Sick of 'toxic black mould'? Quantifying mycotoxins in New Zealand's leaky buildings

Benjamin Clarke,[1,2,] Joanne Harvey,[1] Julian Crane,[3] Caroline Shorter,[3] Nick Waipara[4] and Simon Hinkley[2]*

[1]School of Chemical and Physical Sciences, Centre for Biodiscovery, Victoria University of Wellington, PO Box 600, Wellington 6140

[2]Ferrier Research Institute, Victoria University of Wellington, PO Box 33436, Petone 5046

[3]Wellington Asthma Research Group, Department of Medicine, University of Otago, PO Box 7343, Wellington

[4]Plant and Food Research, Mt Albert Research Centre, Private Bag 92169, Auckland Mail Centre, Auckland 1142

Email: simon.hinkley@vuw.ac.nz

Keywords: *mycotoxin, analytical chemistry, leaky building, house dust, black mould*

Health in New Zealand's leaky buildings

The quality of our indoor environment is a significant determinant of our quality of life. As we spend around 90% of our lives indoors, these spaces naturally influence our wellbeing, with warm, dry homes free of noxious agents being conducive to good health.[1] Conversely, poor quality housing has been consistently linked with poor health outcomes, with over thirty years of research evidencing a robust positive correlation between living in cold, damp, mouldy housing and the development of respiratory disorders.[2-5] Such disorders result in 4 million premature deaths annually, yet despite widespread attention, the exact mechanism by which these environments give rise to respiratory illness remains to be elucidated.[3-9]

New Zealand has notoriously poor housing stock with regards to damp and mould. Unsurprisingly, this correlates with a high incidence of respiratory disorders, and in 2015 alone the wider cost of respiratory disease in NZ was estimated to be $7.05 billion.[1,10,11] Some of the worst examples of this housing stock have been exemplified through our 'leaky buildings' (or 'leaky homes') crisis, which developed through the construction of buildings with weathertightness issues from the 1990s through to the mid-2000s. Subsequent moisture ingress into timber framing and wallboard resulted in widespread fungal growth, with many anecdotal reports of respiratory & other illness that occupants believed to be linked to living in their now damp and mouldy homes.[1,12] The impacts of the resulting leaky building crisis range from the substantial economic losses, estimated to be over $11.3 billion to date, through to mental health, with affected homeowners being driven into suicide, marriage breakups, anxiety and depression.[1,12,13] It may seem clear that something in these houses is making people sick, but, as with the thirty-plus years of research into damp, mouldy housing and respiratory illness in general, exactly what agent or agents are responsible for such building-related illness (BRI) remains a mystery.

In studies of housing quality and respiratory health, the strongest correlations to illness are the presence of active leaks and dampness, the presence of visible mould, and the presence of mould odour.[2-5] It is unsurprising then that fungi and the bioactive secondary metabolites they produce – mycotoxins – continue to

be the one of the most often proposed and studied potential causal agents of BRI. In 2002, the landmark Hunn report commissioned by the NZ Government into the leaky homes crisis was published.[14] With concern surrounding the potential health effects of mould, it recommended an investigation into the health risks associated with fungal decay in these buildings.

In a survey of wet rots in NZ's water damaged buildings, the most commonly identified microorganism was the notorious *Stachybotrys chartarum*, which produces some of the most potent mycotoxins yet discovered – the macrocyclic trichothecenes (MCTs). Selected MCTs [satratoxins G & H (SATG & SATH), roridin E (ROE)] and the 'pendant' variant roridin L2 (ROL2) are shown in Fig. 1.[1,15,16] Depending on sampling methods and the source of data, *S. chartarum* in NZ's leaky buildings may be found in anywhere from 49% to 77% of water-damaged building materials, in 20–45% of air samples from indoor locations with 'elevated' spore counts (*c.f.* outdoor levels), and in 13% of tape-lift samples (targeted sampling of buildings with suspected mould contamination, although this is a poor method for detecting *S. chartarum*).[1,17] In light of the reports of illness, the particularly high potential toxicity of this mould and its high prevalence, a second major leaky homes inquiry commissioned by the NZ Government in 2003 specifically recommended an investigation into "the extent of the *Stachybotrys* problem".[18]

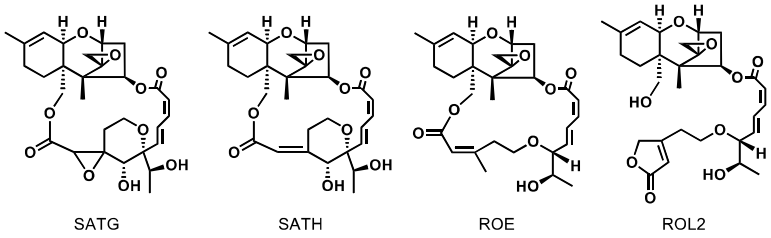


Fig. 1. Key trichothecenes (KTCs) produced by S. chartarum, including the MCTs satratoxins G & H and roridin E, and the non-macrocyclic roridin L2

Toxic black mould

S. chartarum (historically *S. atra*) came to prominence in the late 1980s, where it had been known as a contaminant of animal feed and was subsequently recognised growing in water-damaged buildings.[16,19-21] In agriculture the trichothecenes had earlier been established as the cause of stachybotryotoxicosis, an illness characterised by radiation sickness-like symptoms and sometimes leading to death in the animals and farm workers exposed to *Stachybotrys* contaminated hay.[19] The later notoriety of *S. chartarum* in a building context stems largely from its connection to a cluster of acute idiopathic pulmonary haemorrhage cases in infants in Ohio around 1993.[16,22] Initial investigations suggested that a high level of *S. chartarum* in the water-damaged homes of the infants may have been the cause of illness. However, expert review later described significant methodological shortcomings in the investigation, concluding that no causal link between mould infestation and the observed respiratory symptoms could be established.[16,19,20,22] Despite this critical review, the case lead to a public health hysteria concerning 'toxic black mould', resulting in a significant controversy around mycotoxin-induced BRI that continues to this day.[16,19,22-27]

Although space precludes an in-depth discussion of this controversy, a significant body of research has demonstrated both plausible routes of exposure *via* inhalation and established mechanisms of injury at achievable doses for *S. chartarum* in a BRI context. For example, toxigenic particles from *S. chartarum* can be aerosolised, are found in air samples of infested buildings, are respirable and contain mycotoxins.[28-32] Enzyme-linked immunosorbent assay (ELISA) has been used to detect both *S. chartarum* mycotoxins and antibodies to these toxins in exposed people, suggesting that they do enter the body, and of particular concern are the presence of these toxins on highly respirable sub-micron particles.[33-36] *S. chartarum* fungal fragments – pieces smaller than spores – may reach airborne concentrations over 500 times that of spores, and computer modelling suggests that respiratory deposition of these fragments may be over 200 times greater than that of spores.[29,37] Thus, finding spores on air sampling may indicate exposure to a relatively larger concentration of toxigenic particulates.

While studies suggest that the most potent MCTs are rapidly absorbed, distributed and metabolised, they tend to concentrate in specific cell types such as alveolar macrophages, and as the concentration of toxin at the site of deposition in the lungs will be greater locally than systemically, the environmental exposure required to cause local cellular injury will be lower than that required to achieve acute systemic toxicity.[25,38-40] Inhalation of both *S. chartarum* spores and fungal fragments induces pulmonary arterial remodelling in a murine model, while doses of MCTs based on toxin

concentrations in the air of contaminated buildings were found to cause damage and inflammation in neural cells such as astrocytes, neurons and those of the blood-brain barrier, which may further facilitate the infiltration of toxic substances generally into the central nervous system.[41-43] Intranasal installation of these potent MCTs causes olfactory neuron loss in both mice and monkeys, with repeated smaller doses of the toxins producing cumulative damage equal to or greater than the sum given as a single larger dose.[39,44] As it is established that chronic exposure to sub-acute-effect levels of mycotoxins *via* food can cause population-level increases in illnesses, so this research highlights the potential for harm from low-level chronic exposure to *S. chartarum* trichothecenes in the built environment.[45,46]

Despite this evidence, the effect of *S. chartarum* trichothecenes on respiratory health remains equivocal. Some research finds no significant effect from *S. chartarum* exposure, while trichothecenes are likely not the only component in *S. chartarum*, and fungi in general, that may contribute to such disorders.[47] For example, research indicates that proteinaceous components may also cause or contribute to the observed biological responses, and there may be a multi-factorial aetiology as in many diseases.[48,49] However the notoriety of the trichothecenes and the unanswered questions around their health effects demand more research in order to expose exactly what role they play in the health of our leaky building occupants.

