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Determination of ergosterol on mouldy building materials using isotope dilution and gas chromatography–tandem mass spectrometry

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Abstract

Ergosterol content of building materials was quantified using gas chromatography–tandem mass spectrometry (GC–MS–MS) in an ion trap with external ionisation. Hydrolysing the samples by classic extraction at 85°C for 90 min in vials was faster, more precise and safer than microwave assisted extraction. [4-$^{2}H_{2}$]ergosterol was synthesised and used as internal standard, giving method standard deviation of 5–10% from 10 to 30 ng to 10–15 μg ergosterol in the sample. The use of GC–MS–MS meant that no solid-phase extraction clean-up was needed, so one person could easily prepare 40–80 samples per day.

Keywords: Mould; Building materials; Ergosterol

1. Introduction

Frequency of human symptoms in mouldy buildings can be correlated with the area of mould infested materials [1,2], and thus the fungal biomass. Estimation of fungal biomass on solid substrates is a problem usually solved by quantification of chemical markers such as the cell membrane constituent ergosterol (Erg) [3–6] and 18:2ω-phosphorlipid fatty acids [7], or cell wall components as chitin or β-glucans [8], or by determining the activity of β-*N*-acetylglucosaminidase [7]. Other method are to count spores by microscopy or assess the number of viable counts (as colony forming units, CFU) on growth media, although these methods determine sporulation rather than actual biomass [9–11].

Erg is the predominant compound in the cell membrane of all fungi and yeasts, making it a suitable indicator for growth of these organisms, although it should be noted that also some amoebae and green algae are capable of producing Erg [12].

Erg is partially bound as different esters [13], and thus three different extractions can be performed: *Free Erg*, extracted without saponification; *Total free Erg* by saponification of the esters after extraction; and *Total Erg* by saponification of sample during extraction [6,14–16]. In plant tissue extraction has shown some matrix dependency due to instability of Erg under various conditions, as exposure to sunlight, low pH [16,17], problems which would be eliminated using an isotope substituted internal standard.

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Extraction has included reflux in methanol–water with or without NaOH/KOH for 30–90 min, generally being labour intensive compared with the microwave-assisted extraction (MAE) for total Erg developed by Young [6] and extraction method of Larsson and co-workers [18–21], who hydrolyse small samples in small vials.

Several authors have noted that Erg contents decreases after growth has stopped unless samples was stores at temperatures <-20°C [14,22].

The most common method for analysis of Erg has been High Performance Liquid Chromatography (HPLC) with external standard for quantification. Gas Chromatography (GC) has also been used, usually based on an internal standard (I.S.) as cholesterol or dehydrocholesterol [5,18,19], lacking a commercially isotope substituted standard. However, a method of synthesising a double deuterated Erg has been published [23]. Erg has usually been derivatised to its tertiary-butyldimethylsilyl or trimethylsilyl (TMS) ether to reduce decomposition during injection and chromatography. On-column injection can also be used to reduce decomposition even more [6], but this method requires cleaner samples and is not as robust as splitless injection [24,25].

The objective of this study was to develop a fast method for quantitative determination of Erg on mouldy building materials. The method should along with other methods assess the fungal growth on building materials, incubated at different climatic conditions, used for testing the resistance of materials to fungal infestation.

This paper describes a method for quantitative analysis of ergosterol on building materials, based on addition of [4-$^2H_2$]ergosterol as internal standard, hydrolysis of samples in laboratory vials and gas chromatography–tandem mass spectrometric detection of trimethylsilyl–ergosterol. This is, to the authors knowledge, the first method using isotope dilution for ergosterol analysis.

2. Experimental

2.1. Chemicals

Analytical grade silica, toluene, pentane, pyridine, NaOH, HCl, hexane, isopropyl alcohol, ethanol and Gradient Grade methanol were obtained from Merck (Darmstadt, Germany). Erg for synthesis was obtained from Sigma (St. Louis, MO, USA), *N*,*O*-bis(-trimethylsilyl)trifluoroacetamide (BSTFA) and Erg for quantification (82.4 mg ml^{-1} ±0.5% in chloroform) was obtained from Supelco (Bellefonte, PA, USA). I.S. of 7 μg ml^{-1} of [4-$^2H_2$]-Erg in methanol was prepared.

