Mycotoxins in indoor environments. Determination using mass spectrometry.

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Mycotoxins in Indoor Environments

Determination using Mass Spectrometry

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Cover picture by Eric Carleman and Erica Bloom. Scanning electron microscope picture of *Stachybotrys chartarum*.

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"The pure and simple truth is rarely pure and never simple."

Oscar Wilde

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LIST OF PAPERS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals:

I. **Bloom, E., K. Bal, E. Nyman, and L. Larsson.** (2007). Optimizing a GC-MS method for screening of *Stachybotrys* mycotoxins in indoor environments. *J Environ Monit.* 9(2): 151-156.

II. **Bloom, E., K. Bal, E. Nyman, A. Must, and L. Larsson.** (2007). A mass spectrometry-based strategy for direct detection and quantification of some mycotoxins produced by *Stachybotrys* spp. and *Aspergillus* spp. in indoor environments. *Appl Environ Microbiol.* 73(13): 4211-7.

III. **Bloom, E., L. F. Grimsley, C. Pehrson, J. Lewis, and L. Larsson.** (2008). Molds and mycotoxins in dust from water-damaged homes in New Orleans after Hurricane Katrina. *Indoor Air. In press.*

IV. **Bloom, E., E. Nyman, A. Must, C. Pehrson, and L. Larsson.** (2008). Molds and mycotoxins in indoor environments. A survey in water-damaged buildings. *Submitted.*

ABSTRACT

Dampness in indoor environments may cause various health problems. The specific causative agent(s) are unknown but may originate from degradation processes in damp materials, microbial growth, or a combination of these phenomena. The health impact of dampness in buildings is a politically, legally, and economically important question. Scientists at the U.S. EPA and Lawrence Berkeley National Laboratory have estimated that the symptoms of 4.6 of the total of 21.8 million asthmatics in the USA are caused by indoor dampness and mold to an annual cost of 3.5 billion dollars.

Mycotoxins are secondary metabolites produced by molds which may be *e. g.* cytotoxic (*e. g.* macrocyclic trichothecenes produced by *Stachybotrys chartarum*), genotoxic (*e. g.* sterigmatocystin and aflatoxins produced mainly by *Aspergillus* spp. including *A. versicolor* and *A. flavus*), or immunosuppressive and neurotoxic (*e. g.* gliotoxin produced by *Penicillium* spp. and *Aspergillus* spp. *e. g. A. fumigatus*). Airborne mycotoxins have been demonstrated in water-damaged buildings using both ELISA and mass spectrometry. However, whether mycotoxins at the concentrations found in mold-damaged environments represent a health risk upon inhalation is not known. The mechanisms for mycotoxin uptake, metabolism, and interaction *e. g.* with other fungal constituents such as proteins and (1→3)-β-$_D$-glucan (a fungal cell membrane constituent) are poorly understood.

In this project analytical methods for the detection and determination of selected mycotoxins using GC-MS and HPLC-MS were developed. The methods were applied to 167 mold-contaminated building material samples, of which 67 % were mycotoxin positive. Thus, many molds not only posess the genetic capacity to produce mycotoxins but do it regularly in water damaged indoor environments. In addition, we demonstrated mycotoxins in dust settled in the breathing zone in indoor environments where severe mold-contamination was identified on building materials. We thereby confirm that mycotoxins on such materials can become airborne and thus inhalable.

This project is an example of fruitful national and international inter-disciplinary collaboration between the building industry, companies specialized in remediation measures, and universities.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Dålig ventilation, fukt, hög förekomst av partiklar i luften samt damm i inomhusmiljö kan relateras till en rad luftvägsbesvär. Framförallt är fukt i byggnader sammankopplad med ohälsa och obehagssymptom. Fukt kan reagera med olika byggmaterial och bilda irriterande flyktiga produkter samt även resultera i mikrobiell tillväxt, framför allt av mögel. Amerikanska forskare har uppskattat att symptomen hos 4,6 av USA:s 21,8 miljoner astmatiker orsakas av fukt och mögel och att detta kostar det amerikanska samhället årligen 3,5 miljarder dollar. Trots att det finns nästintill otaliga rapporter om samband mellan mögel i inomhusmiljöer och ohälsa har man ännu inte på ett vetenskapligt sätt definitivt lyckats knyta mätbara exakta mikrobiologiska parametrar t. ex. astma/allergi. Det är alltså tyvärr ännu oklart vilka ämnen, som bildas vid förhöjd fukthalt, som ger besvär, bl. a. för att det saknas tillgång till pålitlig detektionsmetodik. I Sverige har en långlivad debatt pågått om mögel, mögelgifter (s. k. mykotoxiner) och dess betydelse för vår hälsa, men det är få i världen som i praktiken forskar på just mögelgifter.

Man vet att mögelsvampar som kan växa till inomhus har en förmåga att producera mykotoxiner, ämnen som är oerhört toxiska (bl. a. cancerframkallande, vävnads-förstörande och inflammationsinducerande). Dock har man ansett att mängden mykotoxin som vi exponeras för (baserat på antal sporer man funnit i mögelskadade hus) är så liten att den rimligen inte kan ha någon inverkan på vår hälsa. Bakgrunden till detta projekt är att nya, omvälvande data har visat att mykotoxiner – förutom att de är direkt toxiska - även påverkar immunceller i en riktning som innebär ökad risk för allergibenägenhet och att det krävs oerhört små mängder (pikogramnivå) för att framkalla dessa reaktioner. Dessutom har det visats att mögelsvampar frigör mycket små partiklar (små hyf-fragment) som aldrig sedimenterar. Dessa partiklar är mycket mindre -och deponeras mycket effektivare i lungorna - än sporer. Därmed kan vår exponering för mögel vara flera hundra gånger större än vad som tidigare beräknats. Denna exponering pågår konstant under en stor del av dygnets timmar (vi vistas inomhus ca 90 % av vår tid), något som inte alltid tagits hänsyn till i tidigare studier.

Målsättningen med detta projekt har varit att utveckla nya analytiska metoder för

bestämning av några utvalda mykotoxiner och att tillämpa dem på en mängd olika prover från mögelskadade inomhusmiljöer. Delar av byggnadsmaterial (synbart angripna lister, trösklar, prov av gipsplattor, tapet etc.) samt dammprover, främst från golvytor och hyllor, insamlades från mögelskadade hus via professionella skadeutredare samt andra universitet som vi samarbetat med, i exempelvis New Orleans (USA). Extrakt av dessa prover renades, separerades med vätske- (HPLC) eller gaskromatografi (GC) och analyserads med masspektrometri (MS), en analytisk kemisk metod som bl a också används för dopinganalyser och för kriminaltekniska ändamål.

I HPLC-MS-analyserna detekterades sterigmatocystin och alfatoxin B_1 (som bildas av *Aspergillus*-arter), gliotoxin (som bildas av *Aspergillus*- och *Penicillium*-arter), samt de makrocykliska trikotecenerna satratoxin G och H (som bildas av *Stachybotrys chartarum*). I GC-MS-analyserna utnyttjades det faktum att de makrocykliska trikotecenerna, som produceras av *S. chartarum* kemotyp S, vid hydrolys bildar verrukarol medan det inflammationsinitierande mykotoxinet trikodermin, som produceras av *S. chartarum* kemotyp A, bildar trikodermol. Genom att bestämma både verrukarol samt trikodermol kan man således ”screena” prover för mykotoxiner producerade av *S. chartarum*. Mängden svampbiomassa mättes också genom att bestämma en svampmarkör, ergosterol, en molekyl som i naturen unikt återfinns i svampars cellmembran.

Våra analyser visar att de mögelsvampar som projektet främst fokuserat på, och som ofta återfinns i inomhusmiljöer i samband med fuktskada, inte bara har förmågan att producera mögelgifter –de gör det regelmässigt. Dessutom har vi bekräftat att mögelgifter som härrör från synliga mögelfläckar på angripna byggmaterial kan bli luftburna och att vi därmed inandas dem. De metoder som utvecklats i projektet kommer i framtiden att kunna användas som verktyg i studier bl. a. syftande till att utröna mögelgifternas eventuella inverkan på vår hälsa. Det tvärvetenskapliga samarbetet inom projektet har varit mycket givande och illustrerar nyttan av kontakter mellan byggbranschen, skadeutredare samt forskare inom olika discipliner i inomhusmiljöforskningen.

ABBREVIATIONS

AFLAB	Aflatoxin B_1
BSTFA	N, O-Bis(trimethylsilyl)trifluoroacetamide
CDC	Center for disease control and prevention
CFU	Colony forming units
CI	Chemical ionization
CID	Collision induced dissociation
DNA	Deoxyribonucleic acid
EI	Electron ionization
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental protection agency
ERG	Ergosterol
ESI	Electrospray ionization
GC	Gas chromatography
GLIO	Gliotoxin
HFB	Heptafluorobutyryl
HFBI	Heptafluorobutyrylimidazole
HPLC	High pressure liquid chromatography
Ig	Immunoglobulin
INF-γ	Interferon gamma
IL-	Interleukine
LPS	Lipopolysaccharide
MEA	Malt extract agar
MS	Mass spectrometry
MSMS	Tandem mass spectrometry
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
MTR	Macrocyclic trichothecene
MVOCs	Microbial volatile organic compounds
NICI	Negative ion chemical ionization
PBMCs	Periferal blood mononuclear cells
(q)PCR	(quantitative/real-time) Polymerase chain reaction
PICI	Positive ion chemical ionization
RCS	Reuter centrifugal sampler
RESP	Reserpine
RNA	Ribonucleic acid
SATG	Satratoxin G
SATH	Satratoxin H

SIM	Selected ion monitoring
sp. / spp.	Species (singular form/plural form)
STRG	Sterigmatocystin
TCMS	Trimethylchlorosilane
TFA	Trifluoroacetic acid
Th cell	T-helper cell
TMS	Trimethylsilyl
TNF-α	Tumour necrosis factor alpha
TRID	Trichodermol
TSIM	N-trimethylsilylimidazole
VER	Verrucarol
q / Q	Quad

INTRODUCTION

Indoor air quality and health

We spend up to 90% of our time indoors. The indoor air quality has been recognized to have a great impact on our health and well-being. During the energy crisis in the 70's construction procedures changed and buildings were tightly sealed, ventilation rates reduced, and new materials introduced to minimize energy, and financial, loss. The resulting changes in indoor air quality, together with other characteristics of our western lifestyle, have been associated with the significant increase of allergic diseases in Sweden (Aberg 1989, Aberg et al 1995) and the world (Beasely et al 2003) over the last decades.

In buildings, moisture and dampness (Bornehag et al 2001), poor ventilation, dust, and high concentration of particles in the indoor air is associated with a number of respiratory problems (Mommers et al 2005). Still, the specific causes of the adverse health effects are unknown. However, it is likely that the components involved in health related symptoms originate from:

- chemical processes catalyzed by water causing degradation of indoor materials resulting in production of potentially irritating volatile organic compounds.
- growth of microorganisms which contain, or produce, bioactive compounds.
- a combination of the above.

Dampness, and the combination of water leakage and PVC flooring in the bedroom, was shown to be associated with increased prevalence of symptoms in airway, nose and skin among over 10,000 preschool children in Sweden (Bornehag et al 2005). Further, there are studies linking chemical exposures such as formaldehyde, molds and bacteria, and furry pet allergens (Daisy et al 2003, Tranter et al 2005) to health effects including asthma and allergic symptoms, airway infections, and impaired learning ability (Daisy et al 2003, Mendell and Heath 2005). Also, phthalate plasticizers in dwellings have been associated with asthma and allergic symptoms in children (Bornehag et al 2004, Kim et al 2006).

The hygiene hypothesis speculates that a lack of early childhood exposure to infectious agents, symbiotic microorganisms, and parasites increases susceptibility to allergic diseases by impairing immune system development. Factors as pet keeping, day-care attendance, family size, geographical differences, home building construction, urban vs. rural home environment etc, may thus influence health status later in life. Some studies support this hypothesis as pet exposure during the first year of life seems to be health protective (Hesselmar et al 1999). On the other hand, a study on 10,851 1-6 year-old children in Sweden showed that day-care attendance was not protective against any classical allergic symptoms, *e. g.* wheezing, cough, asthma, rhinitis, eczema etc (Hägerhed-Engman et al 2006). Thus, some studies tend to support the hygiene hypothesis and some seem to contradict it. One explanation may be that the hygiene hypothesis is valid or not depending on the microbial flora that we are exposed to. Interestingly, short chain lengths of 3-hydroxy fatty acids from LPS (of Gram-negative bacteria) have related to protective effects for asthmatic symptoms while longer chain lengths have related in the opposite way. Short carbon chain lengths of 3-hydroxy fatty acids (C10, C12 and C14) are found in, *e.g. Escherischia coli* and *Pseudomonas* spp., while long carbon chain lengths (C16 and C18) are found in *Actinobacteria* (Hyvärinen et al 2006, Zhao et al 2008). However, to the best of the author's knowledge there is no support for anything equivalent to the hygiene hypothesis concerning molds or mycotoxins since there are no studies demonstrating that any level of mold exposure could be to advantage in health development.