Is toxic black mould making leaky building occupants sick?

In response to the recommendation of the Government inquiry, the relationship between the prevalence of *S. chartarum* mycotoxins in our leaky buildings and the adverse respiratory symptoms reported by occupants is being investigated.[50] To this end, a matched case-control study will be conducted comparing the levels of trichothecenes in house dust swabbed from surfaces and flooring against measures of specific respiratory symptoms in 100 defined leaky homes and 100 control homes. This research hypothesises that with the observed high prevalence of *S. chartarum* in our leaky buildings, low levels of respirable trichothecenes may be present with significant frequency, and in consideration of research demonstrating a plausible route of exposure and mechanism of injury, that low-level chronic exposure to these mycotoxins may be contributing to the prevalence of respiratory disorders reported by leaky building occupants. In order to conduct this study, a comprehensive measurement of the mycotoxins present is required, for which three analytical measures are proposed.

Firstly, the collected dust samples will be subject to a semi-quantitative screen for over 300 microbial secondary metabolites utilising high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) to give information about general microbial toxin exposure.[7] Secondly, the samples will be hydrolysed to convert the majority of trichothecenes produced by *S. chartarum* into the parent diol, verrucarol (VER, Fig. 2) which will be quantified by an ultra-sensitive, isotope-assisted, gas chromatography-mass spectrometry (GC-MS) method, giving a measure of 'total' trichothecene exposure. Thirdly, where trichothecenes are found, quantification of four key trichothecenes (KTCs) – SATH, SATG, ROE and ROL2 (Fig. 1) – will be completed by HPLC-MS/MS. These KTCs represent two of the most potent MCTs (SATH & SATG) and two chromatographically useful proxies for total trichothecene production (ROE & ROL2). While the initial wider microbial toxin screen will be conducted by collaborators in Vienna using established methodology, the research currently proposed here focuses on the development of the ultra-sensitive GC-MS method for quantification of total trichothecene

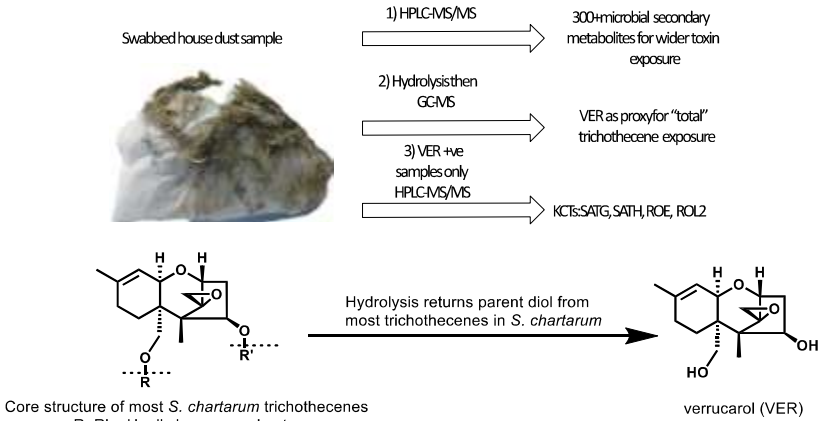


Fig. 2. Proposed analytical approaches to measurement of mycotoxins

exposure and the development of the HPLC-MS/MS method for the quantification of KTCs.[7]

Stachybotrys chartarum & the trichothecenes

S. chartarum has been proposed through chemotaxonomic and genomic analysis to comprise three taxa – *S. chartarum* chemotype (ct.) S, producing the highly potent macrocyclic trichothecenes (Fig. 1), *S. chartarum* ct. A, producing atranones and dolabellanes (Fig. 3), and *S. chlorohalonata*, which also produces atranones and dolabellanes but which was sufficiently different in morphology and genetics to have a unique classification proposed.[20,51-54] A recent genetic analysis of satratoxin and atranone producing *S. chartarum* has suggested that these can be represented by three genotypes; S, which contains the required gene cluster for satratoxin production but not for atranone production, A, which contains the gene cluster for atranone production but not for satratoxin production, and H, which contains the cluster for atranone production in addition to an incomplete set of satratoxin producing genes.[55]

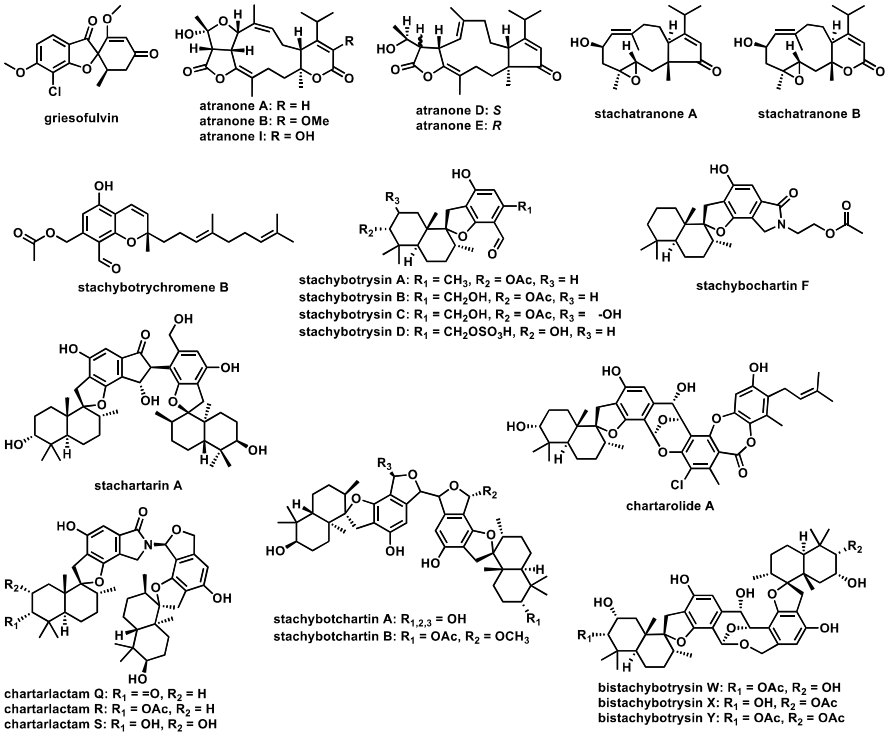


Fig. 3. A selection of non-trichothecene secondary metabolites produced by Stachybotrys

S. echinata (previously *Memnoniella*) grows in the same environments as *S. chartarum* and produces its own mycotoxin profile including simple trichothecenes but in which the metabolite distribution is dominated by griseofulvins (Fig. 3).[9,20,56] The true genetic diversity of *Stachybotrys* remains a matter of debate, and the plethora of compounds it produces is far from extinguished. It may currently comprise perhaps seventy-four species and novel bioactive compounds continue to be reported. These include the stachybotrychromenes and a large number of spirocyclic phenylspirodrimane derivatives; the stachartarins, stachytbotrysins, bistachybotrysins, stachybochartins, chartarolides and chartarlactams (Fig. 3).[56-63]

Over 200 trichothecenes have been identified from a variety of natural sources.[64] They are commonly grouped into four types, A, B, C and D, based on the functionalisation of the core 12,13-epoxytrichothec-9-ene (Fig. 4).[65] Type A trichothecenes are characterised by the presence of either hydroxyl or ester groups at C8, for example in T2, or by lack of substitution as in VER, diacetoxyscirpenol (DAS), trichodermin (TRDM) and trichodermol (TRID) (Fig. 4). Type B have a ketone at C8, for example nivalenol (NIV) and deoxynivalenol (DON), while type C have a C7-8 epoxide such as in crotocin (Fig. 4). Type D trichothecenes contain a macrocyclic ring structure between C4 & C15 as in SATG, SATH and ROE (Fig. 1).