2.2. Synthesis of [4-$^2H_2$]ergosterol

A sample of [4-$^2H_2$]-Erg (Fig. 1) was prepared from ergosterone enol acetate using the method of Barton et al. [23], including purification by chromatography on silica (eluent hexane/isopropyl alcohol 9:1), and recrystallised from 95% ethanol, m.p. ≈150°C.

2.3. Sample preparation

Using a cork bore a 10 mm disk (1–2 mm thick) was cut in the surface of the building material. The disk was transferred to a 14 ml vial, and 2.0 ml methanol, 0.50 ml 2.0 *M* NaOH, and 100 μl [4-$^2H_2$]-Erg (I.S.) was added. Then the sample was ready for classic or microwave assisted extraction.

2.3.1. Microwave assisted extraction

The vial, 14 ml, was sealed with a PTFE lined 20 mm screw cap with a 17-mm hole, so the PTFE membrane would be blown out instead of the vial exploding in case of overheating, placed in a 500-ml

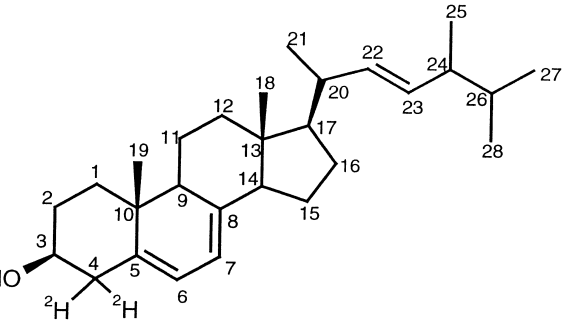

Fig. 1. Structure of [4-$^2H_2$]ergosterol.

container of soft plastic and heated in the microwave oven at 100 W (actual effect) for up to 24 s and allowed to cool to room temperature.

2.3.2. Classic extraction

The 14-ml vials (usually 40–60) were sealed with a PTFE lined screw caps, heated in a oven at 85°C for 90 min and allowed to cool to room temperature.

2.3.3. Derivatisation

Pentane, 2 ml, was added and the 14-ml vials shaken vigorously for 5 s. After 2 min the upper pentane phase was transferred to 2 ml vials and evaporated to dryness at 35°C and 1 mbar in a Rotational Vacuum Concentrator (RVC) from Christ (Osterode, Germany) equipped with a rotor capable of holding 65 vials.

Pyridine, 50 μl, and 50 μl BSTFA was added and the vials were screw capped and allowed to react at room temperature for 30 min. The mixture was evaporated to dryness in the RVC, redissolved in 50 μl toluene and transferred to a 700-μl vial fitting the GC autosampler.

2.4. Instrumentation

A GCQ® (Finnigan Corporation, Austin, TX, USA) integrated GC–MS–MS system (ion trap with external ionisation) fitted with the high temperature ion source was used. The system was controlled from a PC with the Xcalibur 1.1 software (Finnigan Corp.).

2.4.1. Gas chromatography

A Finnigan A200S autosampler was used to inject samples of 1.0 μl splitless at 300°C (split after 30 s, 45 ml/min) in a 4-mm ID Focusliner with glass wool (SGE, Ringwood, Australia).

Separation was performed on a 30 m×0.25 mm (ID), 0.25 μm film thickness, ZB-35 fused-silica column (Phenomenex, Torrance, CA, USA) with the following temperature program: 80°C for 1 min, then 40°C/min to 260°C, then 2°C/min to 320°C holding 2 min. Pressure programming gave a constant linear gas velocity of 35 cm/s. Transfer line temperature was 300°C.

