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Determination of ergosterol in organic dust by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric method was developed for the determination of ergosterol in organic dust. Samples were hydrolyzed under alkaline conditions, and the hydrolysate was extracted, purified on a silica-gel column, and subjected to derivatization. The limit of detection of the trimethylsilyl ether derivative of ergosterol was approximately 10 pg and that of the *tert.*-butyldimethylsilyl ether derivative was approximately 20 pg (injected amounts). House dust contained 6–45 μg ergosterol/g and air from a pig barn contained 0.2–0.3 ng ergosterol/liter. The proposed method can be used as a complement or alternative to microscopy and culturing for measuring fungal biomass in air-borne organic dust.

1. Introduction

Inhalation of air-borne microorganisms has been associated with the development of several clinical symptoms, including pulmonary irritation and a wide spectrum of inflammatory reactions. Clearly, specific and sensitive methods for measuring and characterizing air-borne microorganisms are needed to establish a correlation between the different microbial structures that are inhaled and the individual symptoms. Cultivation of microorganisms after air sampling is of limited value since only viable microbes are detected, and microscopic methods are non-specific and, at best, semi-quantitative. Alternatively, specific microbial constituents can be quantified by using chemical-analytical methods. Thus, air-borne endotoxin–the lipopolysaccharide (LPS) of Gram-negative bacteria–has been determined with gas chromatography–mass spectrometry (GC–MS) to measure long-chain 3-hydroxy fatty acids [1–3], and peptidoglycan–the backbone of both Gram-positive and Gram-negative eubacteria–has been determined using GC–MS to measure muramic acid [3,4]. This analytical approach represents a sensitive, specific, and reproducible way of characterizing the bacterial composition of air-borne organic dust.

Ergosterol has been widely used as a chemical marker for measuring fungal biomass in environmental samples, primarily by using high-performance liquid chromatography (HPLC) [5–8]. Because of its inherent high degree of sensitivity and specificity, it should be advantageous to use GC–MS for measuring ergosterol. The present communication describes a GC–MS method for the determination of ergosterol in organic dust,

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including conditions for sample hydrolysis, post-hydrolysis purification, and derivatization.

2. Experimental

2.1. Chemicals and glassware

Ergosterol, dehydrocholesterol, and *tert.*-butyldimethylsilylchloride were purchased from Sigma (St. Louis, MO, USA), N-methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide from Janssen Chimica (Beerse, Belgium), and N,O-bis-(trimethylsilyl)trifluoroacetamide and 1-*tert.*-butyldimethylsilylimidazole from Fluka Chemie (Buchs, Switzerland). Solvents were of analytical-reagent grade and were used without further purification. All glassware was acid-washed and heated overnight at 350°C prior to use. Glass test tubes were equipped with PTFE-lined screw caps.

2.2. Microorganisms

The following microorganisms were studied: *Candida albicans* (ATCC 2091), *Aspergillus fumigatus* (DSM 819), *Aspergillus flavus* (DSM 1959), *Aspergillus niger*, *Alternaria* sp, *Cladosporium* sp, and *Penicillium* sp (clinical isolates). The microorganisms were cultivated on Sabouraud dextrose agar medium: *C. albicans* for 48 h at 37°C, and the remaining species for 3–4 days at 25°C. Cultures were then transferred to glass test tubes, washed with water, and freeze-dried. Amounts of 5–10 mg of the dried fungal biomass were analysed.

2.3. Dust samples

Seven house dust samples (50–300 mg), collected from a vacuum cleaner on seven different occasions, and two samples of air-borne dust collected by respectively pumping 60 and 195 liters of air in a pig barn through polycarbonate filters (0.4-μm pore size, 37 mm I.D.; Poretics Corp., Livermore, CA, USA), were studied.

2.4. Sample treatment

The standard sterols, cultivated fungi, house dust and air-filter samples were heated at 80°C for 90 min in 3 ml of 10% methanolic KOH. A 1-ml volume of water was added and the aqueous methanolic solution was extracted with 2 × 2 ml of hexane. The hexane phases were pooled, transferred to a new test tube, and evaporated to dryness using a stream of nitrogen. Samples were then dissolved in 1 ml of dichloromethane–hexane (1:1) and applied on a disposable silica-gel column (100 mg Si/1 ml; Analytichem, Harbour City, CA, USA) which had been preconditioned with 1 ml of ether and 1 ml of dichloromethane–hexane. The column was washed with two 1-ml portions of dichloromethane–hexane before eluting sterols/polar compounds with two 1-ml portions of diethyl ether. After evaporation of the solvent, dehydrocholesterol was added as an internal standard, and derivatization was performed.