The trichothecenes produced by *S. chartarum* include the type D MCTs; satratoxin F, SATG, SATH, isosatratoxins F, G & H, ROE, and verrucarin B & J (VEB, VEJ), and the 'pendant' type A trichothecenes – non-macrocycles esterified at one or both of C4 & C15; ROL2 (Fig. 1), trichoverrols A & B (TVLA, TVLB) and trichoverrins A & B (TVNA, TVNB) (Fig. 4).[56,66,67] Hydrolysis of the C4 and C15 esters in these trichothecenes returns the parent simple trichothecene diol – VER – the total synthesis of which, as the racemate, was first reported in 1982.[68,69] Other notable trichothecene producers include the agriculturally-relevant plant pathogens *Fusarium* and *Myrothecium*, a toxic mushroom from Japan, *Podostroma cornu-ame*, and the only known plant sources *Baccharis sp.* and *Holarrhena sp.*, although the source in plants may yet be found to be a co-occurring fungus.[64,67,70-72]

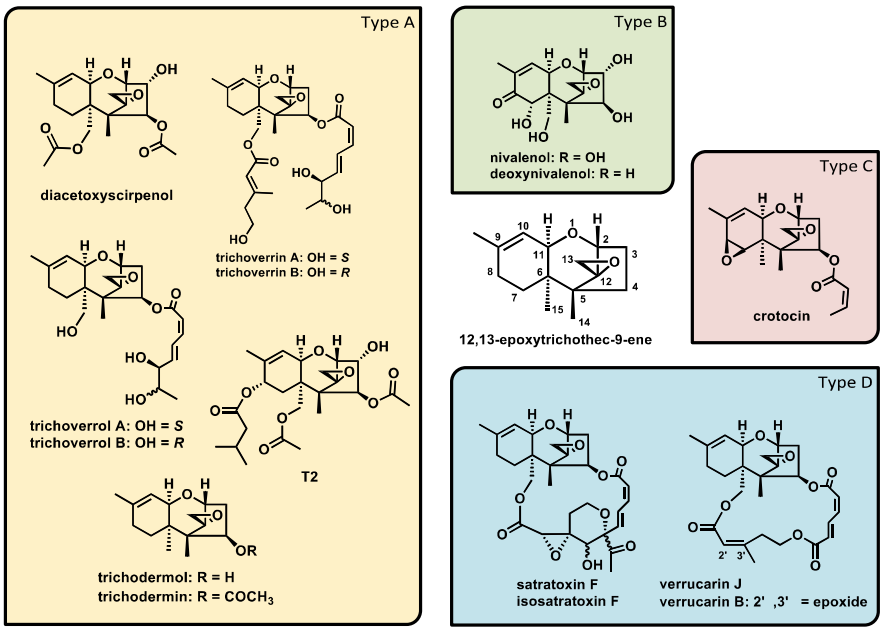
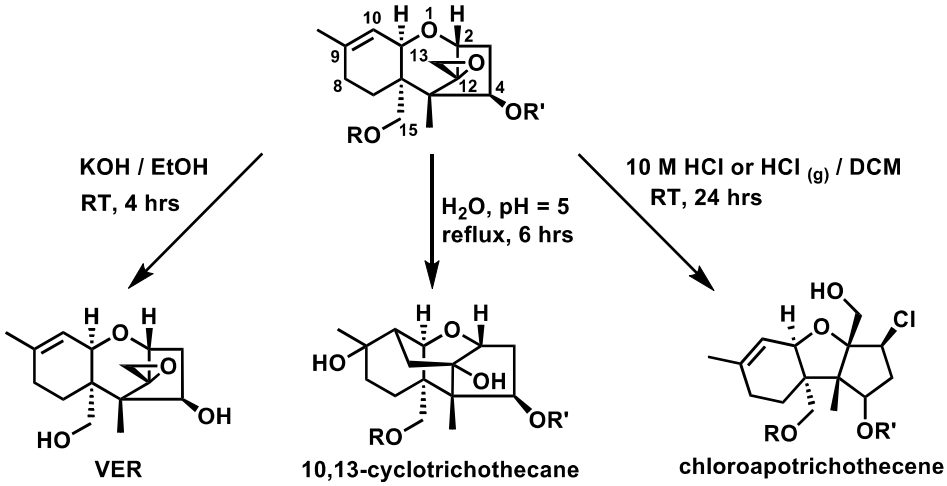


Fig. 4. Representative trichothecenes of each type. The stereochemistries of satratoxin F & its presumed epimer isosatratoxin F remain unresolved.[64]

The chemistry of the MCTs, focusing on the macrocycle, has been the subject of a comprehensive review by Grove (1993).[73] Key reactivities include the base-catalysed hydrolysis of the C4 & C15 esters and the acid catalysed rearrangements of the 12,13-epoxide (Scheme 1). In weakly acidic media, rearrangement gives a 10,13-cyclotrichothecane, presumably *via* protonation of the terminal 12,13-epoxide followed by attack by the 9,10-alkene to form a bridge with concomitant addition of water at the tertiary centre.[74] The propensity for the trichothecene core to undergo this rearrangement is modulated by the functionality of the ring system. For example, substitution of OR at C8 prevents this rearrangement, likely by reducing the nucleophilicity of the 9,10-alkene or by sterically hindering its attack on the epoxide. The presence of a 4,15-macrocycle also prevents such a rearrangement, probably through conformational effects. In strongly acidic media, *e.g.* 10 M HCl or HCl in DCM, an alternative acid-catalysed rearrangement can occur *via* attack of the 1,2-CO bond on the protonated epoxide to give an apotrichothecene.[75] In this scenario, attack from the 9-ene system is suspected to be prevented by its protonation, and the presence of a 4,15-macrocycle does not prevent rearrangement.[73]



Scheme 1. Representative base hydrolysis and acid-catalysed rearrangements of the core trichothecene structure

Matrix effects in mycotoxin analysis

The benchmarks for routine, sensitive and accurate quantification of mycotoxins in the environment are HPLC- & GC-MS based analyses. A major concern in their application is the presence of so called matrix effects (MEs); analyte signal enhancement or suppression caused by other components of the parent material the analyte was extracted from (the 'matrix'), which cause the instrument to over- or under-report the actual analyte concentration. MEs can be divided into two types – effects which affect the slope of a concentration / response curve (rotational effects), caused by other components of the matrix proportionally affecting the ionisation of the analyte, and those which affect the intercept but not the slope of a curve (translational effects) due to a high background / baseline interference (Fig. 5). Three main techniques are used to overcome matrix effects in quantification using MS: matrix-matched calibrations, standard addition, and stable-isotope labelled internal standards.[76]

Matrix-matched calibration uses an external calibration curve prepared in the same or a very similar matrix to the sample. In theory this applies the same MEs to both the calibration and analyte, but it does not correct for inefficiencies in sample extraction or losses during processing. The success of this approach is reliant on how well matched the calibration and sample matrices are, and it ideally uses both a blank matrix and a separate calibration curve for each unique sample matrix, a requirement which can significantly increase the number of analyses conducted when the matrices vary between samples. Standard addition in contrast attempts to correct for MEs by effectively creating the calibration curve within the sample through a series of standard additions of the analyte. In simple terms, by constructing a curve using the data of the known additions and calculating the slope, the background concentration of the analyte can be obtained (Fig. 6). This approach does not require a blank matrix but several standard additions are normally required for each sample, multiplying the number of samples to be analysed. Both of these methods still require considered application, as although they correct for rotational effects they do not necessarily correct for translational effects.[77] Such translational effects must additionally be managed through appropriate sample preparation.