In 2004 the Institute of Medicine (in the USA) published a review article –*Damp Indoor Spaces and Health*- summarizing the evidence of associations between damp indoor environments and mold and health outcomes. According to the Institute there is sufficient evidence for an association between exposure to dampness and mold or other agents in indoor environments and upper respiratory tract symptoms (including nasal congestion, rhinitis, allergic rhinitis, sneezing, runny or itchy nose, sinusitis, and sore throat), cough, wheeze, chest tightness, shortness of breath, and asthma symptoms in sensitized persons with asthma. Moreover, there is sufficient evidence for an association between such exposure and hypersensitivity pneumonitis in susceptible persons as well as severe respiratory infections in immunocompromised patients (*e.g.*

persons undergoing high-dose cancer chemotherapy, recent recipients of solid-organ transplants) and colonization and potential lung infection in patients with chronic pulmonary disorders (*e. g.* cystic fibrosis, asthma, and chronic obstructive pulmonary disease). However, there is limited evidence for associations with respiratory illness in otherwise healthy children, and inadequate or insufficient evidence for associations with respiratory illness in healthy adults. There is also inadequate or insufficient evidence for associations between dampness and mold exposure and pulmonary hemorrhage, neurological effects, or cancer (Institute of Medicine 2004).

Microorganisms in water damaged indoor environments

The specific features of a moisture intrusion problem vary according to building construction practices and climate differences. Moisture and dampness indoors mainly result from construction flaws and inadequate maintenance of the building. Sources of moisture can be intrusion of rain, flood or snow in a leaky basement, wall or attic, leaky waste or water pipes or condensation problems such as faulty window framing and inappropriate location of the vapor barrier in the wall cavity. Another example is lack of ventilation in attics and crawl spaces or behind closets and drawers. Occupant behavior may also contribute to the problem. Bad practices are, *e. g.* not venting dryers to the outside, and not maintaining plumbing, heating, ventilation or air/conditioning systems (Dillon et al 1999).

We are daily exposed to various microorganisms. However, there are some specific microorganisms that especially thrive, and thus have found their niche, in moist and water damaged indoor environments, *viz.* the indoor microflora shift when a building is damaged by water or moisture intrusion. A variety of microorganisms has been found in water-damaged indoor environments, *e. g.* amoebas (Yli-Pirilä et al 2006), mycobacteria (Torvinen et al 2006), actinomycetes (Hyvärinen et al 2006), other opportunistic bacteria, and a diversity of fungi (Dillon et al 1999, Hyvärinen et al 2002). Nevalainen and Seuri (2005) suggest that the microflora of each moisture-damaged building may be unique. Fungal growth mainly relates to building dampness (Meyer et al 2004) while the inhabitant themselves can be a major source of gram-positive bacterial exposure (Fox et al 2005).

Exposure assessment methods for mold

Fungi are prevalent indoors as well as outdoors and culture is the most common method for detection and identification of the mycoflora. The culture media most commonly used vary between laboratories and may not be optimal for water-damaged building related fungi as they were originally designed for fast-growing food-borne fungi (Andersen and Nissen 2000). Typing is generally performed to genus level and more seldom to species level. The latter analysis is demanding to the mycologist, *e. g.* the *Penicillium* family consists of hundreds of species, with the subgenus *Penicillium* alone constituting at present of 58 species (Samson and Frisvad 2004). Fungal identification can also be performed chemically based on metabolic profiles (Nielsen et al 2003).

In one strategy for the interpretation of culture results it is assumed that it is primarily soil fungi that grow on building materials, thus the ratio of the sum of the concentrations of soil fungi vs. phylloplane fungi should be near 1 if the mycoflora is not being amplified indoors (Dillon et al 1999). The extent of fungal damage on surfaces indoors has been well correlated to the number of cultivable spores in the indoor air (Hunter et al 1988, Miller et al 2000, Rao et al 2007, Schwab et al 2007).

One weakness of using culture is that it merely illustrates which live mold spores grow on a specific medium used under the conditions provided during a certain amount of time (generally 5-7 days). Only a small fraction of the mold spores in indoor environmental samples are alive and cultivable. Yet, dead microbial material can still contain toxic metabolites, allergens etc (Green et al 2006, Wilson et al 2004). In a culture medium or a water-damaged building material the secondary metabolite production, even within a single isolate (Jarvis 1995), may vary over time due to fluctuations in water-activity, nutrition and coexisting microbial flora. Slow growing species like *Stachybotrys* spp. are frequently highly underrepresented using culture (Miller et al 2000). Pietarinen et al (2008) found that qPCR determination of fungi and *Streptomyces* generally showed higher concentrations and prevalence than culture.

PCR has been used to identify microorganisms in indoor environments. This method can also determine if an organism has the genetic ability to produce a toxic metabolite, *e.g.* by detecting the *Tri5* gene encoding trichodiene synthase essential in the early

synthesis pathway of trichothecene production in *Stachybotrys* spp. (Land et al 2003, Peltola et al 2002), without showing if the genes in question are actually expressed. Also, there are concerns of cross-reactivity, *e.g.* the primers and probes used by Haugland (1999) did not differentiate *S. chartarum* from *S. chlorohalonata* and *S. yunnanensis* (Li and Yang 2005).

Other PCR methods use primers directed at the nuclear ribosomal RNA operon, *e.g.* the Mold Specific Quantitative PCR method (MSQPCR) developed by Haugland and Vesper (2002, US patent 6,387,652) which was partly funded by (and approved as an analytical tool at) the U. S. Environmental Protection Agency (EPA). This method was developed to be used for indoor environmental samples, *e. g.* airborne particles (Chew et al 2006), house dust, and other bulk samples (Paper III, Vesper et al 2004 and 2007). Over 130 different indoor fungi can be identified and quantified by this technology, and detection sensitivity is high, *e. g.* for *S. chartarum* 2 fungal genome copies (Haugland et al 1999, Roe et al 2001). It is recommended to include a reference sequence and to dilute the DNA extract prior performing the analysis (samples should not contain >0.2 mg of dust/ml) for monitoring and preventing PCR inhibition (Haugland et al 1999, Keswani et al 2005, Roe et al 2001). The MSQPCR method has been found to be reproducible and more robust than conventional PCR in overcoming inhibition (Keswani et al 2005). Using MSQPCR analysis of house dust, asthmatic children in North Carolina has showed higher indoor mold burdens than US median homes (Vesper et al 2007).

By using ELISA, in a study comprising of 831 housing units and 2,456 individuals in the USA, exposure to *Alternaria alternata* was associated with active asthma symptoms. In this study specific *A. alternata* -antigens in vacuumed house dust were measured using a polyclonal anti- *A. alternata* assay (Salo et al 2006). Other ELISA assays for mold antigens in indoor environmental samples are being developed, *e. g.* based on human monoclonal antibodies to an extracellular protein (SchS) of *S. chartarum* (Xu et al 2008). Furthermore there are also immunoassays using antibodies directed against *e.g.* stachyhemolysin and stachyrase-A (Vojdani 2005), and the spore-wall antigen SchS34 of *S. chartarum* (Rand and Miller 2008). In ELISA, however, possible cross-reactivity among fungi has to be taken into account (Schmechel et al 2006).

The endotoxin of Gram-negative bacteria and the cell wall component (1-3)-β-D-glucan of fungi can be measured by *Limulus* Amebocyte lysate assays. (1-3)-β-D-glucan has been used as a measure of fungal exposure. However, the (1-3)-β-D-glucan content in fungal spores differs widely between species, *e. g. Cladosporium* spp. and *Aspergillus* spp. produce relatively large amounts whereas *A. alternata* produce very low levels (Iossifova et al 2008). In addition, there are also other sources of (1-3)-β-D-glucan, such as pollen, plant material and soil bacteria (Lee et al 2006). Therefore, the use of (1-3)-β-D-glucan as an exposure marker for fungi has been questioned (Iossifova et al 2008). Nevertheless, airborne (1-3)-β-D-glucan, indicated to be of fungal origin using size exclusion chromatography, has been strongly correlated to ergosterol (ERG) and visible mold. ERG on the other hand was more highly correlated to visible mold than (1-3)-β-D-glucan (Foto et al 2005).

ERG is a sterol exclusively found in fungal cell membranes and can be a measure of fungal biomass (Larsson and Saraf 1997, Sebastian and Larsson 2003). Spore content of ERG is generally about 1±0.25 μg/mg among common indoor fungi (Miller and Young 1997) although it may vary between species (Bermingham et al 1995, Robine et al 2005). ERG concentrations in solid materials has been related to active biomass (Bjurman 1994), and been shown to be in agreement with viable fungal concentrations in building material samples (Pasanen et al 1999). Interestingly, the indoor air exposure to microorganisms, measured by determination of microbial chemical markers in airborne dust using gas chromatography- mass spectrometry (GC-MS), vary markedly between countries, seasons, and between urban and rural regions (Wady et al 2004).

Molds and their biologically active metabolites.

Hundreds of fungal taxa have been observed indoors (Scott 2001). Normally, the most common airborne fungal genera found in indoor environments are *Cladosporium* spp., *Penicillium* spp, *Aspergillus* spp. and non-sporulating molds (Hunter et al 1988, Shelton et al 2002). If the normal indoor mycoflora is shifted however, as in case of water-damage, a relatively small number of species commonly dominate as fungi are predominately restricted by water-activity. The building materials most prone to mold

contamination in general are water-damaged and aged organic materials such as wooden materials, jute, wallpaper, and cardboard (Gravesen et al 1999).

In water-saturated building materials *S. chartarum*, *C. globosum*, and *M. echinata* are associated with materials containing cellulose (most often the paper layers of gypsumboard), *Penicillium* spp., yeasts, and rot fungi occur in wood (including particleboard and plywood), and ceramic products, paints and glue seem to favor *Acremonium* spp. and *A. versicolor* (Dillon et al 1999, Hyvärinen et al 2002). The xerohilic mold *A. versicolor* (as well as *P. aurantiogriseum*, *P. viridicatum*, *P. brevicompactum*, and *Paecilomyces variotti*) may also contaminate wetted or damp wallboard if water activity decreases. An additional 20 species (approximately) have been regarded as important indoor contaminants of water-damaged housing (Dillon et al 1999).

The biomechanics of mold spore dispersal varies with type of substrate and mold species. In general *S. chartarum* spores are not easily released at airspeeds common in indoor environments (Tucker et al 2007), although more spores are dispersed from building materials than from culture media. In contrast, the aerosolization of particles from *A. versicolor* cultured on malt extract agar (MEA), ceiling tiles, and gypsumboard is fairly constant (Seo et al 2008). Spore emissions at low air velocity flow have shown to be directly proportional to airflow and indirectly proportional to relative humidity (Menentrez and Foarde 2004). Thus, spore aerosolization may increase when mold contaminated material is dried. Even after long periods of water deprivation molds may still be cultivable and toxic (Wilson et al 2004), and both spores and fragments, also from previously overlooked genera, have been identified as sources of allergens (Green et al 2006).

Health effects of exposure to molds have been reviewed in literature, *e. g.* (Curtis et al 2004, McGinnis 2004, Mazur et al 2006, Simon-Nobbe et al 2008). Both single substances and synergistic effects contribute to the toxicity (Shulz et al 2004), *e.g.* bacterial LPS significantly increases the toxic effects of trichothecenes (Zhou et al 2000). The biological activity of microbial spores may vary depending on type of growth substrate; *viz.* inflammatory response and cytotoxicity in cell cultures have shown to differ with building material on which the microorganisms grow (Murtoniemi et al 2001, 2002, 2003a and b, Roponen et al 2001).

Mold levels in dust were found to be associated with new-onset of asthma in office employees in damp indoor environments (Park et al 2008) and the severity of asthma was associated with sensitization to mold (Zureik et al 2002). The risk of developing asthma in young children has shown to increase with the severity of moisture damage and visible mold in the main living quarters (Pekkanen et al 2007), and mold odor was associated with eye symptoms in day-care centers (Ruotsalainen et al 1995). Furthermore, chronic indoor exposure to mold was associated with inflammatory markers in nasal lavage fluid and high prevalence of respiratory symptoms in school employees (Hirvonen et al 1999). In the Leipzig Allergy Risk children Study (LARS) a significant association was found between the incidence of respiratory tract infections and exposure to *Penicillium* spp. spores. In addition, exposure to *Aspergillus* spp. was associated with allergic rhinitis and related symptoms, and significantly lower numbers of Th1 cytokine-producing (IFN-γ, TNF-α, IL-2) T-cells (Müller et al 2002).

(1-3)-β-$_D$-glucan appers to affect the immune system, *e.g.* in non-atopic individuals a dose-response relationship was found between levels of (1-3)-β-$_D$-glucan in house dust and the IFN-γ/IL-4 ratio in serum of the exposed residents (Beijer et al 2003). Immediate toxicological effects in cell cultures on the other hand, have predominately been induced by the growth medium, thus exometabolites/toxins are likely to be responsible for lung damage than *e. g.* fungal cell wall components (Piecková et al 2006).

Many molds that thrive in damp indoor environments are potent mycotoxin producers and may play a role in the reported adverse health effects (American Academy of Pediatrics: Committee on Environmental Health 1998, Bush et al 2006, Müller et al 2002, Nevalainen and Seuri 2005, Robbins et al 2000, Salo et al 2006). Mycotoxins have been extensively reviewed in literature (Bennett and Klich 2003, Jarvis 2002, Jarvis and Miller 2005, Miller 1992, Nielsen 2003, Samson 1992), along with their *e.g.* immunomodulatory and neurotoxic effects (Bondy and Pestka 2000, Campbell et al 2004, Kilburn 2004, Kuhn and Ghannoum 2003). Experiments in animal models show that inhalation of mycotoxin is several times more toxic than ingestion (Cresia 1987 and 1990). However, the most important question - if airborne mycotoxins *at concentrations found in mold damaged indoor environments* make us sick - is yet to be answered.