2.4.2. Mass spectrometry

Tune settings: Ion source temperature of 200°C; electron multiplier at 1450–1800 V (Auto tuned to a gain of $3–4\times10^5$); filament emission current 250 mA at 70 eV; High Mass adjustment 100%; and Automatic gain control 50 (arbitrary unit, range 1–300), for MS–MS the parent ion was selected $m/z\pm0.5$, using a Collision Induced Dissociation (CID) voltage of 1.3 V, and a *q*-value of 0.445 (see *ion stability diagram for ion traps*).

For routine analyses the MS was operated in the multi-scan mode: averaging two full scans m/z 100–500 (0.2 s); then averaging two MS–MS scans m/z 468.3 ± 0.5 at 1.3 V (0.4 s), then averaging two MS–MS scans m/z 470.3 ± 0.5 at a CID voltage of 1.3 V (0.4 s), giving approximately one scan each second (Fig. 2).

2.5. Quantitation of samples

The standard curve was obtained by using 0.050, 0.250, 0.825, 2.50, 8.25 μg Erg. From the data-files the peak area of TMS–Erg and TMS-[4-$^2H_2$]-Erg were derived from the m/z 378 (from MS–MS on m/z 468) and m/z 380 (from MS–MS on m/z 470) respectively, using the automated peak detection and integration part of the Xcalibur software.

2.6. Method evaluation

The extraction efficiency was evaluated by spiking 10 mm disks (1–2 mm thick), of six new building materials (each in triplicate) with 100 μl Erg solution (100 ng/ml) and allowing the samples to dry 1 h before extraction. The extraction efficiency was evaluated on four naturally infested wallpapers (in triplicates) from a previous study. The materials were examined under a stereo microscope to find uniformly infested areas.

2.7. Evaluation of mould growth on building materials at different humidities

A mixture of *Aspergillus versicolor*, *A. ustus*, *Chaetomium* spp., *Cladosporium sphaerospermum*, *Penicillium chrysogenum*, *Stachybotrys chartarum*, *Trichoderma harzianum*, and *Ulocladium* spp. was

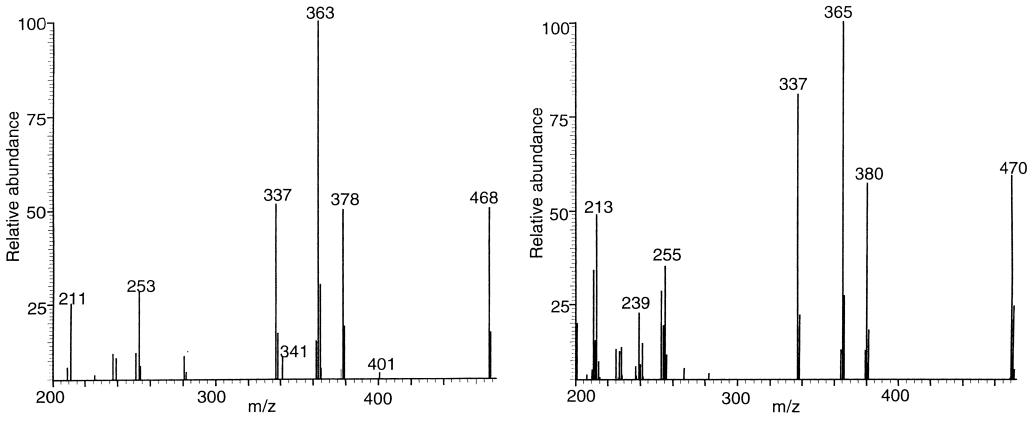

Fig. 2. The mass spectra of trimethylsilyl derivatives of ergosterol (right) and [4-$^{2}H_{2}$]ergosterol.

incubated on 27 different building materials (15×20 cm) at 25°C and 70, 80 and 90% relative humidity (RH) over a 7-month period [26]. The spores were applied by a dry cotton swap gently touched the material at ten different location, this was done to avoid changing the water activity of the material sample by using a spore suspension. From each material, two to three samples were analysed for Erg. On the materials with visible growth samples were taken from high and low infested areas. On the very hard materials such as concrete, a cotton swap was used to sample fungal material from 2 cm^2, as it was impossible to cut discs in the surface.