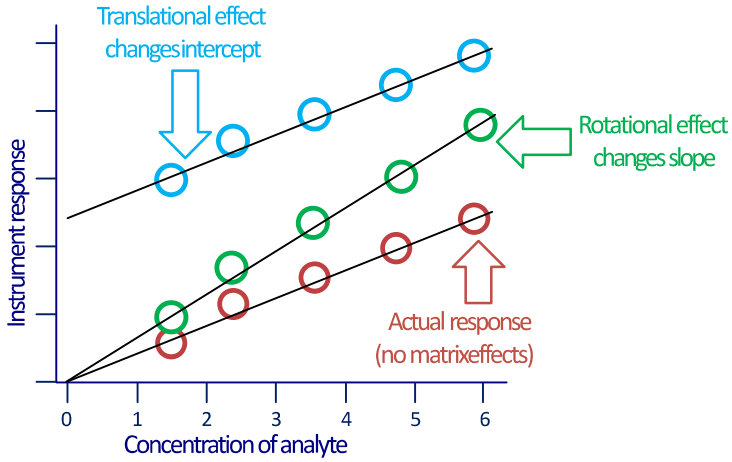


Fig. 5. Rotational & translational matrix effects

Adding a known amount of internal standard (ISTD) to samples before processing can overcome MEs if the ISTD has the same response to processing and instrumental analysis as the analyte. The ideal standard in this approach therefore has exactly the same chemical properties as the analyte. When MS is used as a detection method, this is possible by using a stable isotope (SI) labelled analyte as the ISTD – chemically identical, yet distinguishable upon MS detection due to its mass difference. This approach is known as stable isotope dilution analysis (SIDA, Fig. 7). As the amount of SI labelled ISTD (SI-ISTD) is known prior to analyte extraction, the ratio of SI-ISTD to analyte found *via* MS gives the analyte concentration with compensation for both MEs and processing losses.[76,78] SIDA represents the gold standard in the analysis of mycotoxins in the environment, generally giving excellent quantification of analyte recovery with a very low limit of quantification (LOQ) and limit of detection (LOD).[78]

The main challenges facing SIDA include the stability of labelling and isotope effects (IEs). For example, deuterium placed at labile sites can participate in $^{1}H/^{2}H$ exchange resulting in loss of label, while the mass difference of isotopologues can result in slightly different retention times in GC or HPLC, or changes in fragmentation patterns.[79] This effect is most notable with ^{2}H as opposed to with ^{13}C or ^{15}N labelling as the relative change in mass to the nucleus is significantly greater. IEs can be minimised by only introducing one or two labels, however sufficient difference in mass between analyte and SI-ISTD, ideally ≥ 3 Da, is desired to avoid spectral overlap in the mass spectrum resulting from

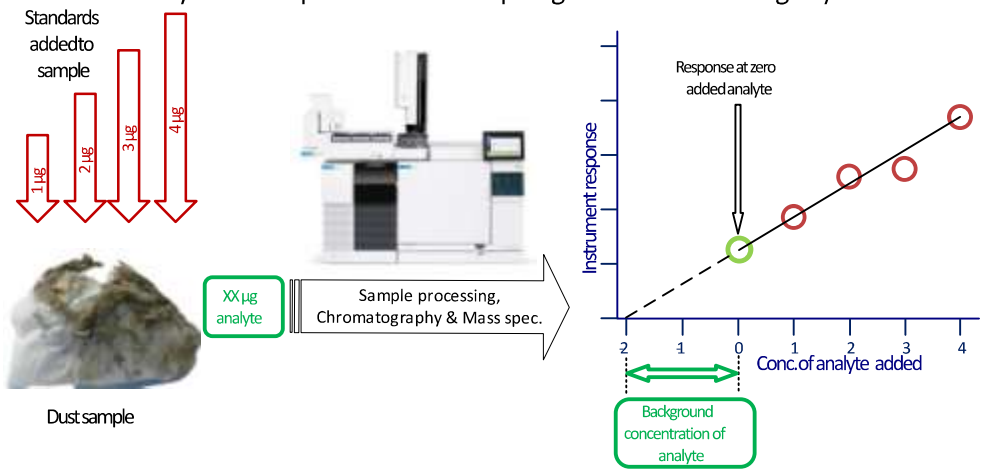


Fig. 6. The standard addition method workflow

naturally occurring isotopologues of either the SI-ISTD or analyte inflating the signal of one another.[78-80]

Isotope assisted trichothecene quantification

The SIDA analysis of trichothecenes in the environment is dominated by measuring simple agriculturally relevant trichothecenes such as DON and NIV in food and biological fluid due to their established health risks. In this context, the use of SIDA is increasing in order to meet food production regulations and more accurately track mycotoxin exposure in populations. Due to this demand, SI-labelled standards of agriculturally relevant trichothecenes are becoming more widely available and utilised, and this has enabled the development of highly sensitive analyses in previously challenging matrices.

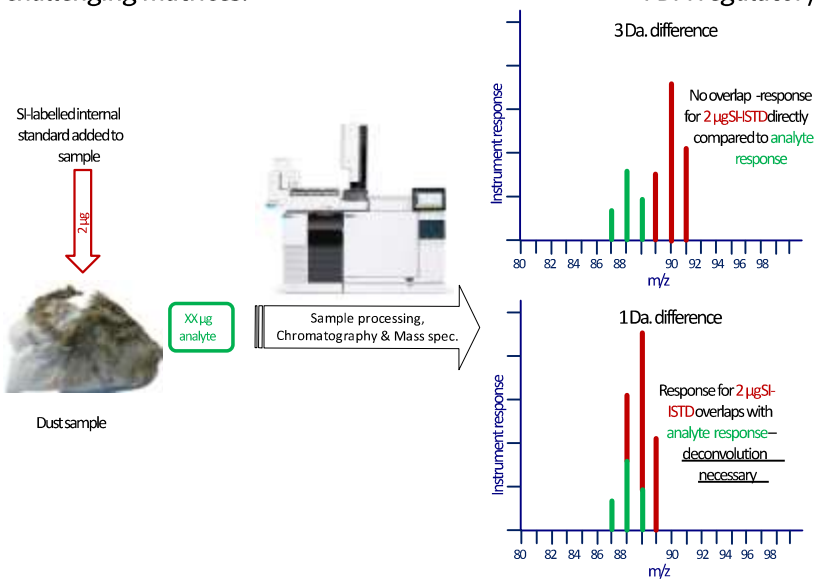


Fig. 7. Simplified SIDA workflow. An insufficient mass difference between standard and analyte can cause isotope overlap, requiring mathematical correction.

Commercially labelled standards are typically fully ^{13}C labelled, although as noted this could lead to undesirable IE, while laboratory-prepared standards are often labelled through facile synthetic steps at just a few sites, *e.g.* a single deuteration or the introduction of two ^{13}C through acetylation with an SI-labelled reagent (Fig. 8). For example, in a report of a SIDA method for the detection of type-B trichothecenes, Bretz *et al.* prepared deuterated DON (15-[$^{2}H_1$]-DON) by semi-synthesis from its natural precursor 3-acetyl-DON (3-Ac-DON).[81] With the SI-ISTD, they were able to mitigate MEs in the HPLC-MS/MS analysis of DON in food samples. Labelling was carried out by oxidising the C15 primary alcohol of 3-Ac-DON to an aldehyde and reducing this with $NaB^{2}H_4$ to return the $^{2}H_1$ labelled C15 carbinol. Deacetylation then gave 15-[$^{2}H_1$]-DON. An analysis of fragmentation patterns *via* MS gave the authors confidence that the chemically feasible mono-labelling at the C15 primary alcohol would be sufficient for use as an SI-ISTD. With only a single mass unit difference, some spectral overlap was found in the mass spectrum, however, these effects were adequately managed through mathematical correction.