Mycotoxins are secondary metabolites produced by molds, *e.g.* to gain strategic advantages over encroaching organisms, and have been suspected of being used in chemical warfare (in "Yellow rain" in Laos, Watson et al 1984). The genes for synthesis of mycotoxins, *e.g* aflatoxin and sterigmatocystin (STRG) among Aspergilli, are well conserved. Mycotoxin production in *Aspergillus* spp. is coupled to the sporulation process and is influenced by water activity, temperature, pH, carbon and nitrogen source (Calvo et al 2002). The higher the water activity is in a substrate the higher the secondary metabolite and mycotoxin production (Nielsen et al 2004). A given mycotoxin may be produced by different molds and one single mold species can have the ability to produce several mycotoxins. For example, in *A. nidulans*, penicillin and STRG production are oppositely regulated by pH (penicillin is favored in alkali and STRG in acidic environments, reviewed by Calvo et al 2002).

Based on spore counts, the airborne mycotoxin concentrations found in damp buildings have been speculated to be insufficient for causing adverse health effects (Bush et al 2006, Kelman et al 2004). However, indoor molds may fragment into very small airborne mycotoxin-containing particles, resulting in up to a 500-fold larger exposure than assumed previously (Brasel et al 2005, Górny et al 2002, Kildesø et al 2003, Sørenson et al 1987). In addition, Cho et al (2005) showed that the respiratory deposition of *S. chartarum* fragments was over 200-fold higher than of spores in adults and an additional 4 to 5 times higher in infants.

Stachybotrys grows on materials rich in cellulose such as bedding straw for domesticated animals (Harrach et al 1983) and on the paper lining of gypsum boards (paper I, II and IV, Gravesen et al 1999, Nielsen et al 1998a and b). This mold needs high water activity to be established, *viz.* if a gypsum board is wetted *Stachybotrys* will likely start to grow (Menentrez et al 2004) even without artificial inoculation (Price and Ahearn 1999).

Stachybotrys spp. produces a vaste array of toxic compounds, *e.g.* atranones (Hinkley et al 1999 and 2003), spirocyclic drimanes (Jarvis et al 1995), MVOCs (Gao and Martin 2002), proteinases (Yike et al 2007), siderophore and hemolysins (Vesper et a 2000), MTRs (El-Maghraby et al 1991, Grove 1993, Jarvis et al 1995), and simple

trichothecenes such as trichoverrol A, trichoverrol B, trichodermin and trichodermol (Nielsen 2002).

S. chartarum (Figure 1 and 2) has been involved in disease outbreaks (Croft et al 1986, Hodgson et al 1998, Johanning et al 1996) and linked to pulmonary hemorrhage in animals and humans (CDC 1995, Elidemir et al 1999, Flappan et al 1999, Jarvis et al 1998). This mold -the "toxic black mold"- has attracted media interest (New York Times 2001) and also been extensively reviewed in scientific literature (Hintikka 2004, Jarvis 2003, Masten 2004, Pestka et al 2008). The pulmonary effects have been reviewed by Yike and Dearborn (2004). The effects of airway exposure to *S. chartarum* and its mycotoxins are dose-dependent (Flemming et al 2004, Leino et al 2003, Rand et

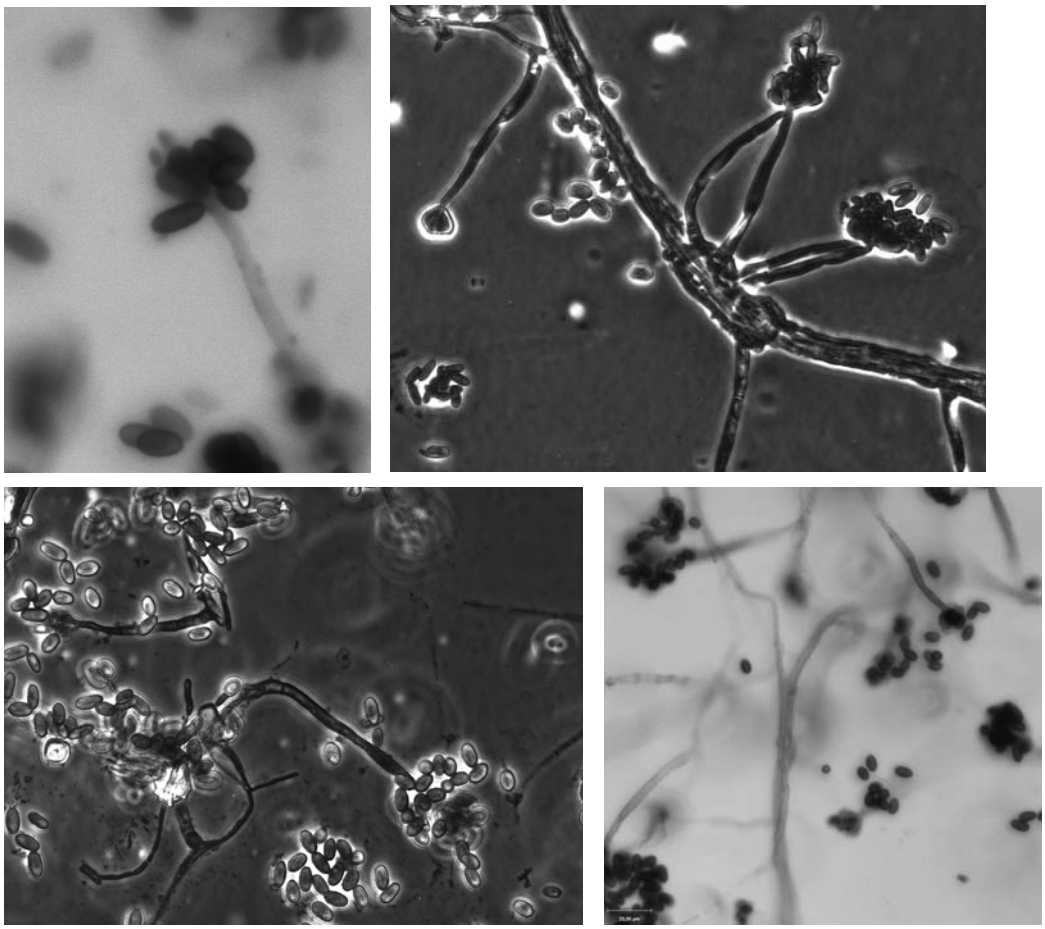


Figure 1. Light and phase-contrast microscope photographs of *S. chartarum*.

al 2006, Rao et al 2000) even at low spore doses (1 to 1.5 x 10^4 spores/kg bodyweight, Flemming et al 2004). The effects seem related to alcohol-soluble toxins in the spores (Rao et al 2000, Yike et al 2001). Exposure to *Stachybotrys* combined with other atypical fungi in occupational environments is associated with lower respiratory-, dermatological-, eye-, constitutional-, and chronic fatigue symptoms, as well as fewer T-cells and dysfunction (Johanning et al 1996). There has been no correlation found between IgE or IgG antibodies against *Stachybotrys* and disease (Johanning et al 1996, Hodgson et al 1998, Savilahti et al 2002). Antibodies to mold and satratoxins in individuals exposed in water-damaged indoor environments have been reviewed by Vojdani et al (2005).

There are at least 3 different species of *Stachybotrys* based on metabolite production: *S. chlorohalonata*, *S. chartarum* chemotype S, and *S. chartarum* chemotype A (Andersen et al 2002 and 2003). There seem to be no correlation between these species and geographic area (Andersen et al 2002, Cruse et al 2002, Elanskii et al 2004). *S. chartarum* chemotype A produces inflammatory atranones and simple thrichothecenes such as trichodermin (Rand et al 2006), whereas chemotype S produces cytotoxic MTRs which have been detected in sera from individuals and pets exposed to *S. chartarum* in water-damaged indoor environments (Brasel et al 2004, Mader et al 2007, Yike et al 2006). The MTR SATG can also, at low levels, specifically target olfactory sensory neurons in mice initiating inflammatory response (rhinitis) in the nose which further extends into the brain resulting in mild focal encephalitis (Islam et al 2006).

The toxicological properties are different between *Stachybotrys* strains (Hudson et al 2005, Nielsen et al 2002, Nikulin et al 1996). Murine lung responses generated by spores from *S. chartarum* evoke rapid inflammation which is sustained 4 times longer for chemotype A than for chemotype S (Flemming et al 2004). The cytotoxic effects observed seem to derive from the MTRs of *S. chartarum* chemotype S (Nielsen et al2002), although the liquid culture medium of indoor-originated *S. chartarum* chemotype A has also been observed to be cytotoxic when intratracheally instilled in Wistar rats (Piecková et al 2006).

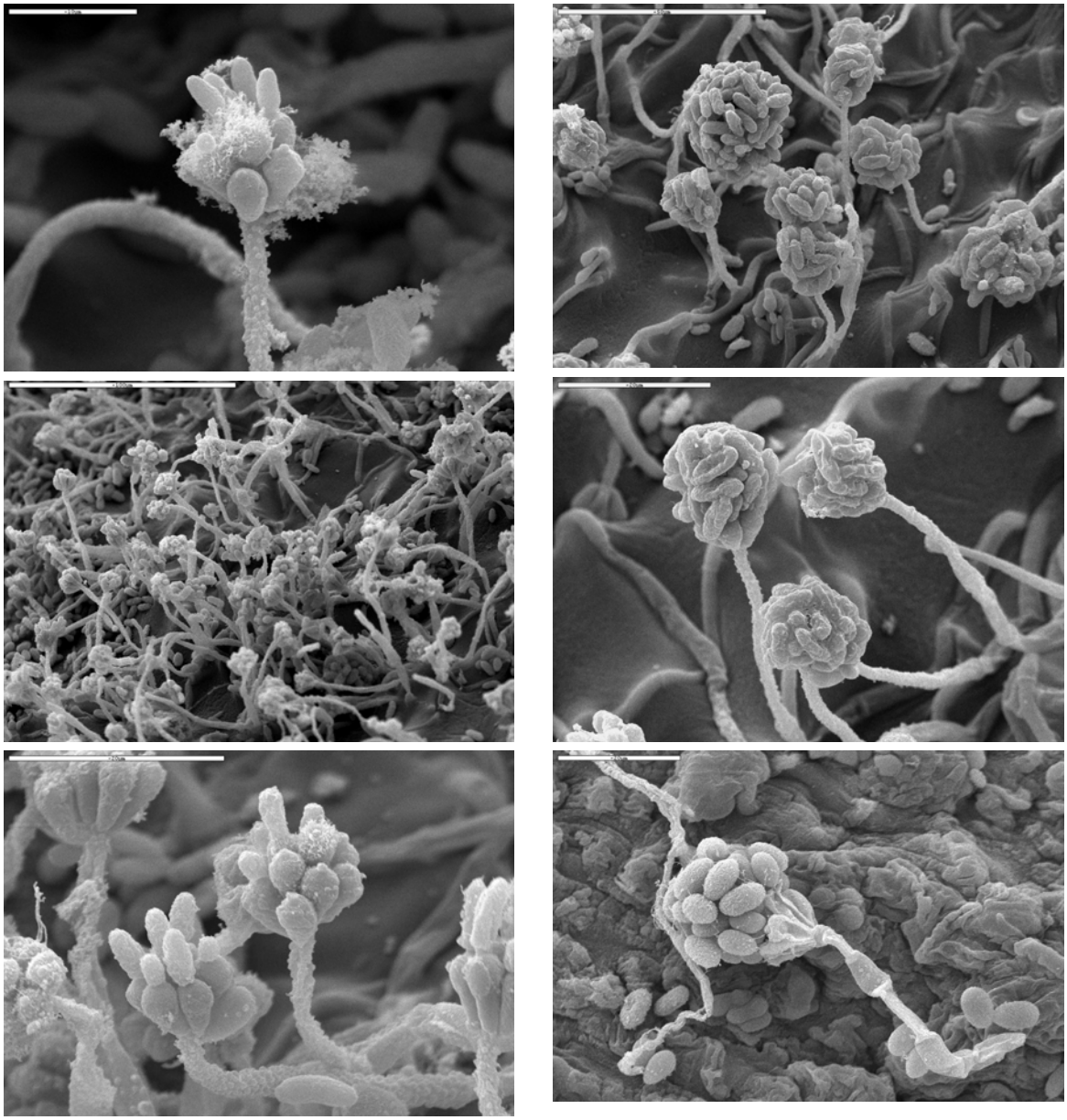


Figure 2. Scanning electron microscopy pictures of *S. chartraum* chemotype S. The samples were fixed with osmium tetroxide vapour and critical point dried.

Co-cultivation of *S. chartarum* and *Streptomyces californicus* has shown to increase the ability of spores to cause apoptosis (by > a factor 4) and cell cycle arrest, and affects the inflammatory response in mouse macrophages (Penttinen et al 2005a and b). Other synergistic effects have also been observed between *Streptomyce*s spp. and *Stachybotrys*, (Huttunen et al 2004, Penttinen et al 2006, Penttinen et al 2007), between TRID and

the bacterium *Streptomyce*s spp. (Huttunen et al 2004), and between *Stachybotrys* sp and *A. versicolor* (Murtoniemi et al 2005).

The actinomycete *Streptomyces* is a gram-positive soil bacterium often isolated from moisture-damaged buildings. Spores of *S. californicus* has been shown to provoke toxic effects in the lungs of mice at the same level of exposure as spores of *S. chartarum* after repeated intranasal instillations (Nikulin et al 1997). The toxic effects of *S. californicus* seem not however be limited to the lungs of mice but also include exposure-induced effects in spleen and lymph nodes (to where intense recruitment of neutrophils, macrophages and lymphocytes is seen which suggests that both adaptive and non-adaptive immunological responses are involved). The immunostimulation in the lungs and the systemic immunotoxicity, especially in the spleen are effects which resemble those caused by chemotherapeutic agents (Jussila et al 2003).