On a daily basis 40–60 samples (using the classic extraction method) were analysed together with five calibration standards and two blanks.

3. Results and discussion

3.1. Sample preparation

For the MAE it was not possible to use more than 24 s at 100 W=2.4 kJ (calibration of effect gave 100±1 W), and the solvent came to a vigoros boil after 10–14 s. Using the same energy input as Young et al. [6], 35 s at 375 W=13 kJ, almost all solvent escaped from the vial or the vial exploded. The MAE extraction efficiency did not increase after 2×24 s but the standard deviation (SD) increased with about 50% (results not shown).

Comparison of extraction efficiencies on the four mould infested wallpaper materials (Table 1) showed that MAE did not differ significantly from the classic

Table 1
Extraction of ergosterol from mould infested materials by microwave assisted extraction and classic extraction

Material	Wallpaper A[a]		Wallpaper B[b]		Wallpaper C[c]		Wallpaper D[d]	
Extraction method	MAE[e]	Classic	MAE	Classic	MAE	Classic	MAE	Classic
Area of 363/365 amu	0.34	0.40	0.58	0.92	1.28	1.0	0.31	0.25
SD[f] (%)	11	9	34	12	39	8	44	4

[a] Infested with *Ulocladium* sp.
[b] Infested with mixture of *Ulocladium* sp. and *Penicillium chrysogenum*.
[c] Infested with *Aspergillus versicolor*.
[d] Infested with *Chaetomium* sp.
[e] Microwave assisted extraction.
[f] Standard deviation, based on three extractions.

Table 2
Recovery of ergosterol from spiked new building material after classic extraction

Material	Plywood	Chip board	Gypsum board	Cardboard	Wallpaper 1	Wallpaper 2
Recovery	93	105	81	106	92	83
SD[a] (%)	4	1	1	2	3	3

[a] Standard deviation, based on three extractions.

method with respect to degree of extraction, but with respect to SD. The poor SD compared to Young et al. [6] might be due to the microwave oven not being able to heat the small sample uniformly, or the extraction time being too short.

The classic method showed an extraction efficiency of Erg from the spiked materials of 83–106% of the spiked amounts (Table 2).

Using splitless injection no problems with column deterioration were seen, in contrast to Young et al. [6] who experienced deterioration (using on-column injection) if they did not neutralise the samples prior to extraction with pentane.

We tried to neutralise the hydrolysed samples with 1.0 *M* HCl before adding the pentane, but only 10–30% of the Erg could be extracted if pH<7 after neutralisation, which might be due to chemical break down at low pH [17].

Derivatisation gave one product and no unsilylated Erg was detected in any sample. Early in the study the production of TMS–Erg at different reaction times was studied, and it was observed that after 14–16 min, no free Erg could be detected in the samples. Samples kept at 5°C were stable for several months.

The Focus liner and a double goose neck liner (J&W Scientific, Folsom, CA, USA) gave 20–30% higher peak areas compared with straight liners. Careful deactivation of the liners after cleaning gave 10–30% higher peak areas.

Peak tailing was not observed on the 35% phenyl–methyl silicone column, compare with a 5% phenyl methyl silicone column (30 m×0.25 mm, film thickness 0.1 μm) (Hewlett-Packard, Avondale, PA, USA) tried early in the study.