The same authors had also previously prepared [$^{2}H_3$]-3-Ac-DON through mono-[$^{2}H_3$]-acetylation of DON.[82] McMaster recently also used SIDA with a singly deuterated [$^{2}H_1$]-DON in a GC-MS method to quantify DON in 196 sorghum samples, after a traditional non-SI assisted method produced inconsistent recoveries.[83] The SIDA method found more samples exceeding the FDA regulatory limits than the traditional method, including thirteen that would have otherwise passed the 1.0 µg g^{-1} advisory limit under the non-SI assisted method, and six that would have otherwise passed the 5.0 µg g^{-1} advisory limit. Spectral overlap resulting from the small mass difference of the analyte and standard was observed but was again mitigated through mathematical correction.

In another example of facile labelling, Asam *et al.* reported on the use of ^{13}C labelled ISTDs in the HPLC-MS/MS analysis of type-B trichothecenes in food.[84] The authors stated that the accuracy of MS analysis for these compounds had been restricted by the lack of an SI-ISTD, and noted that research using a chemically related surrogate would have suffered from MEs and variation in extraction efficiencies. Three ^{13}C labelled trichothecene standards, [$^{13}C_2$]-3-Ac-DON, [$^{13}C_2$]-15-Ac-DON, and [$^{13}C_2$]-4-Ac-NIV, were prepared by $^{13}C_2$-acetylation of the commercially available deacetylated precursors. The standards were found to be isotopically stable at pH 3 & 7 over 2 days. This was an advantage over the previously synthesised ^{2}H-labelled [$^{2}H_3$]-3-Ac-DON by Bretz *et al.* where the location of the ^{2}H label in the acetyl group could undergo $^{1}H/^{2}H$ exchange during sample clean-

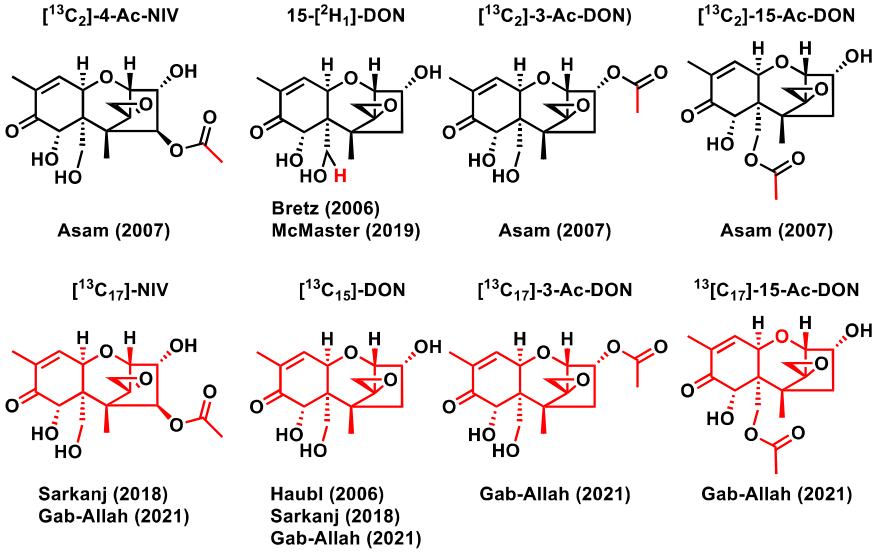


Fig. 8. Isotopically labelled standards used for trichothecene analysis in food via SIDA. Red areas indicate label locations, with the bottom row representing fully ^{13}C labelled standards.

up.[82] A commercially available, fully ^{13}C labelled [$^{13}C_{15}$]-DON was used as a standard for DON. In this case, a significant IE was found where the calibration slope between SI-ISTD and unlabelled analyte was much greater than unity (1.7 *versus* 1). Whereas the mass difference (15 Da) was too high to give isotope overlap, the ratio of [^{13}C]-formaldehyde to water fragments in the SI-ISTD was different to the ratio of formaldehyde to water fragments in the analyte. The higher abundance of [^{13}C]-formaldehyde fragments had thus resulted in a higher slope. Overall the standards allowed excellent sensitivity with low LOD & LOQ and good precision.

Despite the potential for IE with fully labelled standards they have proved quite useful. Häubl *et al.* illustrated the power of using SI-ISTDs in circumventing MEs when in 2006 they used the commercially available [$^{13}C_{15}$]-DON to analyse maize and wheat for DON by HPLC-MS/MS without sample clean-up.[85] Simple acetonitrile / water extracts with no ISTD gave apparent analyte recovery of 29±6% in wheat and 37±5% in maize, while use of the SI-ISTD gave recoveries of 95±3% and 99±3% respectively, illustrating the potential for SIDA to provide reliable and accurate quantification with even minimal sample clean-up.

More recently, in a study which demonstrates the state-of-the-art, Šarkanj and co-workers in 2018 developed a robust and ultra-sensitive UHPLC- (Ultra-HPLC-) MS/MS method for the detection of multiple mycotoxin biomarkers in urine using SIDA and a range of 12 SI-labelled mycotoxin ISTDs.[86] Urine is particularly susceptible to MEs, and as SIDA is considered an ideal way to quantify the recovery of analytes from varied matrices, it was hoped that this method would be applicable to the wide range of urines and mycotoxins expected in epidemiological studies. Compared with the same samples previously analysed in a 'dilute and shoot' (minimal sample clean-up) method using un-labelled standards, Šarkanj's method found more positive samples (above LOD) and a greatly increased number of quantifiable samples (above LOQ) for all comparable analytes, including the type-B trichothecenes NIV, DON and de-epoxy-DON (Fig. 9). It was proposed that the SIDA method would allow a realistic assessment of mycotoxin exposure in large scale epidemiological studies.

Finally, Gab-Allah *et al.* used SIDA in the development of an ultra-performance liquid chromatography- (UPLC-) MS/MS reference method for the analysis of type-B trichothecenes in cereal grains using fully ^{13}C labelled [$^{13}C_{15}$]-DON, [$^{13}C_{15}$]-NIV, [$^{13}C_{17}$]-3-Ac-DON and [$^{13}C_{17}$]-15-Ac-DON.[88] Excellent accuracy, high reliability and low LODs & LOQs were achieved, with the authors noting the LODs and LOQs were the lowest amongst a selection of recent SIDA LC-MS trichothecene analysis reports.[87] These studies are but a few that have leveraged the power of SIDA to overcome the challenges associated with the highly sensitive detection of mycotoxins in complex matrices.

Quantifying trichothecenes in indoor matrices

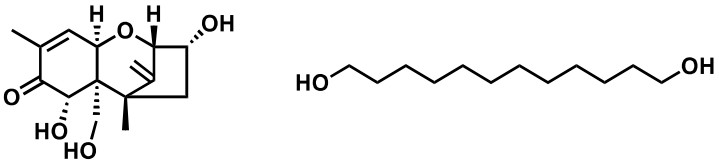
The analysis of trichothecenes in buildings typically focuses on house dust, as particulate inhalation represents the expected route of exposure. House dust has been found to be particularly prone to MEs and so analyses need to be carefully approached in this regard. Unfortunately, the application of SIDA to the quantification of building related trichothecenes (*e.g.* VER, SATG, SATH & ROE) is decidedly lacking due to an absence of suitable SI-labelled standards.[78] It is likely that this absence of standards reflects low demand due to the lack of regulation (*c.f.* agriculture), which in turn is rooted in a lack of knowledge about the risks from trichothecenes in these environments, as well as the tech-

nical challenges associated with preparing SI labelled standards. The mitigation of MEs in building-related matrices has thus typically used matrix-matched calibrations and internal standards which differ from the analyte. Compared with the results seen using SIDA in an agricultural context, this can severely limit the quality of analysis.

Such situations are illustrated across the literature. A 2009 paper by Vishwanath *et al.* describes the simultaneous detection of 186 microbial secondary metabolites including trichothecenes in indoor matrices by HPLC-MS/MS.[88] For a dust matrix, analyte signal was supressed by more than 50% in a third of the analytes, and apparent recoveries of below 50% for half of the analytes was observed. It was proposed that this was due to incomplete extraction and the severe MEs presented by house dust. This was noted to be an extremely challenging matrix, and a significant difference in MEs between dust samples was found. It was proposed that matrix-matched calibrations would likely be unsuitable for dusts due to the severe MEs, and that SI-ISTDs would be required to overcome these.