Well-known mycotoxin producers in the *Aspergillus* family are *A. flavus* and *A. parasiticus* who produce the highly carcinogenic aflatoxins (IARC 2002) via the precursors STRG (aflatoxin B_1, AFLAB, aflatoxin G_1) and dihydro-STRG (aflatoxin B_2, aflatoxin G_2). AFLAB-production by *A. flavus* may be inhibited though antagonistic interactions with a variety of other mold strains (including non-AFLAB-producing *A. flavus* strains, Cvetnic´ and Pepeljnjak 2007). Aflatoxigenic strains of *A. flavus* and *A. fumigatus* who produce mycotoxins in culture may not do so on building materials even without competition (Ren et al 1999). *A. versicolor* on the other hand, one of the most commonly encountered molds in water-damaged indoor environments, produces STRG and 5-methoxy-STRG (Gravesen et al 1999). *A. versicolor* lacks the enzymatic pathway necessary to convert these precursors to the corresponding aflatoxins; hence, it emits large amounts of the carcinogenic compound STRG (Frisvad 1989). The biological activity of STRG, measured as its ability to initiate bile duct hyperplasia in ducklings, is found to be 125 times lower than of AFLAB (Lillehoj and Ciegler 1968). In the study of Sumi et al (1994) inhalation of *A. versicolor* spores over a period of 6 months resulted in granulomatous lesions in the lungs of exposed germ-free rats.

Another member of the *Aspergillus* family is *A. fumigatus*, often the cause of aspergilliosis in immunocompromised patients. The mycotoxin GLIO is suspected to be a virulence factor of *A. fumigatus* and has been determined in the serum of patients

suffering from aspergilliosis (Lewis et al 2005). GLIO is also produced by species of the *Penicillium* genus, including *P. citrinum*, and by other *Aspergillus* spp and *Monascus* spp (Kupfahl et al 2008). The toxicity of GLIO has been shown to be due to the presence of a disulphide bridge (Müllbacher et al 1986), capable of inactivating proteins via reaction of thiol groups and of generating reactive oxygen species (Gardiner et al 2005). GLIO was first considered as an antibiotic (Timonin 1942) but was later excluded from medical use as it was found to be neurotoxic (Axelsson 2006) and immunosuppressive (Orciuolo et al 2007), *e.g.* genotoxic to human lymphocytes (Dönmez-Altuntas et al 2007). In addition, GLIO has, along with the mycotoxins citrinin and patulin (also produced by *Aspergillus* and *Penicillium* spp), been shown to be immunomodulatory at picogram levels in CD3-CD28-stimulated human peripheral blood mononuclear cells (PBMCs). IFN-γ production was inhibited, an effect caused by a reduction of the number of IFN-γ-producing T lymphocytes rather than by a reduced functional capacity of the individual cells, thus, causing a T-cell polarization towards a Th2 phenotype (Wichmann et al 2002). This Th2-polarization has been shown to be accompanied by the up-regulation of IgE-syntesis (Wichmann et al 2003). When exposing human monocytic cells to low doses of GLIO (100 ng/ml) and citrinin (less than 10 μg/ml) a cytokine imbalance was observed with a reduction of Il-10 concentrations compared to those of TNF-α and IL-6, potentially resulting in an increased risk of an inflammatory response (Johannessen et al 2005). Citrinin exposure to human alveolar epithelial cells, at nontoxic concentrations, also causes depletion of intracellular glutathione suggesting an increased susceptibility to inflammatory trigger agents in the environments, such as LPS or other microbial components (Johannessen et al 2007)

Detection of mycotoxins

Today, standardized methods for determination of microbial constituents and products are lacking. There are over 200 mycotoxins identified to date, as well as fungal proteinases (Yike et al 2007) including ribotoxins (Lacadena et al 2007) and hemolysins (Van Emon et al 2003, Vesper et al 2001, Vesper and Vesper 2002), and toxins and uncharacterized biologically active compounds from bacteria, *e. g. Streptomyces*

(Andersson et al 1998). The biologically active mold components determined so far are few and merely the tip of the iceberg.

One approach of measuring mycotoxins indoors is to determine their toxic activity, *e.g.* as in the luciferase translation bioassay which is based on trichothecenes inhibition of firefly luciferase in cell culture (Yike et al 1999). However, the toxic effects of *S. chartarum* seem to be related to methanol-soluble toxins in the spores (Rao et al 2000a) and in the *in vitro* luciferase protein translation assay the presence of alcohol is not desirable as it has been shown to inhibit the reaction in a dose dependant manner (Black et al 2006). Toxic substances may however be removed from the spore surface by washing in aqueous solution (Karunasena et al 2004) and the luciferase translation bioassay has shown to be a convenient method giving reproducible results. Nevertheless is does not demonstrate the toxin composition of environmental samples (Yike et al 1999).

ELISA incorporated with macrocyclic trichothecene-specific antibodies has been used in detecting MTRs in airborne dust (Charpin-Kadouch et al 2006, Brasel et al 2005) and in serum samples from individuals exposed to mold (primarily *Stachybotrys* spp., Brasel et al 2004). In addition, antibodies produced against the hemolytic agent stachylysin (produced by *Stachybotrys* spp.) were successfully used in an ELISA analysis of serum samples from human adults working in water-damaged buildings with *Stachybotrys*-contamination (Van Emon et al 2003).

Mass Spectrometry

Mass spectrometry (MS) is a chemical analytical technique which has both qualitative and quantitative uses, such as identifying unknown compounds, determining a compounds' isotopic element, structure (by observing its fragmentation), amount in a sample, or other physical, chemical, or biological properties. In MS the composition of a compound or sample is identified on the basis of the m/z ratio of charged particles. A MS system has three essential modules: an ion source which transforms the molecules in a sample into ionized fragments; a mass analyzer, which sorts the ions by applying electric and magnetic fields; and a detector, which measures the value of an indicator quantity and thus provides data for calculating the abundances each ion

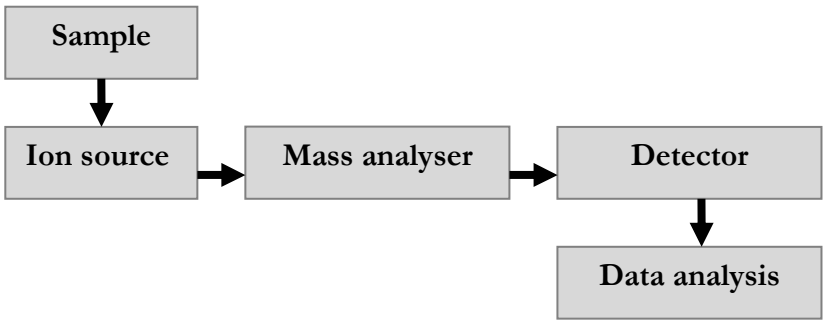


Figure 3. The principle construction of a mass spectrometer.

fragment present (http://wikipedia.org/) (Figure 3). There are different types of mass analyzers such as magnetic sector analyzers, quadrupole mass filters, quadrupole ion traps, time-of-flight analyzers, and ion cyclotron resonance instruments (Chapman 1993).

In the ion trap all analyses events may be separated in time but not in space. Ions may be ejected using resonance excitation, whereby a supplemental oscillatory excitation voltage is applied to the end cap electrodes and the trapping voltage amplitude and/or excitation voltage frequency is varied to bring ions into a resonance condition in order of their m/z ratio. Also a non-resonance mode may be applied (Chapman 1993).

Quadrupole mass analyzers use radio frequency and oscillating electrical fields between four parallel rods to selectively stabilize or destabilize ions passing through to the detector, *i. e.* it acts as a mass selective filter (Odham and Larsson 1984). A common variation of the quadrupole is the triple quadrupole (papers I-IV), in which a linear series of three separate quadrupoles is used. The first (Q1) and third (Q3) quadrupoles act as mass filters, and are separated by the middle non-mass filtering (q2) quadrupole, which acts as a collision cell (using argon gas, paper I-IV). When collecting data in the full scan mode, a target range of mass fragments is determined, a method useful in determining unknown compounds in a sample. During instrument method development chemical standards are first analyzed in full scan mode to determine the retention time and the mass fragment fingerprint before moving to a SIM method. SIM only monitors selected peaks associated with a specific substance entered into the

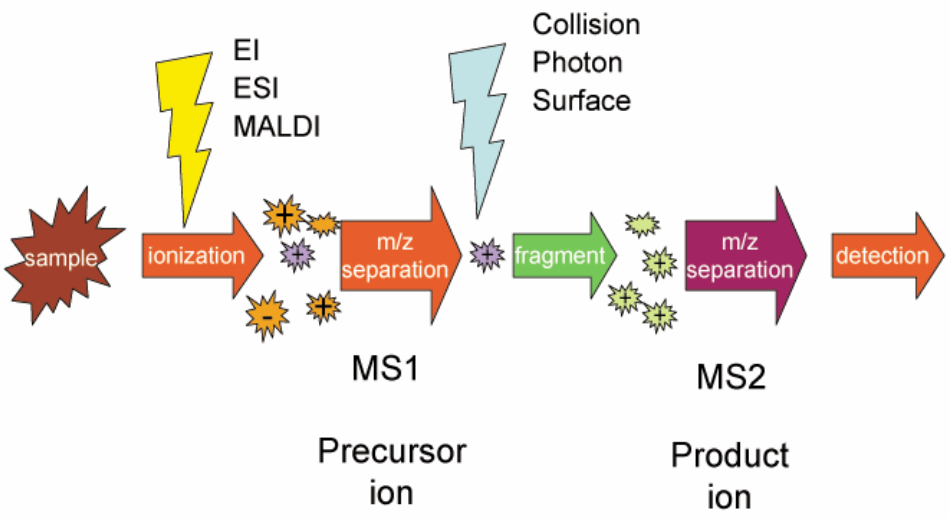


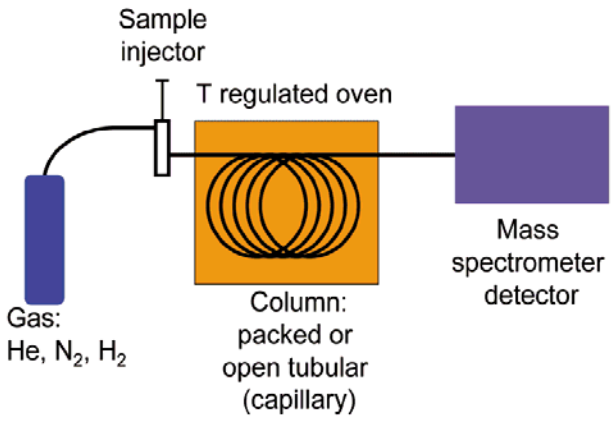
Figure 4. Simplified scheme of the principle of tandem mass spectrometry.

instrument method. One advantage of SIM is that the detection limit is lower since the instrument is only monitoring at a small number of fragments during each scan, thus, more scans can take place per time unit. In addition, since only a few mass fragments of interest are being monitored, matrix interferences are generally lower. In tandem mass spectrometry (MSMS), Q1 is set to a specific mass, *i. e.* a precursor ion, and q2 is filled with gas (*e. g.* argon) with which the selected ions collide resulting in fragmentation. Q3 can then either be set to scan all generated fragment ions in a spectrum (MS scan) or to monitor a single/few selected fragment ion(s) (MSMS) (Figure 4). In this method, the tallest peak is assigned 100% of the value, and the other peaks are assigned proportionate values, which are used in identification of specific compounds. In quantitative MS it is essential to use SIM or MSMS results in relation to an internal standard (paper I-IV) (Chapman 1993).

GC-MS

GC is used in, mainly, analytical work to separate different compounds in gas phase (Figure 5). A stream of heated carrier gas called the mobile phase carries the sample molecules through the gas chromatograph. The sample is introduced into an injector

where it is volatilized and then swept through the chromatographic column (the stationary phase). Here the sample molecules distribute between the stationary (liquid or solid) and the mobile (gas) phase according to their partition coefficients. The sample molecules are selectively retarded according to their interactions with the stationary phase, *i. e.* the greater the solubility in the stationary phase, and the higher the partition coefficient, the longer the time from injection until the sample molecule reaches the MS detector. This elapsed time, the retention time, is determined by the carrier-flow rate, the type of column and the temperature. If analysis is performed appropriately each peak in the recorded chromatogram (by the MS) represents each of the separated sample components. The area under each peak can be correlated to the concentration of the corresponding component in the injected sample (Odham and Larsson 1984). All compounds with relatively high vapor pressure can be analyzed by GC. Molecules with low or no volatility may also be analyzed with GC after derivatization, a process where one or more hydrogen atoms bound to an oxygen or nitrogen atom are replaced with an atom or molecule which reduces the polarity/boiling point of the molecule. Macromolecules may be degraded into smaller units and derivatized before analysis, *e. g.* as in the case of verrucarol (VER) and TRID (paper I-IV, Ohham and Larsson 1984).



T denotes temperature.

Figure 5. Principles of gas chromatography (here exemplified with a MS detector).

The most common form of ionization in GC-MS is electron ionization (EI). Separated gaseous compounds are fed online from the GC into the MS ion source where they are bombarded with free electrons emitted from a charged metallic filament, causing an ionization that fragments the molecules. In chemical ionization (CI) (positive ion CI, PICI, or negative ion CI, NICI) a reagent gas, *e. g.* methane (paper I) or ammonia (paper II-IV), is introduced into the ion source. The molecules of the reagent gas are ionized by the electrons omitted by the filament and the formed ions then react with the introduced sample molecules causing an ionization of the analyte molecules. CI is a softer ionization technique than EI. One of the main benefits of using CI is that a mass fragment closely corresponding to the molecular weight of the analyte of interest is produced (Chapman 1993).