3.2. Mass spectrometry

The high mass parts of TMS–Erg and TMS-[4-$^2H_2$]-Erg are shown in Fig. 3. Prominent peaks produced by loss of TMS–OH followed by loss of $CH_3^{\cdot}$. The peak at *m/z* 337, representing a loss of 131 u, in the spectrum of TMS–Erg has been suggested to come from a loss of a moiety including C1, C2 and C3 [27] but the corresponding loss in the spectrum of TMS-[4-$^2H_2$]-Erg is 133 u, giving the same peak at *m/z* 337, meaning that the two ^{2}H-atoms at C4 are lost. The use of MS–MS using collision induced dissociation (CID) on TMS-[4-$^2H_2$]-Erg showed that M^+ (*m/z* 470) could be fragmented into *m/z* 380, 365 and 337. MS–MS showed that *m/z* 380 could be fragmented to *m/z* 365 and 337. MS–MS–MS showed that *m/z* 380 could be fragmented in to *m/z* 365, which then could be fragmented to *m/z* 337.

On standards full-scan and MS–MS was compared using the same datafiles. Full-scan MS (integrating *m/z* 363/365 or 378/380) gave higher, although not statistically significant (SD 3–6%) than MS–MS on the M^+(integrating *m/z* 363/365 or 378/380) giving SD (4–8%), but on real samples MS–MS gave better defined peaks than full-scan MS, and the automated peak detection routine in the Xcalibur software gave no false negatives compared with manually inspection of the datafiles

It was tried to do MS–MS on the range *m/z* 469±2 as both the I.S. and analyte ions would be present at the same time, but ion to molecule reactions specially seen as deuterium migration made quantification impossible, even when low CID reaction times were used.The MS–MS fragmentation patterns seem to be affected by four factors:

- Ion to molecule reactions, which is a real problem in ''thick'' samples giving problems both for full-scan and MS–MS, high air and/or water levels enhances this problems.
- Dirty ion volume, facilitated the loss of one methyl group from the mother ion.
- Width with which the mother ion is selected. The narrower the band the less energy is required, e.g.