Two different derivatives were used: trimethylsilyl (TMS) derivatives, formed by adding 50 μl of N,O-bis(trimethylsilyl)trifluoroacetamide and 15 μl of pyridine to the sample and then heating for 30 min at 60°C; *tert.*-butyldimethylsilyl (tBDMS) derivatives, prepared by adding 50 μl of N-methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide–*tert.* - butyldimethylsilylchloride–*tert.*-butyldimethylsilylimidazole–acetonitrile (50:0.5:5:100, v/v) to the sample and then heating for 2 h at 60°C. The preparations were dissolved in hexane and analysed the same day as they were prepared. The sterol standards were analysed as both TMS and tBDMS derivatives, whereas the fungi and the dust samples were analysed only as TMS derivatives.

2.5. Gas chromatography

A Varian Model 3500 gas chromatograph (Los Altos, CA, USA) was used for analysis of the derivatives of the sterol standards. Injections were made in the splitless mode; nitrogen (1 ml/min) was used as the carrier gas. The fused-silica capillary column (SE-54, 0.25 μm film

thickness, 25 m × 0.25 mm I.D.) was temperature programmed at 20°/min from 170 to 290°C. The injector was kept at 290°C and the flame ionization detector at 300°C. The chromatograms were evaluated using a Chrompack integration system with an IBM PS/2 Model 30 and a Chrompack BD 70 printer/plotter.

2.6. Gas chromatography–mass spectrometry

A VG Trio-1 S mass spectrometer connected to a Hewlett-Packard Model 5890 gas chromatograph (Fisons, Manchester, UK) was used for analysis of the sterol standards, fungi, and dust samples. Injections were made in the splitless mode using a Hewlett-Packard Model 7673 autosampler; helium, at a flow-rate of 1 ml/min, was used as carrier gas. The column, injector temperature, and temperature program were the same as for the GC analyses. The interface temperature (between the GC and the MS systems) was 290°C, and the ion source temperature was 220°C. The MS was operated in both scanning and selected-ion monitoring (SIM) modes; the ionization energy was 70 eV.

2.7. Stability of derivatives, recovery

A stock solution consisting of a mixture of ergosterol and dehydrocholesterol (40 μg of each, 1 μg/μl) was distributed in 1.5-ml glass bottles, evaporated to dryness, and converted to TMS and tBDMS derivatives (samples prepared in duplicate) according to the procedures described above. Samples were diluted in hexane to a final volume of 500 μl; the concentration of the sterol derivatives was approximately 80 ng/μl. The ergosterol/dehydrocholesterol area ratios were determined by GC on days 1,2,4,5,8 and 11.

The recovery of ergosterol in the silica-gel purification step was evaluated. Three 40-μg samples of ergosterol were allowed to pass through separate silica-gel columns, and 40 μg of dehydrocholesterol was then added to each of the six eluates (three obtained by elution with hexane–dichloromethane, and three by elution with diethyl ether). In addition, three 40-μl samples containing a mixture of ergosterol and dehydrocholesterol (1 μg/μl of each, in methylene chloride) were also prepared, without any silica-gel column purification. All nine samples were TMS derivatized and analysed by GC.

2.8. Detection sensitivity, quantitation

GC–MS detection sensitivity was evaluated by injecting 1-μl portions of preparations of TMS and tBDMS derivatives of ergosterol. The preparations contained 100, 50, 10, and 5 pg of derivative/μl.

To construct a standard curve, TMS derivatives of ergosterol and dehydrocholesterol were separately prepared and the preparations were combined and diluted to the desired concentrations for GC–MS analysis. The dehydrocholesterol derivative was injected in 200-pg amounts, whereas injected amounts of the ergosterol derivative varied from 10 to 2000 pg (n = 8).