HPLC-MS

Similar to GC-MS liquid chromatography MS (LC-MS) separates compounds chromatographically, however, the mobile phase is liquid, usually a mixture of water and organic solvents, *e. g.* methanol or acetonitrile. The column holds a chromatographic packing material (the stationary phase), and a pump moves the mobile phase(s) through the column. The sample analyzed is introduced into the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase as it traverses through the column. The amount of retardation generally depends on the nature of the analyte, stationary phase and mobile phase composition. The use of high pressure (high pressure LC, HPLC) increases the linear velocity which gives the sample molecules less time to diffuse within the column, leading to improved resolution in the resulting chromatogram. A refinement in HPLC is to vary the mobile phase composition during the analysis, *i. e.* to create a gradient elution. The gradient separates the analyte molecules as a function of the affinity of the analyte for the current mobile phase composition relative to the stationary phase. The mobile phases may contain buffers or salts to assist in the separation of the analyte components or compounds such as TFA which acts as an ion pairing agent. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte.

The most commonly used form of HPLC is reversed phase HPLC in which a non-polar stationary phase (*e. g.* silica treated with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}) and an aqueous, moderately polar mobile phase are used. With this type of stationary phase, retention time is longer for molecules which are non-polar (*e. g.* C-H, C-C), while polar molecules (-OH, $-NH_2$, COO- or $-NH_3^+$) elute more readily. Due to the overall decrease in surface area branched chain compounds (or organic compounds with single C-C-bonds) elute more rapidly than their linear isomers. Organic compounds containing a C=C or C-C-triple bonds also tend to have relatively shorter retention times. The retention time is increased for an analyte by adding a polar solvent to the mobile phase, or decreased by adding a more hydrophobic solvent. The pH value of the mobile phase(s) changes the hydrophobicity of the analyte, and thus influence the separation process. To control the pH a buffering agent, such as sodium phosphate, formic acid or TFA is often added to the mobile phase. The buffers also neutralize charges on any residual exposed silica on the stationary phase and, in addition, act as ion pairing agents to neutralize charge on the analyte (http://wikipedia.org/).

In electrospray ionization (ESI) mode the analyte and mobile phase mixture is pushed, at a flow rate of a few microlites per minute, through a very small needle capillary together with a nebulizing gas (nitrogen for positive ESI and air for negative ESI). The outlet of the capillary, the capillary tip, has a charged potential (typically 3-5 kV) and when the analyte and mobile phase mix enters through the capillary tip into the ion source (spray chamber) the liquid flow convert into a mist of small droplets. Heated nitrogen (drying gas) is often used to facilitate the nebulizing and evaporation process, *e. g.* to minimize vacuum chamber contamination by solvent molecules. This produces a stable analyte ion current which communicates between atmosphere pressure and the first vacuum stage of the MS guided by a charged shield and passed through a long metal capillary tube that (Champman 1993). A schematic overview of the HLPC-MS interface is provided in Figure 6.

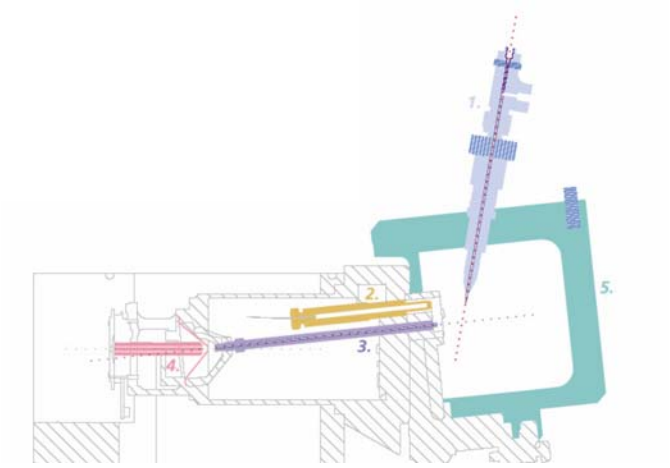


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Figure 6. A schematic view of an API interface between a HPLC and a MS detector. The mobile phase moves through the needle capillary (1) and enters the spray chamber (5) where a counter flow of heated, drying gas is introduced (2). The high temperature and high voltage of the needle results in rapid droplet desolvation of the mobile phase and the formed charged ions are guided by a high voltage shield through an inert, large diameter capillary (3) which leads to a skimmer and hexapole ion guide (4) inside the MS.

MS detection of mycotoxins

There are many studies conducted where mycotoxins were measured in dust collected in occupational environments, *e. g.* simple trichothecenes in grain dust (Nordby et al 2004), aflatoxins and ochratoxin A in air samples in a coffee factory (Tarín et al 2004), and ochratoxin A in airborne dust from Norwegian cow farms (Skaug et al 2000). However, there are only a handful of studies where mycotoxins have been determined in samples collected in (water-damaged) residential environments, and in some of these studies molds from such indoor environments were first inoculated onto culture media or building materials prior chemical analysis of mycotoxins (Nielsen et al 1998b, Nieminen et al 2002, Nikulin et al 1994, Tuomi et al 2001). In direct analysis, without prior culture, HPLC-MSMS has been used to demonstrate satratoxins and STRG in indoor building material samples (paper II and IV, Tuomi et al 1998 and 2000), STRG in carpet dust (Engelhart et al 2002), and SATG and SATH in air samples (Gottschalk et al 2008).

For *Stachybotrys* trichothecenes a convenient GC-MS screening method was applied (paper I, II, and IV, Croft et al 1986, Gravesen et al 1999, Nielsen et al 1998a). This method provides information of the total trichothecenes content derived from *Stachybotrys* spp. (Hinkley and Jarvis 2001, Nielsen et al 1998a and b). In this analysis VER and TRID, hydrolysis products of *S. chartarum* MTRs and trichodermin respectively, are determined (Bata et al 1985, Harrach et al 1981, Jarvis et al 1984, Krishnamurthy et al 1986, Szathmary et al 1976). The deacetylation process of trichodermin also occurs naturally when entering, *e. g. Mucor* cells (Fonzi and Sypherd 1986).

Interestingly, SATG covalently bound to lysine, cysteine, and histidine has also been detected by MS as adducts in serum samples of human and cat residents in *S. chartarum* -contaminated buildings (Mader et al 2007, Yike et al 2006).

AIMS OF THE STUDY

The aims of this thesis were:

1. To develop GC-MS and HPLC-MS analytical methods for determination of selected mycotoxins typically produced by molds encountered in water-damaged buildings.
2. To apply these methods for direct mycotoxin analysis of building materials and dust samples from authentic water-damaged environments.
3. To study the prevalence of selected potent mycotoxins in samples from indoor environments with verified mold damage.
4. To initiate studies on potential associations between mycotoxins and fungi as analysed by culture, PCR, and determination of ergosterol. .

MATERIAL AND METHODS

Samples

All studied samples were collected in dwellings with a history of water damage; the types of buildings/locations (for samples analysed in paper I, II and IV) are summarized in Table 1. Pieces of gypsum board, linoleum flooring, wall paper, tile, concrete, isolation, wooden skirting boards etc were collected by company personell specialized in remediation measures. In total 167 building material samples were analyzed for mycotoxins (paper I, II, IV); one hundred of these building materials were also analyzed for ERG (paper IV).

Settled dust samples (n=32) were collected on cotton swabs (n=4) and ALK filters (ALK-ABELLÒ A/S, Denmark) (n=28) in Swedish water-damaged dwellings (paper I, II, and IV), *viz.* in single-family houses (n=3), offices (n=3), apartments (n=2), churches (n=2), schools (n=2), and care facilities (n=2). Eighteen of these samples were analyzed for ERG (paper IV). In addition, bulk dust samples (n=7) were collected in 5 flood-damaged houses in New Orleans using a brush and pan (paper III).

Table 1. Houses/locations (n=88, n=14, and n=30) where samples (building materials, settled dust, and RCS cultures respectively) were collected.

Location/house where samples were collected	Type of collected sample		
	Building material[3]	Settled dust	RCS culture
Govermental building	2	0	1
Office building	19	3	6
Shop	0	0	1
Care facility (*e. g.* hospital)	2	2	3
School building	19	2	4
Apartment building	18	2	6
Singel family house	11	3	1
Children's daycare center	10	0	3
Sport facility[1]	3	0	2
Church	0	2	0
Others[2]	4	0	3

[1]Sport facility denotes for building materials: a swimming hall and two gyms; and for RCS cultures: an indoor icehockey rink and a swimming hall.
[2]Others denotes for building materials: two cinemas, an atelier, and a hotel; and for RCS cultures: an emergency exit tunnel, a public transporation vehicle, and a greenhouse.
[3]The 4 building materials in paper I are not included.

Cultures (n=45) of airborne fungal particles were collected using a Reuter Centrifugal Sampler (RCS) during a sampling time of 4 min (paper II and IV) in apartments (n=6), offices (n=6), schools (n=4), care facilities (n=3), kindergartens (n=3), and a single family house, a shop, a municipal hall, an indoor ice rink, a swimming hall, a green house, a public transportation vehicle, and an emergency exit tunnel.

Sampling and Cultivation

Building material samples and dust were cultured on MEA (a nutrient rich agar medium) (paper I-IV). Aliquots from serial dilutions of dust collected i New Orleans (paper III) were also plated on czapek yeast extract agar (CYA, a medium which promotes sporulation of aflatoxigenic fungi) and dichloran 18% glycerol agar (DG18, high water content medium). All plates were incubated at 23 °C for a minimum of 5-7 days and the colony forming units (CFUs) were identified to genus level according to Samson et al (2004).

Airborne dust samples were collected using an RCS (from Biotest Diagnostics, NJ, USA), an air sampler which collects airborne microorganisms quantitatively onto a culture medium according to the impaction principle (paper II and IV). Air (40 l/min) was sucked into the sampler from a distance of at least 40 cm by means of an impeller, entering the impeller drum, and set in rotation (average rotational speed of 4096 rpm ± 2 %). The contained particles in the air were impacted by centrifugal force onto a plastic strip containing Rose-bengal agar. After sample collection, the agar strips were incubated at 23 °C for 5-7 days, and colonies were counted as CFU/m^3 and identified according to Samson et al (2004).

Microscopy

Phase-contrast microscopy was used to study mold cultures and tape-lift samples (paper I-IV). Tape-lifts were collected by pressing tape onto a moldy surface. A drop of lactic phenol was then put on a microscope slide, the tape was pressed onto the slide, and another drop of lactic phenol was added. Finally a coverslip was overlaid and the slide was put under the microscope. The mold spores were examined and identified to

genus level according to Samson et al (2004). In absence of spores the mycelium was denoted *mycelia sterila.*

QPCR

Dust samples (paper III) were sent to Anozona (Uppsala, Sweden), contract partner of Fugenex (Yorkshire, UK), who are licensed to use DNA extraction and MSQPCR analysis according to Haugland and Vesper (2002, US patent 6,387,652). In summary, dust sample extracts were diluted, and the DNA was extracted by a rapid bead-milling method (Dneasy Plant Mini Kit; tungsten carbide bead, Qiagen, Hilden, Germany). DNA sequences were amplified using fluorescent labeled probes (TaqMan™ system) producing real time detection of PCR products (Haugland et al 2004) on a 7300 Real-time PCR Instrument (Applied Biosystems, Foster City, USA). The amplified DNA sequences were then counted, compare to DNA standard curves, and quantified as number of DNA-sequences (gene copies) per milligram dust. Detailed information on the primer and probe sequences (*i. e* .directed at the nuclear ribosomal RNA operon, internal spacer regions, ITS1 or ITS2) is provided at http://www.epa.gov/microbes/moldtech.htm.

Chemical experiments

The sample preparation process is illustrated in Figure 7.

Extraction

The samples were weighted, covered with methanol in glass test tubes with Teflon-lined screw caps, and stored in the dark, overnight or for 72 h, at room temperature. After extraction samples were centrifuged and the supernatants were decanted into new tubes. The extraction method was then elaborated with to improve recovery of the analytes, *e. g.* sterile water were added and the mixtures were extracted twice with 2 ml heptane (paper I-II), or the extraction was repeated with dichloromethane after which the methanolic and dichloromethane phases were pooled (and heptane-extraction omitted, paper III-IV).