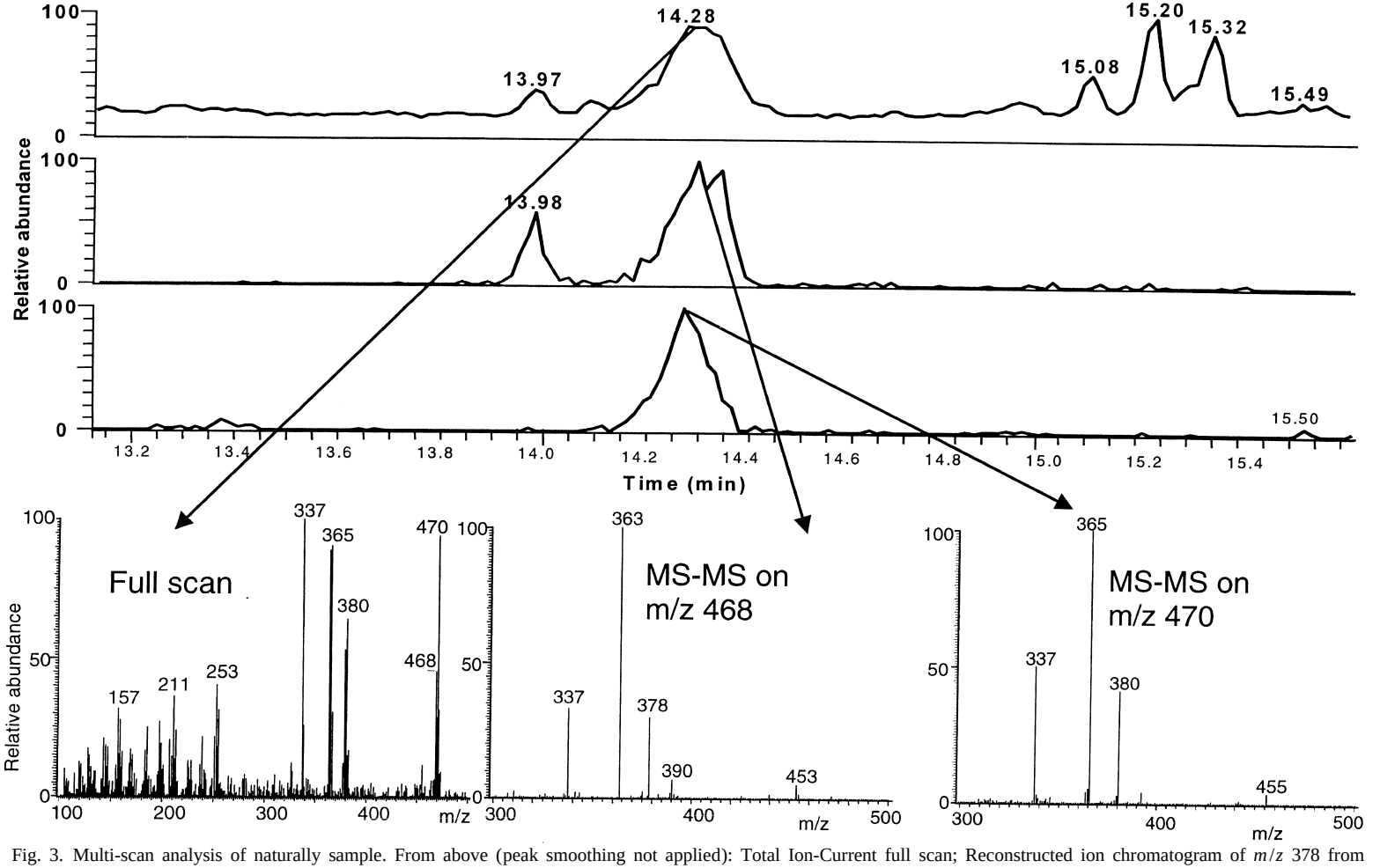

Fig. 3. Multi-scan analysis of naturally sample. From above (peak smoothing not applied): Total Ion-Current full scan; Reconstructed ion chromatogram of m/z 378 from MS–MS scans on m/z 468 amu (TMS–ergosterol); Reconstructed ion chromatogram of m/z 380 amu from MS–MS on m/z 470 (TMS-[4-$^{2}H_{2}$]ergosterol); and lowest, the peak apex spectra from each scan event (from left); Full scan, MS–MS on m/z 468; and MS–MS ON m/z 470.

468±0.5 at 1.3 V gives approx. the same MS–MS spectra as 468±2 at 1.8 V.

- Reaction time during CID seemed to be important for production of ions, which was only produced when long reaction times were used.

3.3. Quantification

As seen in Table 2, the SD ranged from 1 to 4% in spiked samples (triplicate). In contrast to real samples (Table 2), in which the SD ranged from 4 to 12% (triplicate). Detection limits in the sample of 2–10 ng (s/n 5) using the m/z 378 ion were recorded, and 1–10 ng (s/n 5) using m/z 363 ion, but some interference was seen from the internal standard on the m/z 363 ion.

Linear calibration curves (5 points and 1 blank, $y=0.0008x-0.01$) could be obtained from the detection limit to 10–15 μg Erg in the sample. The calculated ratio of 378/380 u as function of Erg shows that quantification should be possible up to 40–60 μg Erg in a sample, which is not practically possible due to ion-to-molecule reactions in the ion trap.

Detection limits could probably be lowered if the full-scan segment (Fig. 2) is removed from method, as more scans could be performed giving a better defined peaks, but then it would be impossible to see a coeluting compound, which can give problems with ion to molecule reactions.

3.4. Evaluation of mould growth on building materials at different humidities

No visible growth of moulds were observed at 70% RH, and there was a very large variation on the Erg content on the material surfaces at 80 and 90% RH as the materials could be heavily infested on

Table 3
Ergosterol content of building materials incubated with at mixture of moulds at 70, 80 and 90% relative humidity for 7 months

