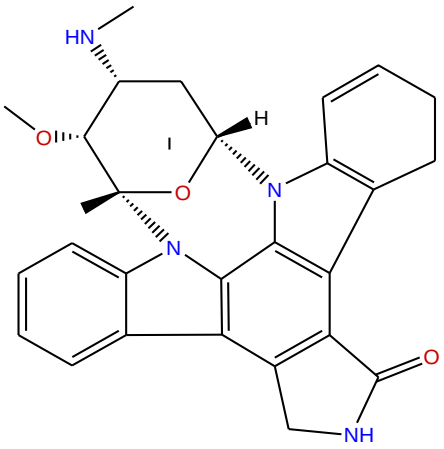
MW- 293.146



Monactin

$C_{41}H_{66}O_{12}$,

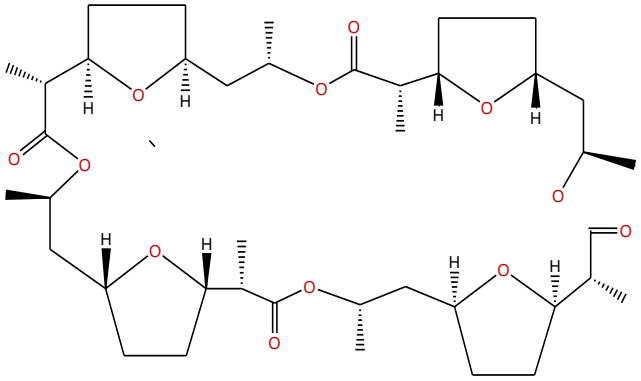
MW- 750.956



Staurosprin

$C_{28}H_{26}N_4O_3$

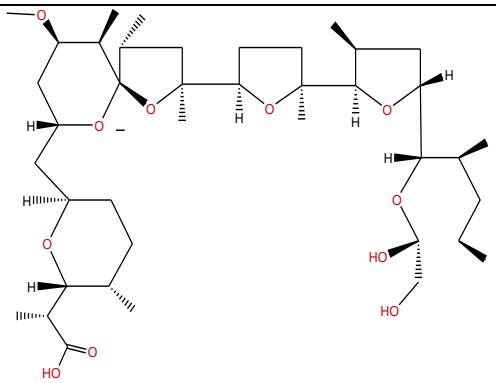
MW-466.531



Nonactin

$C_{40}H_{64}O_{12}$,

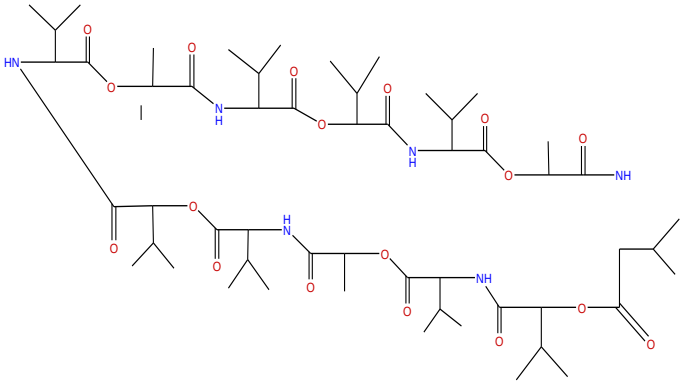
MW- 736.929



Nigericin

$C_{40}H_{68}O_{11}$,

MW- 724.961



Valinomycin

$C_{54}H_{90}N_6O_{18}$

MW- 1111.32

Curriculum vitae

Personal information	
First name/surname	Vinay Vishwanath
Address	Haus Panorama, Brigittenauer Lände 224, Zimmer-6152, Wien 1200
Contact number	+43 - 6803185333
E-mail	vinsringeri@gmail.com
Age	30
Gender	Male
Nationality	Indian
Education and training	
Date	March 2008 - January 2012
Degree to be awarded	Dr.nat.tech. (Ph.D.)
Thesis title/occupational skills	**"Development, validation and application of mass spectrometric methods for indoor environmental quality evaluation with special emphasis on microbial metabolites"**. Method development, validation and application, small molecules, LC-MS/MS, GC-MS.
Affiliation	Center for Analytical Chemistry, Department IFA-Tulln, University of Natural Resources and Life Sciences Vienna, Konrad-Lorenz-Straße 20, A-3430 Tulln, Austria.
Date	June 2001 - July 2003
Degree awarded	Master of Science Biotechnology (M.Sc)
Thesis title/technical skills	**"Conformational study of proteins/peptides using CD and Fluorescence spectroscopy as measuring tools"**. Protein purification, stability measurement.
Affiliation	Department of Biotechnology, University of Mysore, Mysore-570 006, India.
Date	June 2001
Degree awarded	Bachelor of Science (B.Sc)
Major	Botany, Biochemistry and Microbiology
Previous occupations	
Research trainee	Institute of bioinformatics (IOB), Bangalore, India
Date/Duration	Six months, July - December 2003
Job profile	Data mining and curation (www.hprd.org)

Research fellow	Avestha Gengraine Technologies Pvt. Ltd. Bangalore, India
Date/Duration	1.8 years (January 2004 - August 2005)
Job profile	Genomics & Molecular biology
Internship	Gwangju Institute of Science and Technology (GIST). Gwangju, South Korea.
Date/Duration	Six months, September 2005 - February 2006
Job profile	Construction of virtual 2D maps for human brain proteome
Tool	JVirGel (www.jvirgel.de)
Researcher	Medical University of Vienna (MUW).
Date/Duration	1.8 years, June 2006 - February 2008
Job profile	Neuroproteomics and mass spectrometry
Technical details	MALDI-TOF/TOF & 2D electrophoresis.

List of publications

Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry. **Vishwanath V**, Sulyok M, Labuda R, Bicker W, Krska R**. Anal Bioanal Chem**. 2009 Nov; 395(5):1355-72.

Differential protein levels and post-translational modifications in spinal cord injury of the rat. Afjehi-Sadat L, Brejnikow M, Kang SU, **Vishwanath V**, Walder N, Herkner K, Redl H, Lubec G.J **Proteome Res**. 2010 Mar 5;9(3):1591-1597

Co-occurrence of bacterial toxins and mycotoxins in moisture damaged indoor environments. Täubel M, Sulyok M,**Vishwanath V**, Bloom E, Turunen M, Järvi K, Kauhanen E, Krska R, Hyvärinen A, Larsson L and Nevalainen A. Täubel M, Sulyok M, **Vishwanath V**, Bloom E, Turunen M, Järvi K, Kauhanen E, Krska R, Hyvärinen A, Larsson L, Nevalainen A. **Indoor Air**. 2011 Oct; 21(5):368-75.

Evaluation of settled floor dust for the presence of microbial metabolites and volatile anthropogenic chemicals in indoor environments by LC-MS/MS and GC-MS methods. **Vishwanath V**, Sulyok M, Weingart G, Kluger B, Täubel M, Mayer S, Schuhmacher R, Krska R. **Talanta**. 2011 Sep 30; 85(4):2027-38.