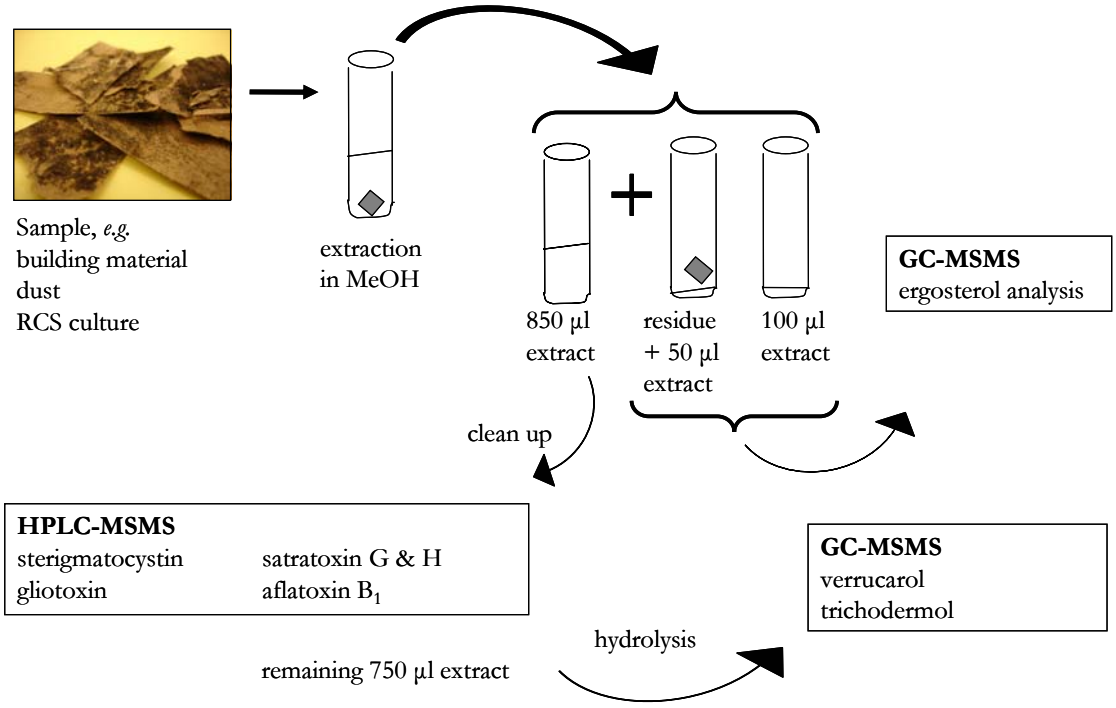

Figure 7. Overview of the sample preparation process (MeOH denotes methanol).

The extracts were then evaporated under a gentle stream of nitrogen and dissolved in 1 ml dichloromethane.

In paper I the 1 ml dichloromethane extract was solely used for GC-MS analysis, whereas in paper II and III the extract was divided into two equal parts where one half was used for GC-MS and the other half for HPLC-MS analysis. In paper IV, the sample and the sample extract were divided into three parts; 1) the sample and 50 µl of sample extract, 2) 100 µl of sample extract, and 3) the remaining 850µl sample extract. Portions 1-2) were used for ERG analysis and portion 3 for analysis of mycotoxins.

Purification and hydrolysis

In paper IV, the first two sample extract divisions were analyzed for ERG according to Sebastian and Larsson (2003). Briefly, samples were dried, hydrolyzed in methanolic potassium hydroxide, mixed with dehydrocholesterol (internal standard in GC-MS), partioned twice with water and heptane, after which the heptane phases were

evaporated to dryness. The samples were then re-dissolved in heptane-dichloromethane (1:1, vol/vol), applied onto silica gel columns, eluted with diethyleter and again evaporated before derivatization.

The remaining sample extract (3) designated for mycotoxin analysis was purified using solid phase extraction. Several columns were tested *e. g.* containing silica gel, propylsulfonic acid, primary and secondary amines, and aminopropyl groups. However, the polyethyleneimine (PEI) column gave the best recovery and reproducibility (unpublished results). After pre-conditioning the columns with methanol and dichloromethane, samples were applied and analytes were eluted with dichloromethane (heptane, chloroform, diethyleter, ethyl acetate and acetonitrile were also tested but were not equally efficient). After elution samples were evaporated under nitrogen, re-dissolved in methanol, filtered through 0.45 μm Millex syringe filters into new Teflon-capped analysis vials, and kept at -20°C pending HPLC analysis. After HPLC analysis the remaining methanolic extracts were mixed with internal standard (1, 12-dodecanediol), evaporated under nitrogen, hydrolyzed in methanolic sodium hydroxide, and extracted twice with water and dichloromethane. The organic phases were taken to new tubes, evaporated to dryness and placed in a desiccator overnight.

Derivatization (for GC-MS)

For ERG, derivatization was carried out by adding N, O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and pyridine to the dried samples followed by heating at 60°C for 30 min. Heptane was then added to each tube and samples were stored overnight in room temperature prior to analysis. Trimethylsilyl (TMS) derivatization of ERG and dehydrocholesterol is illustrated in Figure 8.

For mycotoxins, two derivatization approaches were applied, *viz.* obtaining TMS-derivatives using a mix of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), N-trimethylsilylimidazole (TSIM), and trimethylchlorosilane (TCMS) (paper I) and hepta-fluorobutyryl-derivatives by using heptafluorobutyrylimidazole (HFBI) (paper I-IV). TMS and HFB derivatization of VER and TRID is illustrated in Figure 9.

TMS derivatization was performed by adding a derivatization mixture of MSTFA : TSIM: TCMS (3: 3: 2, v: v: v) and pyridine, and heating the tubes at 60 °C for 30 min,

after which dichloromethane was added and samples were transferred to autosampler vials. HFB-derivatization was made by adding acetonitrile-toluene (1: 4, v: v) and HFBI followed by heating at 70°C for 60 min. In paper I samples were washed with sterile distilled water and the upper phase was transferred into auto sampler vials. In paper II-IV samples were not washed but left standing in excess of derivatizing agent in room temperature at minimum of 4 h before analysis. The derivatized samples were all stored at 4 °C pending analysis.

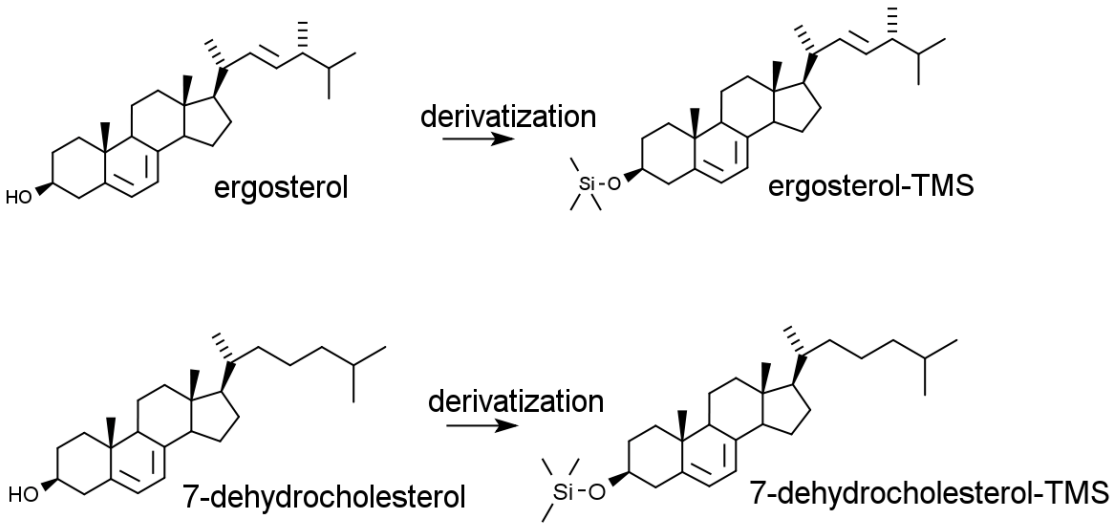

Figure 8. TMS derivatization of ERG and dehydro-cholesterol.

GC-MS

Derivatives of ERG were analyzed using a Saturn 2000 ion trap GC-MS system (Varian Inc., Palo Alto, USA.). Sample volumes of 1 µl were injected onto a FactorFOUR™ fused-silica capillary column (VF-5ms, 30 m x 0.25 mm i.d) (Varian Inc.) and analyzed in the electron impact mode at 70 eV and a trap temperature of 220°C, according to Sebastian and Larsson (2003).

Derivatives of VER and TRID were analyzed by GC-MSMS using a CP-3800 GC triple quadrupole MSMS system (Varian Inc.) at an energy of 70 eV. Analyses were

performed in EI mode with an ion source temperature of 250°C (TMS-derivatives) and 200°C (HFB-derivatives) (paper I), or in negative CI mode at an ion source temperature of 200°C or 150°C using methane (0.4 kPa, paper I) or ammonia (0.8 kPa, paper II-IV), respectively, as ionization gas. Sample volumes of 1-2 µl were injected onto a FactorFOUR™ fused-silica capillary column (VF-5ms, 30 m x 0.25 mm i.d) in the splitless mode with a helium carrier gas pressure of 69 kPa, using a CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland). The injector syringe was washed 5 times with acetone and toluene, respectively, before and after each sample injection.
A mix of HFBI and acetone (1:3, v:v) was injected in between samples to eliminate any trace of un- or semi-derivatized VER/TRID. The temperature of the column was programmed from 90 to 280°C at 20°C per minute (the injector and transfer line temperature was 280°C). The MSMS conditions were optimized by repeatedly injecting 0.1–1 ng amounts of standards at different collision energy, ion source temperature,and argon pressure in the collision cell. The parameters that gave the largest product ion peak area were selected. Detection sensitivity, defined as amount of standards injected with a signal-to-noise ratio ≥4 (software calculated peak-to-peak values), was determined by analyzing derivatized standard preparations diluted in dichloromethane (TMS- derivatives) or acetonitrile–toluene (1:4, v:v) (HFB-derivatives). The instrument performance was ensured by including TRID/VER standards and 1,12-dodecanediol (internal standard) in each batch of samples analyzed. Two calibration curves were constructed for VER/TRID (n=3) (0, 25, 50, 100, 250, 500, 1000 pg and 0.5, 1, 2.5, 5, 10, 25 ng respectively) together with internal standard (250 pg and 2.5 ng respectively). The coefficient of variation was calculated by dividing the standard deviation by the mean peak area ratio of VER/TRID standard to the internal standard, and the recovery was calculated by dividing the mean peak area from 1-ng injections of VER/TRID standards that had passed the sample preparation procedure with corresponding VER/TRID standards that did not pass this procedure.

Ions used in SIM analysis of VER–TMS_2 were m/z 320 (probably representing M–TMSOH), m/z 307 (M–CH_2OTMS), m/z 277 (M–CH_2OTMS–$2CH_3$), and m/z 217

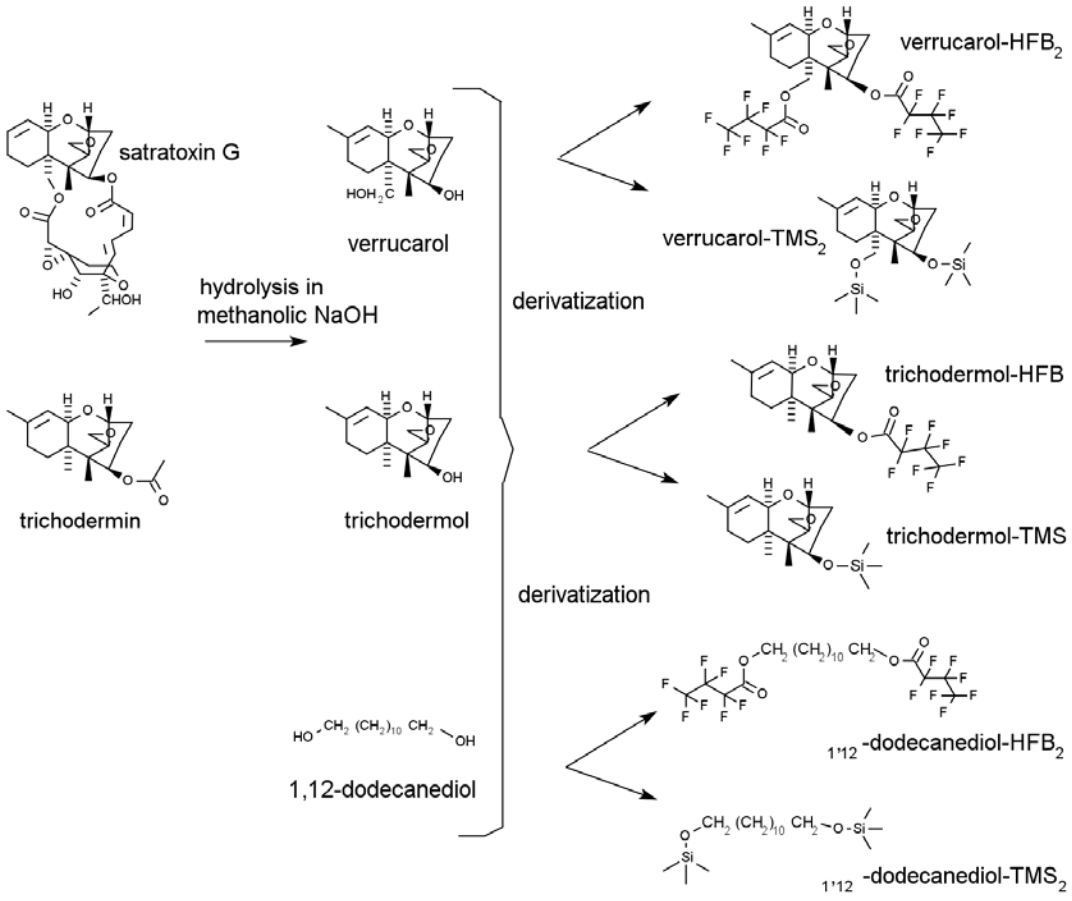

Figure 9. The hydrolysis of *S. chartarum* MTRs (exemplified by SATG) and trichodermin into VER and TRID which are then derivatized into HFB-esters or TMS-derivatives.

(M–CH_2OTMS–TMSOH). Fragment ions of *m/z* 217 and product ions of *m/z* 174 were used for monitoring in MSMS; with a detection limit of 20 pg.

Ions used in SIM analysis of TRID–TMS were *m/z* 217 (probably M–TMSOH–CH_3), *m/z* 176, and *m/z* 161. Of these, *m/z* 176 and product ions of *m/z* 161 were used in MSMS monitoring. The detection limit was 10 pg both in MSMS and SIM (for all ions monitored).