In 2012, Pietzsch *et al.* reported a follow-up analysis of schools in Europe again for 186 microbial secondary metabolites using HPLC- and GC-MS/MS.[89] Approximately 10 samples each of settled dust and swabs of mouldy surfaces were collected preferentially from locations where dampness or moisture damage had been noted. HPLC-MS/MS was used to screen for the metabolites using external calibrations prepared with a multi-analyte standard. No attempt was made to assess or correct for extraction losses or MEs as in previous research[89] they had found matrix-matched calibrations unsuitable for correcting these effects in extremely heterogeneous matrices such as dust. A GC-MS/MS method was used specifically for the detection of trichothecenes, where hydrolysis of samples prior to analysis generated VER and TRID as proxies for total trichothecene exposure. An ISTD of 1,12-dodecanediol (Fig. 9) was added to the samples prior to hydrolysis. Trichothecenes, as measured by the detection of VER, were found in only 4 of 675 samples. The use of unsuitable ISTDs and the presence of MEs make it difficult to draw any conclusions with regards to the presence of trichothecenes, again underlining the desirability of SI-ISTDs.

These researchers would go on to conduct one of the most comprehensive studies of its type, when Kirjavainen *et al.* surveyed dust samples using HPLC-MS/MS for the presence of 333 microbial secondary metabolites in 93 homes of 1-year old children in Finland.[7] Microbial secondary metabolites were ubiquitous, being found in all houses. A moderate but significant negative correlation was found between overall metabolite abundance and the prevalence of asthma symptoms at 6 years of age. External calibration curves derived from serial dilutions of a multi-analyte standard were used with no matrix-matching, and no S. chartarum trichothecenes were found. Authors from this group had in previous research[88,89] stated the particular difficulty that dusts present in terms of MEs, both within the individual sample and due to the great variability that is expected between samples. It has been argued that such multi-analyte approaches, while valuable for qualitative screens, can only be considered semi-quantitative due to the inability to apply MEs mitigation such as matrix-matching to the large range of analytes.[76]



de-epoxy-DON 1,12-dodecanediol

Fig. 9. Standards used in building-relevant trichothecene analysis

The difficulties presented by MEs in such heterogeneous indoor environmental samples were further highlighted by Saito et al. in a 2016 analysis of MCTs in the floor dust of water damaged buildings using GC-MS/MS.[90] By subjecting dust samples to hydrolysis conditions, trichothecenes were converted to VER which was used as a proxy for total MCT content. Previously established GC-MS/MS methods had used an ISTD of 1,12-dodecanediol, and this was examined for its ability to correct for MEs alongside a matrix-matched calibration method. With no ISTD adjustment, a 280% enhancement of the analyte signal was observed. However, the ISTD was found to suffer severe MEs compared with the VER analyte, with the consequence that, in 21 dust samples analysed with ISTD correction applied, VER was not found. In contrast, with a matrix-matched calibration curve and no ISTD adjustment, VER was detectable in 38% of samples, and a 94% recovery was observed in spiking experiments. The authors proposed that the use of this particular standard

for VER analysis could therefore result in a significant over- or under-estimation of trichothecene content in dust. Attempts at preparing stable isotope labelled VER, considered to be the ideal ISTD, were fruitless, and it was suggested that matrix-matched calibrations may provide a useful alternative to overcome MEs when a suitable ISTD is not available.

The ability of matrix-matching to correct for MEs in these contexts was examined specifically by Jaderson & Park who investigated MEs in the measurement of microbial secondary metabolites, including VER, in dust using UPLC-MS/MS.[91] They compared the use of the trichothecene de-epoxy-DON as an ISTD against the use of matrix-matched calibration curves. By comparing the results of standards prepared in neat solvent, standards prepared in a solvent extract of the dusts (matrix-matched), and standards prepared by spiking dusts with a known amount of standard prior to extraction, they were able to gauge MEs and extraction efficiency. Signal suppression due to MEs was found in all 31 microbial secondary metabolites studied. MEs varied significantly by the building from which the dust was collected, the analyte of interest, and the concentration of the spike. It was concluded that MEs could result in a significant underestimation of microbial secondary metabolites in dust samples. Matrix-matched calibrations were found to provide acceptable compensation for MEs and extraction loss, and it was suggested they could provide reasonable results when it was practical to prepare them. However, the increased number of samples required to generate a calibration curve for each matrix and analyte, and the availability of a blank matrix for each sample, were both noted as obstacles to the wider application of the technique. De-epoxy-DON was not found to be a suitable universal ISTD, and where an SI-ISTD for each analyte would be ideal, the impracticalities of achieving this meant that investigations into other correction methods such as standard addition were advised.

In other instances the use of matrix-matching has been reported as inadequate in accounting for MEs. For example, Došsen et al. developed a semi-quantitative UHPLC- (Ultra-HPLC-) QqQ (triple quadrupole MS) method for the detection of S. chartarum secondary metabolites in dust and wall swabs from a water damaged building.[92] UHPLC-qQTOF (quadrupole-quadrupole-time-of-flight MS) analysis of S. chartarum culture extract as a preliminary screen identified several novel potential biomarkers as well as satratoxins and atranones through dereplication against mass-spectral databases of known S. chartarum compounds. These biomarkers were then quantified using UHPLC-QqQ and calibrations based on similar compounds for which reference materials were available. MEs were assessed for the Kimwipes™ used to collect the dust but not for the dust itself, and ion enhancement and suppression were observed even in this presumably less-complex matrix. Thus it was concluded that matrix-matched calibrations would not be adequate to account for the observed MEs and that SI-ISTDs would be preferable.

Next steps

The lack of an ideal analytical tool for the measurement of S. chartarum mycotoxins in indoor environments compels us to develop GC-MS and HPLC-MS/MS techniques using SIDA and standard addition quantification methods respectively. To this end we are preparing an SI labelled VER standard via semi-synthesis from the closely related DAS and pure examples of KTCs by extraction from natural sources. In ongoing work, strains of S. chartarum from the Manaaki Whenua International Collection of Microorganisims from Plants have been cultivated on potato-dextrose agar before inoculation onto Uncle Ben's par-boiled rice, a medium which is known to facilitate the production of MCTs.[93] After 4–6 weeks incubation at room temperature, extraction with 1:1 chloroform:methanol has yielded a crude extract suitable for purification using established techniques.[93] In this way, the KTCs SATG, SATH and ROE have been prepared successfully, while we hope to soon isolate ROL2.[66,94,95]

With regards to the GC-MS method, synthesis of the proposed SI-VER has commenced with the production of a mono-labelled variant in which we hope to shortly introduce further isotopic labelling. These approaches to trichothecene quantification, along with the high mould contamination rates in our leaky buildings, a large sample size, well-matched controls and objective measures of illness, should provide us with a particularly powerful method with which to investigate the relationship between the prevalence of these potent mycotoxins and the health of NZ's leaky building occupants. Overall, this study will further inform the wider controversy surrounding the role of S. chartarum tri-

chothecenes in the development of respiratory disorders, while the SIDA technique will facilitate future investigation into the presence of these building-relevant trichothecenes in a variety of contexts.