Material	70% RH (ng/cm^2)			80 RH (ng/cm^2)		90 RH (ng/cm^2)		
Calcium silicate plate	ND	ND	ND	ND	ND	ND	ND	ND
Painted glass fibre WP[a] on wet zone GB[b]	ND	ND	ND	ND	ND	467	590	ND
PVC floor on wet zone GB	ND	ND	ND	ND	ND	ND	ND	
Wallpapered chipboard	ND	ND	ND	500	ND	780	977	ND
Painted, wallpapered, gypsum board	ND	ND	ND	370	147	1112	5883	4167
Painted gypsum board	ND	ND	ND	ND	ND	256	ND	
Gypsum board (fibre gypsum)	ND	ND	ND	ND	72	473	478	478
MDF plates	ND	ND	ND	615	190	1477	1760	1851
Beech wood floor boards[c]	ND	ND	ND	893	1593	1297	2869	358
Old plywood (25 years)	ND	ND	ND	ND	224	792	1018	269
Previously mould contaminated plywood[c]	262	356	368	612	1132	1480	1657	1776
Plywood	ND	ND	274	ND	ND	2008	633	409
Paper wool insulation	ND	ND	ND	ND	ND	144	438	769
Mineral wool (MMMF)	ND	ND	ND	84	79	47	656	
Pine wood on mineral wool (MMMF)	ND	ND	ND	616	43	1162	1987	3974
Pine wood, contaminated with earth	44	ND	ND	2012	ND	1601	912	800
Pine wood	ND	ND	ND	1413	791	2698	5817	3397
Planed pine wood	ND	ND	ND	525	ND	120	6543	1735
Wallpapered levelling layer	ND	ND	ND	2025	127	1505	330	1015
Levelling layer	ND	ND	ND	ND	ND	ND	ND	ND
Levelling layer (concrete)	ND	ND	ND	ND	ND	ND	ND	
High density concrete	ND	ND	ND	ND	ND	ND	ND	
Wallpapered low density concrete	ND	ND	ND	ND	ND	2241	901	1742
Low density concrete	ND	ND	ND	ND	36	ND	ND	
Wallpapered gypsum board	ND	ND	ND	106	99	2601	961	1010
Gypsum board (fabricate 1)	ND	39	ND	ND	ND	496	ND	
Gypsum board (fabricate 2)	ND	ND	ND	58	ND	600	549	

[a] WP: wallpaper.
[b] GP: gypsum board.
[c] previously mould contaminated. ND: not detected. Experimental details, see Section 2.7.

some parts whereas some parts could be without growth [26]. No Erg was detected when analysing new building materials, but as seen in Table 3, three of the materials incubated at 70% RH: *Pine wood, contaminated with earth*, *Gypsum board (fabricate 1)*, and *Plywood* contained detectable quantities of Erg, although no growth seemed to occur a 70% RH. These samples were presumable taken at points with very little infestation or in the inoculation points, which we have made visible in later studies.

Generally there were correlation between the Erg content, the degree of infestation assessed by visual inspection, inspection by a stereo microscope, and β-*N*-acetylglucosaminidase (correlation to Erg, $R^2 =$ 0.75 based on 54 samples) determined by the Mycometer Test (MycoTec, Copenhagen, Denmark) (result not shown) [26]. Both Erg analysis and the Mycometer Test showed growth on materials with no visible signs of growth [26].

All 200 materials, 40 standards and blanks were analysed during 1 week. During this time the only maintenance performed on the GCQ system was changing of the septa three times (routine after ca. 100 injections), and after 160 analyses the ion-volume was cleaned and 10 cm was cut from the column, as the lowest calibration level, 25 ng, was not detected.

4. Conclusion

The high SD of MAE, compared with the classic extraction method (Table 1) and especially the occasionally exploding vials made the classic extraction method our choice. By hydrolysing and extracting small samples in vials, it was possible for one person to make up to 80 samples, including 5 calibration levels, ready for GC–MS–MS analysis per day.

By monitoring the product ions m/z 378 and m/z 380 originating from the molecular ions, of TMS–Erg and TMS-[4-$^2H_2$]-Erg respectively, the datafiles could be analysed automatic by the Xcalibur software. Due to the isotope substituted standard it was possible to have a very low SD of 4–12% on real samples.

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