Ions used in SIM analysis of VER–HFB_2 in EI mode were *m/z* 445 (M–HFBO), *m/z* 444 (M–HFBOH), and *m/z* 416 (M–CH_2OHFB–CH_3). In MSMS, ions of *m/z* 444 were fragmented, and product ions of *m/z* 123 were monitored. The detection limit both in MSMS and SIM was 10 pg.

In TRID–HFB-analysis ions of m/z 446 (M) and m/z 431 (M–CH_3) were used in SIM. In MSMS, fragment m/z 446 and product ions of m/z 431 were monitored. The detection limit both in SIM and MSMS was 2 pg.

In NICI mode, VER–HFB_2 SIM analysis was perfomed monitoring ions m/z 638, m/z 302, and m/z 213, and parent ion m/z 638 (M–HF) and product ion m/z 213 (HFBO) were used for MSMS monitoring.

In case of TRID–HFB, ions of m/z 426 (M–HF) were used both in MSMS monitoring product ions of m/z 159 (C_3F_5CO) and SIM. The detection limits both of VER and TRID were 0.1 and 0.2 pg in, respectively, MSMS and SIM.

HPLC-MS

In paper II-IV, a ProStar HPLC/1200L triple quadrupole MSMS system (Varian Inc., Walnut Creek, CA, USA) was used. 20 µl of sample was injected using an autosampler (Varian, model 410) into a Polaris 5µM C18-A 150x2.0 mm RP-18 column equipped with a MetaGuard 2.0mm Polaris 5µM C18-A pre-column (Varian). The column was maintained at 25 °C and the flow rate was 0.2 ml/min. A supplement of 10 mM ammonium acetate and 20 µM sodium acetate was added to the methanol-aqueous buffer for increasing cationization in the ESI mode. A variety of modifications in the mobile phase program were applied (paper II-IV). Finally, an initial methanol concentration of 20% was held for 1 min, after which it was raised linearly (9 min) first to 70% and then to 100% (10 min) and held for 9 min before it was lowered (2 min) again to 20% and kept 9 min for stabilization (paper IV). 10 µl of methanol was injected in between samples to minimize cross-contamination. Nitrogen from a nitrogen generator (Domnick Hunter Ltd, Tyne & Wear, UK) was used both as nebulizing gas (50 psi) and as drying gas (20 psi), and argonium (0.23 mPa) was used for collision induced dissociation (CID). The capillary temperature was 310°C, the capillary voltage 40 V, the needle voltage 5000 V, and the electron multiplier voltage 2000V. MS spectra were collected with a scan time of 0.5 s and a scan width of 0.7 s. All these parameters were elaborated with and tested to achieve the highest possible signal in the mycotoxin MSMS- analysis.

The MS was tuned through direct injection of PPG tuning solution with a syringe according to the manufacturer's protocol. Mycotoxin standards and the internal standard reserpine (RESP) were included in each batch of samples analyzed in order to ensure instrument performance. Calibration curves were constructed by injecting mycotoxin together with internal standard at a minimum of seven concentration points. The coefficient of variation was calculated by dividing the standard deviation with the mean peak area ratio of mycotoxin standard to internal standard, and the recovery was calculated by dividing the mean peak area from injections of mycotoxin standards that had passed the sample preparation procedure with corresponding standards that did not pass this procedure.

In STRG-analysis ions of m/z 325 $(M+H)^+$ were used for fragmentation in MSMS, and the product ions of m/z 310 and m/z 281 were monitored and used for identification purposes; the detection limit was 0.2 pg (injected amount monitoring m/z 310, signal-to-noise ratio [peak-to-peak value] ≥4).

For SATG ions of m/z 567 $(M+Na)^+$ were used for fragmentation in MSMS, and its product ions m/z 263 and m/z 231 were monitored.

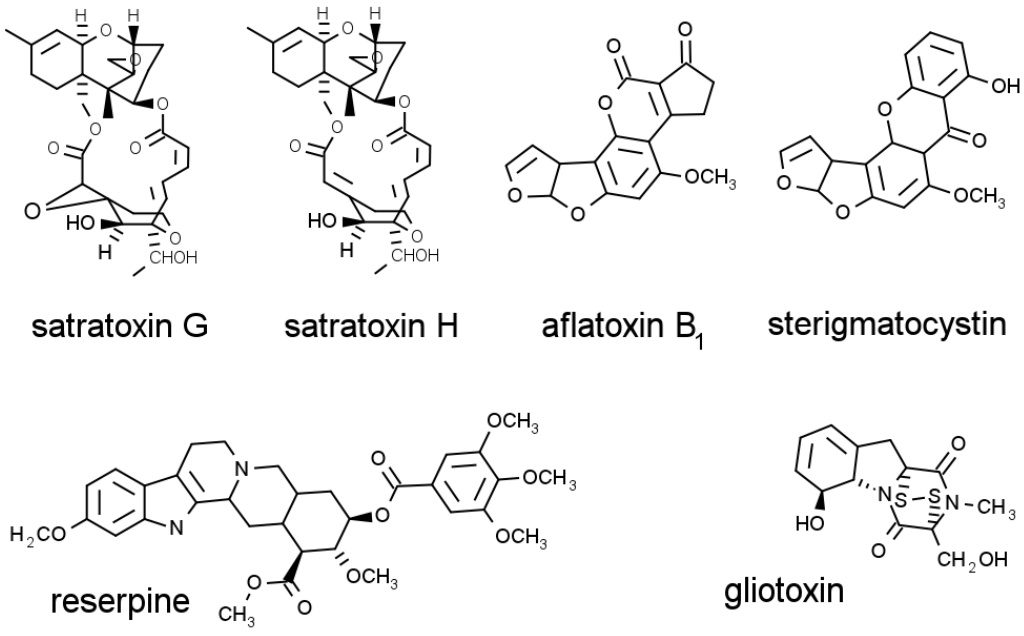

Figure 10. The chemical structure of the internal standard RESP and selected mycotoxins.

In the SATH analysis m/z 551 $(M+Na)^+$ was used for fragmentation in MSMS, and its product ions of m/z 321 and m/z 303 were monitored. The detection limits for SATG and SATH were not set as the crude standards used were unadequate for quantification purposes.

For reserpine, m/z 609 $(M)^+$ was used as the parent ion in MSMS and its product ion m/z 195 was monitored.

For AFLAB, ions of m/z 313 $(M+Na)^+$ were used in MSMS to produce product ions of m/z 241. The detection limit for AFLAB was 5 pg.

In positive ESI analysis of GLIO m/z 349 $(M+Na)^+$ were used in MSMS and product ions of m/z 285 were monitored. The detection limit was 5 pg. Chemical structures of selected mycotoxins analyzed in HPLC-MS is provided in Figure 10.

SUMMARY OF RESULTS

Mass Spectrometry

Details on the ions used in MS are summarized in Table 2 and 3.

Table 2. Overview of all monitored ions in GC-MS analysis.

Analyte	Method	Mass (range) in MS (m/z)	Mass (range) in MSMS (m/z)	CID[b] (V)	Paper
Analysis using triple quadrupole MS		**Q_1**	**Q_3[a]**		
VER/TRID-TMS	EI-scan	70-500	70-500	-	I
VER-TMS	EI-SIM	320,277,217	320,277,217	-	I
TRID-TMS	EI-SIM	217,176,161	217,176,161	-	I
VER-TMS	EI-MSMS	217	**174**	-15	I
TRID-TMS	EI-MSMS	176	**161**	-15	I
VER- HFB_2/TRID-HFB	EI-scan	190-700	190-700	-	I
VER- HFB_2	EI-SIM	445,444,416	445,444,416	-	I
TRID-HFB	EI-SIM	446,431	446,431	-	I
VER- HFB_2	EI-MSMS	444	**123**	-10	I
TRID-HFB	EI-MSMS	446	**431**	-10	I
VER-HFB_2	NICI-MSMS	638	**213**	15	I-IV
TRID-HFB	NICI-MSMS	426	**159**	20	I-IV
1,12 Dodecanediol-HFB	NICI-MSMS	574	**213**	10	I-IV
Analysis using ion trap MS		**Segment range**	**Target range[a]**		
ERG-TMS	EI-MSMS	363	**157**;140-400	-0.3	IV
dehydrocholesterol-TMS	EI-SIS	351+352	300-400	-	IV
VER/TRID-TMS	EI-scan	70-500	70-500	-	[c]
VER-TMS	EI-SIS	320,277,217	200-300	-	[c]
TRID-TMS	EI-SIS	263,189,161	100-300	-	[c]
VER-TMS	EI-MSMS	277	**187**,159; 85-285	-0.48$_{res}$[d]	[c]
TRID-TMS	EI-MSMS	145	**128**,117;70-150	-44	[c]

[a]The values in boldface represent the product ions used for quantification in MSMS.
[b]CID=Collision-induced dissociation value
[c]Unpublished data
[d]Res=Resonance mode

The peak area ratios of ERG standard/internal standard (dehydro-ERG) vs. the amounts of ERG standard followed the equation y=0.0000081x (R2=0.99) for the 0-200 ng amounts.

The peak area ratios of the mycotoxin standards analyzed in GC-MSMS /internal standard (1,12-dodecanediol) vs. the amounts of the mycotoxin standards in the

Table 3. Overview of all monitored ions in HPLC-MS analysis.

Analyte	Method	Q_1 (m/z)	Q_3[a] (m/z)	CID[b] (V)	Paper
Multiple mycotoxins	ESI^+-scan	100-1200	100-1200	-	II
STRG	ESI^+-SIM	671,363,325	671,363,325	-	II
SATG	ESI^+-SIM	1111,567,545	1111,567,545	-	II
SATH	ESI^+-SIM	1079,551,529	1079,551,529	-	II
GLIO	ESI^+-SIM	562,365,349	562,365,349	-	[c]
	ESI^--SIM	325,295,255	325,295,255	-	[c]
AFLAB	ESI^+-SIM	647,325,313	647,325,313	-	[c]
RESP	ESI^+-SIM	609	609	-	[c]
STRG	ESI^+-MSMS	325	**310**,281	-25	II-IV
SATG	ESI^+-MSMS	567	**263**,231	-31	II-IV
SATH	ESI^+-MSMS	551	**321**,303	-31	II-IV
GLIO	ESI^+-MSMS	349	**285**	-13	III
	ESI^--MSMS	325	**262**	2	[c]
AFLAB	ESI^+-MSMS	313	**241**,285,253	-30	III
RESP	ESI^+-MSMS	609	**195**	-45	II-IV

[a]The values in boldface represent the product ions used for quantification in MSMS.
[b]CID=Collision-induced dissociation value
[c]Unpublished data

samples followed the equations y = 0.5082x (VER, R^2 =0.998) and y = 0.3301x (TRID, R^2 = 0.959). The coefficient of variation was 5.3% (5 ng VER), 3.2% (5 ng TRID), 18% (0.5 ng VER), and 33% (0.5 ng TRID).

The peak area ratio of the STRG standard/internal standard (reserpine) versus the amounts of STRG standard followed the equations y=0.0675x+0.714 (R^2=0.992) for 0- to 1,000-pg amounts and y=0.0035x+1.742 (R^2=0.992) for 0.5 to 25 ng amounts; the recovery value was 53%±6%, and the coefficient of variation was 11.2%.

The equations for AFLAB and GLIO standards were y=0.0278x+3.9687 (R^2=0.98) for 0-5000 pg AFLAB injected, and y=0.0162x-1.7385 (R^2=0.99) for 0-5000 pg GLIO injected. The coefficient of variation was 16% for AFLAB and 12% for GLIO.

SATH and SATG could not be quantified, since the purity of these crude mycotoxin preparations was unknown.

Overview of all analyzed samples

Out of 167 building materials, 67% were positive for at least one of the selected mycotoxins. A detailed summary of the analysis of building materials is provided in

Table 4. In addition, approximately a quarter of all studied settled dust samples and about half of all cultured dust samples were positive for at least one of the mycotoxins analyzed (Table 5).

Table 4. Mycotoxins detected in all building material and dust samples studied.

Mycotoxin(s)	No. of samples in the indicated building material in which mycotoxin(s) was detected					No. of dust samples in which mycotoxin(s) was detected	
	gypsum board	wood based	linoleum flooring	wall paper	other[2]	settled dust	RCS culture
	n= 80	*n*= 47	*n*=12	*n*= 13	*n*= 15	*n*= 39	*n*= 45
TRID	13	4	nd	3	4	nd	4
VER	nd	4	1	nd	nd	5	1
STRG	4	7	nd	3	1	1	7
TRID, VER	19	7	nd	3	3	1	2
TRID, STRG	9	1	nd	1	nd	2	nd
VER, STRG	1	nd	nd	nd	1	1	3
STRG, AFLAB	nd	nd	nd	nd	nd	nd	1
TRID, VER, GLIO	nd	nd	1	1	nd	nd	1
TRID, VER, STRG	6	4	nd	1	nd	nd	2
VER, STRG, GLIO	nd	nd	nd	nd	nd	nd	nd
TRID, VER, SATG, SATH	3	nd	nd	nd	nd	nd	nd
TRID, VER, SATG	1	nd	nd	nd	nd	nd	nd
TRID, VER, STRG, SATG	1	nd	nd	nd	nd	nd	nd
TRID, VER, STRG, SATG, SATH	5	nd	nd	nd	nd	nd	1
VER, STRG, SATG, SATH	nd	nd	nd	nd	nd	nd	1

[1] nd= not detected; *n*= number of samples analyzed,
[2] Includes 3 stone/concrete samples, 3 painted wall materials, 2 synthetic materials, 5 flooring materials, 1 wall isolation, and 1 inventory.

Table 5. Overview of the occurrence of mycotoxin(s) in all studied samples.