References

1. Bennett, J.; Howden-Chapman, P.; Siebers, R. *Do damp and mould matter? Health impacts of leaky homes.*; Steele Roberts Publishers: Wellington, N.Z., 2009.
2. World Health Organisation. Germany 2009.
3. Caillaud, D.; Leynaert, B.; Keirsbulck, M.; Nadif, R. *Eur. Respir. Rev.* **2018,** *27*.
4. Mendell, M. J.; Mirer, A. G.; Cheung, K.; Tong, M.; Douwes, J. *Env. Health Persp.* **2011,** *119*, 748-756.
5. Institute of Medicine *Damp Indoor Spaces and Health*; The National Academies Press: Washington, DC, 2004.
6. Forum of International Respiratory Societies. In *The Global Impact of Respiratory Disease*; European Respiratory Society: Sheffield, 2017.
7. Kirjavainen, P. V.; Täubel, M.; Karvonen, A. M.; Sulyok, M.; Tiittanen, P.; Krska, R.; Hyvärinen, A.; Pekkanen, J. *Indoor Air* **2016,** *26*, 448-456.
8. Nevalainen, A.; Täubel, M.; Hyvärinen, A. *Indoor Air* **2015,** *25*, 125-156.
9. Miller, J. D.; McMullin, D. R. *Appl. Microbiol. Biotech.* **2014,** *98*, 9953-66.
10. Keall, M. D.; Crane, J.; Baker, M. G.; Wickens, K.; Howden-Chapman, P.; Cunningham, M. *Environ. Health* **2012,** *11*, 33.
11. Telfar Bernard, L.; Zhang, J.; University of Otago: Dunedin, 2018.
12. Dyer, P. *Rottenomics: The Story of New Zealand's Leaky Buildings Disaster.*; David Bateman Ltd: Auckland, 2019.
13. Leahy, B. In *NZ Herald*: New Zealand, 2019.
14. Hunn, D.; Bond, I.; Kernohan, D.; Building Industry Authority: Wellington, 2002.
15. Wannemacher, R. W.; Wiener, S. L. In *Medical Aspects of Biological Warfare;* Zajtchuk, R. Ed.; Office of The Surgeon General: Washington DC, 1997; pp. 655-676.
16. Jarvis, B. B. *Phytochem.* **2003,** *64*, 53-60.
17. Fletcher, K.; Clarke, B. Ed.; Biodet Services Ltd: New Zealand, 2019.
18. Yates, D.; Forty-seventh Parliament: Wellington, 2003.
19. Barceloux, D. G. *Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants, and Venomous Animals*; John Wiley & Sons, Inc.: New Jersey, 2008.
20. Jarvis, B. B.; Sorenson, W. G.; Hintikka, E. L.; Nikulin, M.; Zhou, Y.; Jiang, J.; Wang, S.; Hinkley, S.; Etzel, R. A.; Dearborn, D. *Appl. Env. Microbiol.* **1998,** *64*, 3620-5.
21. Robbins, C. A.; Swenson, L. J.; Nealley, M. L.; Kelman, B. J.; Gots, R. E. *Appl. Occupat. Env. Hygiene* **2000,** *15*, 773-784.
22. Miller, J. D.; Rand, T. G.; Jarvis, B. B. *Medical Mycology* **2003,** *41*, 271-291.
23. Chang, C.; Gershwin, M. E. *Clin. Rev.Allergy Immunol.* **2019,** *57*, 449-455.
24. Bush, R. K.; Portnoy, J. M.; Saxon, A.; Terr, A. I.; Wood, R. A. *J. Allergy Clin. Immunol.* **2006,** *117*, 326-333.
25. Yike, I.; Dearborn, D. G. *Mycopathologia* **2011,** *172*, 1-3.
26. Straus, D. C.; Wilson, S. C. *J. Clin. Immunol.* **2006,** *118*, 760.
27. Pestka, J. J.; Yike, I.; Dearborn, D. G.; Ward, M. D. W.; Harkema, J. R. *Toxicol. Sci.* **2008,** *104*, 4-26.
28. Sorenson, W. G.; Frazer, D. G.; Jarvis, B. B.; Simpson, J.; Robinson, V. A. *Appl. Env. Microbiol.* **1987,** *53*, 1370-5.
29. Lemons, A. R.; Croston, T. L.; Goldsmith, W. T.; Barnes, M. A.; Jaderson, M. A.; Park, J.-H.; McKinney, W.; Beezhold, D. H.; Green, B. J. *Inhalation Toxicol.* **2019**, 1-11.
30. Aleksic, B.; Draghi, M.; Ritoux, S.; Bailly, S.; Lacroix, M.; Oswald, I. P.; Bailly, J.-D.; Robine, E. *Appl. Env. Microbiol.* **2017,** *83*.
31. Brasel, T. L.; Martin, J. M.; Carriker, C. G.; Wilson, S. C.; Straus, D. C. *Appl. Env. Microbiol.* **2005,** *71*, 7376-7388.
32. Gottschalk, C.; Bauer, J.; Meyer, K. *Mycopathologia* **2008,** *166*, 103-107.
33. Brasel, T. L.; Douglas, D. R.; Wilson, S. C.; Straus, D. C. *Appl. Env. Microbiol.* **2005,** *71*, 114-122.
34. Vojdani, A.; Thrasher, J. D.; Madison, R. A.; Gray, M. R.; Heuser, G.; Campbell, A. W. *Arch. Env. Health* **2003,** *58*, 421-432.
35. Brasel, T. L.; Campbell, A. W.; Demers, R. E.; Ferguson, B. S.; Fink, J.; Vojdani, A.; Wilson, S. C.; Straus, D. C. *Arch. Env. Health* **2004,** *59*, 317-23.
36. Van Emon, J. M.; Reed, A. W.; Yike, I.; Vesper, S. J. *J. Occup. Environ. Med.* **2003,** *45*, 582-91.
37. Cho, S.-H.; Seo, S.-C.; Schmechel, D.; Grinshpun, S. A.; Reponen, T. *Atmos. Env.* **2005,** *39*, 5454-5465.
38. Gregory, L.; Pestka, J. J.; Dearborn, D. G.; Rand, T. G. *Toxicol. Pathol.* **2004,** *32*, 26-34.
39. Carey, S. A.; Plopper, C. G.; Hyde, D. M.; Islam, Z.; Pestka, J. J.; Harkema, J. R. *Toxicol. Pathol.* **2012,** *40*, 887-898.
40. Amuzie, C. J.; Islam, Z.; Kim, J. K.; Seo, J.-H.; Pestka, J. J. *Toxicol. Sci.* **2010,** *116*, 433-440.
41. Karunasena, E.; Larrañaga, M. D.; Simoni, J. S.; Douglas, D. R.; Straus, D. C. *Mycopathologia* **2010,** *170*, 377-390.
42. Nagayoshi, M.; Tada, Y.; West, J.; Ochiai, E.; Watanabe, A.; Toyotome, T.; Tanabe, N.; Takiguchi, Y.; Shigeta, A.; Yasuda, T.; Shibuya, K.; Kamei, K.; Tatsumi, K. *Mycopathologia* **2011,** *172*, 5-15.
43. Croston, T. L.; Lemons, A. R.; Barnes, M. A.; Goldsmith, W. T.; Orandle, M. S.; Nayak, A. P.; Germolec, D. R.; Green, B. J.; Beezhold, D. H. *Am. J. Respir. Cell Mol. Biol.* **2020,** *62*, 563-576.
44. Islam, Z.; Harkema, J. R.; Pestka, J. J. *Env. Health Persp.* **2006,** *114*, 1099-1107.
45. Bryden, W. L. *Asia Pacific J. Clin. Nutr.* **2007,** *16 Suppl 1*, 95-101.
46. Bennett, J. W.; Klich, M. *Clin. Microbiol. Rev.* **2003,** *16*, 497-516.

47. Øya, E.; Becher, R.; Ekeren, L.; Afanou, A. K. J.; Øvrevik, J.; Holme, J. A. *Int. J. Env. Res. Pub. Health* **2019,** *16*, 1085.
48. Yike, I.; Rand, T. G.; Dearborn, D. G. *Toxicol. Sci.* **2005,** *84*, 408-417.
49. Harding, C. F.; Pytte, C. L.; Page, K. G.; Ryberg, K. J.; Normand, E.; Remigio, G. J.; DeStefano, R. A.; Morris, D. B.; Voronina, J.; Lopez, A.; Stalbow, L. A.; Williams, E. P.; Abreu, N. *Brain Behav. Immun.* **2020,** *87*, 218-228.
50. Crane, J.; Shorter, C.; Waipara, N.; Hinkley, S. F.; Douwes, J.; Pierse, N.; Howden-Chapman, P.; Plagmann, M.; Health Research Council of New Zealand: New Zealand, 2017.
51. Andersen, B.; Nielsen, K. F.; Jarvis, B. B. *Mycologia* **2002,** *94*, 392-403.
52. Andersen, B.; Nielsen, K. F.; Thrane, U.; Szaro, T.; Taylor, J. W.; Jarvis, B. B. *Mycologia* **2003,** *95*, 1227-38.
53. Hinkley, S. F.; Jiang, J.; Mazzola, E. P.; Jarvis, B. B. *Tetrahedron Letters* **1999,** *40*, 2725-2728.
54. Hinkley, S. F.; Mazzola, E. P.; Fettinger, J. C.; Lam, Y.-F.; Jarvis, B. B. *Phytochem.* **2000,** *55*, 663-673.
55. Ulrich, S.; Niessen, L.; Ekruth, J.; Schäfer, C.; Kaltner, F.; Gottschalk, C. *Mycotoxin Res.* **2019**.
56. Wang, Y.; Hyde, K. D.; McKenzie, E. H. C.; Jiang, Y.-L.; Li, D.-W.; Zhao, D.-G. *Fungal Diversity* **2015,** *71*, 17-83.
57. Feng, J.; Zhang, M.; Jia, X.; Zhao, J.; Chen, R.; Xie, K.; Chen, D.; Li, Y.; Liu, J.; Dai, J. *Fitoterapia* **2019,** *136*, 104158.
58. Liu, J.; Jia, X.; Zhao, J.; Feng, J.; Chen, M.; Chen, R.; Xie, K.; Chen, D.; Li, Y.; Zhang, D.; Peng, Y.; Si, S.; Dai, J. *Org. Chem. Front.* **2020,** *7*, 531-542.
59. Ding, Z.-G.; Ding, J.-H.; Zhao, J.-Y.; Chunyu, W.-X.; Li, M.-G.; Gu, S.-J.; Wang, F.; Wen, M.-L. *Fitoterapia* **2018,** *125*, 94-97.
60. Liu, D.; Li, Y.; Li, X.; Cheng, Z.; Huang, J.; Proksch, P.; Lin, W. *Tetrahedron Letters* **2017,** *58*, 1826-1829.
61. Jagels, A.; Hövelmann, Y.; Zielinski, A.; Esselen, M.; Köhler, J.; Hübner, F.; Humpf, H.-U. *Mycotoxin Res.* **2018,** *34*, 179-185.
62. Li, Y.; Wu, C.; Liu, D.; Proksch, P.; Guo, P.; Lin, W. *J. Nat. Prod.* **2014,** *77*, 138-147.
63. Zhang, H.; Yang, M.-H.; Zhuo, F.-F.; Gao, N.; Cheng, X.-B.; Wang, X.-B.; Pei, Y.-H.; Kong, L.-Y. *RSC Advances* **2019,** *9*, 3520-3531.
64. Grove, J. F. In *Progress in the Chemistry of Organic Natural Products;* Herz, W.; Falk, H.; Kirby, G. W. Eds.; Springer Vienna: Vienna, 2007; pp. 63-130.
65. McCormick, S. P.; Stanley, A. M.; Stover, N. A.; Alexander, N. J. *Toxins* **2011,** *3*, 802-814.
66. Hinkley, S. F.; Jarvis, B. B. In *Mycotoxin Protocols;* Trucksess, M. W.; Pohland, A. E. Eds.; Humana Press: Totowa, NJ, 2001; pp. 173-194.
67. Shank, R. A.; Foroud, N. A.; Hazendonk, P.; Eudes, F.; Blackwell, B. A. *Toxins* **2011,** *3*, 1518-1553.
68. Schlessinger, R. H.; Nugent, R. A. *J. Am. Chem. Soc.* **1982,** *104*, 1116-1118.
69. Jarvis, B. B.; Yatawara, C.; Greene, S. L.; Vrudhula, V. M. *Appl. Env. Microbiol.* **1984,** *48*, 673.
70. Choe, S.; In, S.; Jeon, Y.; Choi, H.; Kim, S. *Forensic Science International* **2018,** *291*, 234-244.
71. Bertoni, M. D.; Romero, N.; Reddy, P. V.; White, J. F. *Mycologia* **1997,** *89*, 375-382.
72. Loukaci, A.; Kayser, O.; Bindseil, K. U.; Siems, K.; Frevert, J.; Abreu, P. M. *J. Nat. Prod.* **2000,** *63*, 52-56.
73. Grove, J. F. *Nat. Prod. Rep.* **1993,** *10*, 429-448.
74. Grove, J. F. *J. Chem. Soc. Perkin Trans. 1* **1986**, 647-654.
75. Jeker, N.; Tamm, C. *Tetrahedron Letters* **1989,** *30*, 6001-6004.
76. Capriotti, A. L.; Caruso, G.; Cavaliere, C.; Foglia, P.; Samperi, R.; Laganà, A. *Mass Spectr. Rev.* **2012,** *31*, 466-503.
77. Ellison, S. L. R.; Thompson, M. *Analyst* **2008,** *133*, 992-997.
78. Rychlik, M.; Asam, S. *Anal. Bioanal. Chem.* **2008,** *390*, 617-28.
79. Reed, D. R.; Kass, S. R. *J. Am. Soc. Mass Spectr.* **2001,** *12*, 1163-1168.
80. Varga, E.; Glauner, T.; Köppen, R.; Mayer, K.; Sulyok, M.; Schuhmacher, R.; Krska, R.; Berthiller, F. *Anal. Bioanal. Chem.* **2012,** *402*, 2675-2686.
81. Bretz, M.; Beyer, M.; Cramer, B.; Humpf, H.-U. *Molec. Nutr. Food Res.* **2006,** *50*, 251-260.
82. Bretz, M.; Beyer, M.; Cramer, B.; Humpf, H.-U. *Molec. Nutr. Food Res.* **2005,** *49*, 1151-1153.
83. McMaster, N.; Acharya, B.; Harich, K.; Grothe, J.; Mehl, H. L.; Schmale, D. G. *Food Anal. Methods* **2ds019,** *12*, 2334-2343.
84. Asam, S.; Rychlik, M. *Eur. Food Res. Technol.* **2007,** *224*, 769-783.
85. Häubl, G.; Berthiller, F.; Krska, R.; Schuhmacher, R. *Anal. Bioanal. Chem.* **2006,** *384*, 692-696.
86. Šarkanj, B.; Ezekiel, C. N.; Turner, P. C.; Abia, W. A.; Rychlik, M.; Krska, R.; Sulyok, M.; Warth, B. *Anal. Chim. Acta* **2018,** *1019*, 84-92.
87. Gab-Allah, M. A.; Choi, K.; Kim, B. *Food Control* **2021,** *121*, 107557.
88. Vishwanath, V.; Sulyok, M.; Labuda, R.; Bicker, W.; Krska, R. *Anal. Bioanal. Chem.* **2009,** *395*, 1355-1372.
89. Peitzsch, M.; Sulyok, M.; Täubel, M.; Vishwanath, V.; Krop, E.; Borras-Santos, A.; Hyvärinen, A.; Nevalainen, A.; Krska, R.; Larsson, L. *J. Env. Monitor.* **2012,** *14*, 2044-2053.
90. Saito, R.; Park, J.-H.; Lebouf, R.; Green, B. J.; Park, Y. *J. Occupat. Env. Hygiene* **2016,** *13*, 442-450.
91. Jaderson, M.; Park, J.-H. *Safety Health Work* **2019,** *10*, 196-204.
92. Došen, I.; Andersen, B.; Phippen, C. B. W.; Clausen, G.; Nielsen, K. F. *Anal. Bioanal. Chem.* **2016,** *408*, 5513-5526.
93. Hudson, D. Masters Thesis, Victoria University of Wellington, 2019.
94. Hudson, D.; Singh, A. J.; Lewis, A. R.; Sulyok, M.; Hinkley, S. F. R. *Magn. Reson. Chem.* **2020,** *58*, 198-203.
95. Ridge, C. D.; Mazzola, E. P.; Coles, M. P.; Hinkley, S. F. *Magn. Reson. Chem.* **2017,** *55*, 337-340.