Building material	gypsum board	wood based	linoleum flooring	wall paper	other[2]	settled dust	RCS culture
no. of analyzed samples	*n*= 80	*n*= 47	*n*=12	*n*= 13	*n*= 15	*n*= 39	*n*= 45
no. of mycotoxin positive samples	62	27	2	12	9	10	23
% mycotoxin positive samples	78	57	17	92	60	26	51

Additional comments

The MS method development for TRID and VER was started by analyzing TMS-derivatives (Langseth and Rundberget 1998) on a GC ion trap MS instrument. Sensitivity improved considerably when changing to a triple-quadrupole MS instrument, HFB-derivatives (Langseth and Rundberget 1998, Nielsen et al 1998a and b), and NICI (Kostiainen et al 1989, Kostiainen and Rizzo 1988). The reduction in background noise when using MSMS analysis increased sensitivity by a factor of 100 compared to results reported in previous studies (Nielsen et al 1998a).

In HPLC-MS it may seem controversial to use sodium as additive in the mobile phase since formic acid (Wilkes and Lay 2001), TFA (Smedsgaard 1997) or ammonium formate (Gottschalk et al 2008) are commonly used. However, sodium as an additive has been tested in other studies (Demulle et al 2006, Tuomi et al 1998 and 2000) and has been recommended to increase cationization in ESI-MS (Schnieder et al 1991).

DISCUSSION

Mold in buildings - guidelines and regulations

Regardless of the incomplete knowledge we have of the specific agents and mechanisms that are responsible for indoor air related health problems most institutions, agencies and organisations agree that mold exposure in general should be avoided. However, this advice is not new. The noun *tsara'at* mentioned in Leviticus 14:33-45 (3rd book of the Old Testament) has been interpreted as mold (Heller et al 2003). In this text the Lord tells Moses and Aaron how to remediate a mold-affected house. First a priest should be asked to inspect it. Then the mold should be scraped off the building material and dumped outside of town in an "unclean place". The building should then be sealed before the priest returns for inspection after one week. If the mold damage has spread it is advised to tear down the entire building.

Today mold exposure is regarded as a health concern mainly where the exposure is very high, *e. g.* in dusty agricultural occupational environments (Lacey and Dutkiewicz 1994) and during remediation procedures after water- and mold-damage *e. g.* in the aftermath of natural disasters. Recently, the Federal Emergency Management Agency (FEMA), part of the U.S. Department of Homeland Security, issued a warning to residents cleaning homes and buildings damaged by *e. g.* flood and storms, encouraging the use of protective equipment and recommending everyone to avoid unnecessary exposure to mold. Moreover, specific groups, *e. g.* immunocompromised patients and children younger than 12 years are recommended to avoid any exposure to high concentrations of mold in flooded areas (FEMA 2008).

The FEMA warning applies to *e. g.* cleaning management after hurricane Katrina, one of the costliest and deadliest natural disasters in the history of United States. This hurricane struck Louisiana, Mississippi, and parts of Florida, Georgia, and Alabama in August of 2005. In New Orleans, the city levee system catastrophically failed; about 80% of the city flooded to varying depths up to about 6 m, causing a large loss of lives and property damage (Knabb et al 2005). Due to the floods, ca 80% of all houses and their contents were subsequently contaminated by mold growth. The situation and

associated health risks were described *e. g.* in popular science reporting from a mycologist's own home (Bennett 2006), and in reports from the U. S. Center for Disease Control and prevention (CDC) and the National Institute for Occupatinal Safety and Health (NIOSH). The living conditions and health-related needs of returning residents were assessed (CDC 2006) and evaluations of health hazards were made (West et al 2006). Additional quality tests were conducted by the Natural Resources Defense Council (NRDC 2005). In scientific press there were several reports, *e. g.* review papers (Manuel 2006) and cross-sectional studies on chronic conditions among children and adolescents (Rath et al 2007). Several studies were also made to assess bioaerosols with respect *e. g.* to molds and endotoxins (Chew et al 2006, Rao et al 2007, Solomon et al 2006), as well as glucans (Rao et al 2007) in New Orleans. We showed that the molds present in this subtropical climate zone produce mycotoxins (paper III).

Exposure to mold is not only to be avoided in high level exposure environments like in the aftermath of natural disasters. Immunocompromised, allergic, asthmatic patients or people with breathing problems in general are regarded as being especially sensitive to mold (CDC Disaster Safety 2006). The Committee on Environmental Health, in the American Academy of Pediatrics, has released a policy statement which describes guidelines for pediatricians to help reduce infants' exposure to mold (American Academy of Pediatrics: Committee on Environmental Health 1998). Scientists at the U.S. EPA and Lawrence Berkeley National Laboratory have estimated that the symptoms of 4.6 of the 21.8 million asthmatics in the USA are caused by indoor dampness and mold to an annual cost of 3.5 billion dollars (Mudarri and Fisk 2007).

The U.S. EPA and CDC provide guidelines for mold cleanup online. Here residents are advised to resolve the problem which originally caused the water-damage and to clean the moldy surface (if less than ca 0.9 m^2 area) with detergent or bleach while avoiding exposure to the mold (EPA 2008, CDC Disaster Safety 2006). There are also other, more local guidelines in the USA, *e. g.* on assessment and remediation of fungi in indoor environments in New York (The New York City Department of Health and the New York City Human Resources Administration 2008).

In the Canadian Environmental Protection Act 1999 (Government of Canada 2007) Health Canada simply recommends "to control humidity and diligently repair any water damage in residences to prevent mold growth; and to clean thoroughly any visible or concealed mold growing in the residential buildings".

The Swedish National Board of Health and Welfare (1999, The Environmental Code, chapter 9, § 3) recommends to remediate and prevent water damage and not to tolerate visible mold or smell of mold in residential buildings. It specifically mentions to take special consideration to sensitive residents/occupants and advocates investigations of the buildings including both microbial and chemical analyses. If concealed mold is suspected the authorities can demand a more comprehensive investigation of the building according to The Environmental Code (chapter 26, § 22). Section 6:24 of the BFS 2006:22 amendments of the Swedish Board of Housing, Building and Planning states that "buildings and their installations shall be designed in such a way that microorganisms cannot affect indoor air to the extent where they are detrimental to human health or cause unpleasant smell" (BFS 2006).

The World Health Organisation (WHO) has through a working group developed guidelines for indoor air quality taking into consideration pollutants, indoor combustion and biological agents (WHO 2006) including the issues of dampness and mold (WHO 2007). These guidelines mainly recommend prevention of microbial growth through control of moisture and water-damage. If problems of dampness and microbial exposure occur remediation should be performed, not least among low-income populations to prevent additional burdens of poor health on this vulnerable group. Interestingly, the WHO working group also states that "Building owners are responsible for providing healthful workplaces or living environments free of excessive moisture and mold problems by ensuring proper building construction and maintenance. Occupants are responsible for managing water use, heating, ventilation, appliances etc. in a proper manner that does not lead to dampness and mold growth".

In conclusion, guidelines from the Old Testament to the WHO all agree that mold and moisture should be avoided regardless of what causative agent(s) lay behind the reported health problems in damp or water-damaged indoor environments. This statement is clear and provides a general strategy how to handle these problems while

further research is conducted to bring more detailed clarity to this complex phenomenon.

This study in context

In the mid 80s and 90s, mold problems indoors came in focus as several disease outbreaks were detected (Croft et al 1986, CDC 1995, Elidemir et al 1999, Flappan et al 1999, Hodgson et al 1998, Jarvis et al 1998, Johanning et al 1996). Methods for detection of mycotoxins, especially of trichothecenes derived from *Stachybotrys* spp. (Bata et al 1985, Harrach et al 1981, Jarvis et al 1984, Krishnamurthy et al 1987, Szathmary et al 1976) were developed and refined (Hinkley and Jarvis 2001, Nielsen et al 1998a and b). Studies reveald that mold growing on indoor building materials can produce mycotoxins (Engelhart et al 2002, Gravesen et al 1999, Nielsen et al 1998a, Tuomi et al 1998 and 2000). However, based on number of spores found in indoor environments, mycotoxin levels were not considered likely to pose a health threat (Bush et al 2005, Kelman et al 2004).

This thesis project was prompted mainly by two sets of published studies. One set showed that not only spores are released from mold-contaminated building materials but also much smaller fungal fragments (0.3-20 μm, Górny et al 2002, Kildesø et al 2003). These small fragments were found to be released in up to 320-514 times higher numbers than spores, share common antigens with spores (Górny et al 2002), and demonstrate a 230-250 fold higher respiratory deposition than spores in adults and an additional 4-5 times higher deposition in infants (Cho et al 2005). These fungal particles (diameter < 1μm) may contain mycotoxins (Brasel et al 2005). Thus, our exposure to mold and mycotoxins in water-damaged building may be much higher than suggested in previous estimations based merely on spore concentrations (Bush et al 2005, Kelman et al 2004).

Another set of studies showed that mycotoxin challenge, at picogram levels, to PBMCs lead to a polarization towards the Th2 phenotype (mainly caused by a reduction in numbers of IFN-γ-producing T lymphocytes rather than by a reduced functional capacity of a single cell) which suggestes an increased risk for allergy

development (Wichmann et al 2002). Interestingly, in another study, low exposure doses of mycotoxins in human monocytic cells caused a cytokine imbalance with a reduction in IL-10 concentrations compared to those of TNF-α and Il-6. These results imply a risk of inflammatory responses and could potentially partly explain the diffuse general indoor air-related symptoms and worsening of asthmatic inflammatory and allergic reactions experienced in moldy environments (Johannessen et al 2005). These data clearly show that mycotoxins even at very low concentrations may cause immunomodulation.

The results of these studies inspired to the initiation of this project –to improve mass spectrometry methods for selected mycotoxins and to use these methods to study mycotoxins in water-damaged indoor environments. The initiative came naturally since our research group is specialized in mass spectrometry methods and has over 20 years of experience in the indoor environmental research field.

In paper I a GC-MS screening method for analysis of hydrolysis products of mycotoxins produced by *Stachybotrys* spp. was optimized with respect to method performance (sensitivity, selectivity); an important task considering the very low mycotoxin concentration present in indoor environments. The screening analysis was also convenient to use since individual *Stachybotrys* mycotoxins are not commercially available (VER was in this case purchased and TRID was a kind gift from Poul Rasmussen). *Stachybotrys* mycotoxins were specifically selected because of their documented toxic potency (Pestka et al 2008) and suggested link to indoor-environmental disease outbreaks (Dearborn et al 1999 and 2002, Vesper et al 2000). The methods were successfully applied in direct analysis of authentic indoor environmental samples including dust settled in the breathing zone.

In paper II our battery of mycotoxin analytes was expanded to include STRG, SATG and SATH. HPLC was introduced as a separation method, giving the opportunity to measure intact macrocyclic trichothecenes SATG and SATH (kind gift from Prof. Bruce B. Jarvis), in addition to the hydrolysis product VER of *Stachybotrys* (chemotype S) MTRs (Andersen et al 2002 and 2003). The carcinogenic STRG was selected because it is produced by *A. versicolor* and a few other molds frequently encountered in water-damage buildings (Engelhart et al 2002, Frisvad et al. 1989, Hajjar et al. 1989).

These mycotoxin analyses were applied to authentic indoor environmental samples including building materials and dusts. The collection of building material samples were mainly gypsum boards since *Stachybotrys* is commonly found on water-damaged materials rich in cellulose (Miller et al 2003).

In paper III dust samples collected from buildings in New Orleans flooded in the aftermath of hurricane Katrina were analyzed. This is the first time that mycotoxins have been determined in Katrina-associated buildings and the results demonstrate that molds in indoor environments in a subtropic climate may produce mycotoxins.

In paper IV we expanded our battery of mycotoxin analytes even further and incorporated GLIO and AFLAB, mycotoxins produced by various *Aspergillus* and *Penicillium* spp., common in water-damaged indoor environments (Nielsen 2002). AFLAB is highly carcinogenic (Colombe 1993) and is routinely screened for in food and animal feed according to safety regulations around the world (FAO 2004). GLIO is a neuphrotoxic (Axelsson 2007) and immunosuppressive agent which has been shown to cause cell death by means of apoptosis and necrosis (Gardiner et al 2005). Also, GLIO has been linked to the development of allergy (Wichmann et al 2002) and inflammation response (Johannessen et al 2005). The analyzed samples in this study were not emphasized on gypsum boards but were building material samples that had been consecutively collected by personnel specialized in remediation measures during a one-year period. The distribution of the selected mycotoxins found in this study is thus a cross section on mycotoxin prevalence in water-damaged buildings undergoing technical investigation due to reported indoor-related health problems.

CONCLUSIONS

- GC-MSMS analysis of VER and TRID HFB-derivatives in NICI mode and HPLC-MSMS analysis of STRG, SATG, SATH, GLIO, and AFLAB in positive ESI mode, using triple quadrupole MS, are very specific and sensitive methods for analysis of indoor environmental samples.
- The developed integrated HPLC- and GC-MSMS analysis strategy for the combined analysis of all indicated mycotoxins is convenient and applicable especially when sample size is small.
- Molds in water-damaged indoor environments, in both sub-tropic and tempered climatic zones, produce mycotoxins regularly. Mycotoxins were found in settled dust on surfaces above floor level indicating that mycotoxins from mold-contaminated building materials can become airborne and inhalable.
- Mycotoxin production or prevalence does not seem to correlate to microscopy findings, CFU counts, or PCR results for fungi. There is a potential correlation between fungal biomass and mycotoxin production.

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"There is one thing even more vital to science than intelligent methods;
and that is, the sincere desire to find out the truth,
whatever it may be."
Charles Sanders Peirce