2.9. Reproducibility

A 100-mg aliquot of a house dust sample was subjected to the described alkaline hydrolysis procedure. The heterogenous mixture was centrifuged and the supernatant distributed, in equal amounts, into 7 test tubes. Dehydrocholesterol (internal standard, 500 ng in chloroform) was added to each tube whereafter the hydrolysates were extracted, purified, and TMS derivatized. The preparations were diluted 5-fold with hexane before analysed by GC–MS in the SIM mode as described above.

3. Results

3.1. Gas chromatography: derivative formation, stability, and recovery

According to GC results, both ergosterol and dehydrocholesterol were completely derivatized: no peaks corresponding to the underivatized sterols appeared, even when as much as 100 ng

of pure sterol derivatives was injected (detection limit approximately 1 ng). TMS derivatives, as compared to tBDMS derivatives of the same sterols, eluted earlier from the column and showed sharper peaks.

The mean ergosterol/dehydrocholesterol ratios of samples stored at room temperature for up to 11 days were 0.65 (range: 0.62–0.69) for the TMS derivatives and 0.61 (range: 0.56–0.66) for the tBDMS derivatives ($n = 12$ for both). The recovery of ergosterol in the silica-gel column purification step varied between 97 and 102%; ergosterol was not detected in any of the dichloromethane–hexane eluates.

3.2. Mass spectrometry: quantitation and limit of detection

The mass spectrum of TMS-derivatized ergosterol showed two abundant ions in the high-mass range: m/z 337 $[M - 131]^+$, which is due to loss of the trimethylsilanol group and the C_1–C_3 fragment, and m/z 363 $[M - 105]^+$, due to loss of the trimethylsilanol group and one methyl group; these ions were used to determine ergosterol in the dust samples. A small molecular ion peak (m/z 468) was also seen in the spectrum of the TMS derivative. The tBDMS derivative revealed a similar fragmentation pattern, i.e. m/z 337 and m/z 363 dominated in the high-mass region, and a small molecular ion was seen at m/z 510. The TMS derivative of dehydrocholesterol (internal standard) produced dominating ions of m/z 325 $[M - 131]^+$ and 351 $[M - 105]^+$; the molecular ion was m/z 456 (Fig. 1).

The lowest detectable amounts of the TMS and tBDMS derivatives of ergosterol were, respectively, about 10 and 20 pg (signal-to-noise ratio of 4, SIM mode, m/z 363). The standard curve of TMS-derivatized ergosterol was linear over the studied range, i.e. 10–2000 pg (injected amount), and followed the equation $y = 1.32 \cdot 10^{-3}x - 2.29 \cdot 10^{-2}$ ($r = 0.998$; x = picograms of ergosterol derivative injected; y = peak-area ratios of ions 337/325).

3.3. Fungi and dust samples

Ergosterol was the dominating sterol in all fungal strains studied (this is shown for *Aspergillus flavus* in Fig. 2); other identified sterols include zymosterol and episterol (data not shown). *A. fumigatus* contained the largest amounts of ergosterol, i.e. 14.3 μg/mg (dry weight); the other fungi contained between 2.5 and 8.8 μg/mg.

The amounts of ergosterol in the dust samples were determined by SIM analysis using ions m/z 337 and 363. The seven house-dust samples contained between 6 and 45 μg/g, and the two air-filter samples contained 0.2 and 0.3 ng of ergosterol/l, respectively (an analysis example is shown in Fig. 3). After analysis of the pure fungal cultures, a new column of the same type was installed and used to analyse the dust and filter samples, and this change caused the differences in the retention times.

The amounts of ergosterol in the seven hydrolysates from the 100-mg house dust sample used for evaluating the reproducibility were calculated to 45.4 (mean value) ± 1.6 (S.D.) μg/g dust; the coefficient of variation was 3.5.

4. Discussion

Unsatisfactory indoor air quality is suspected to be a major cause of the worldwide increase in the occurrence of asthma, allergies, and related diseases. A correlation between the presence of moulds in indoor environments (as a result of high humidity) and development of symptoms has been demonstrated repeatedly, and the inhalation of both endotoxin and fungal β(1–3)glucan have been shown to elicit several toxic reactions [9–13]. GC–MS has been used extensively in our laboratory to determine endotoxin by quantitation of specific long-chain 3-hydroxylated fatty acids [1–3]. Similarly, muramic acid, a unique compound of peptidoglycan, has been used as a marker for measuring the total bacterial content of, for example, organic dust [3,4].

Ergosterol, a fungus-specific membrane lipid,

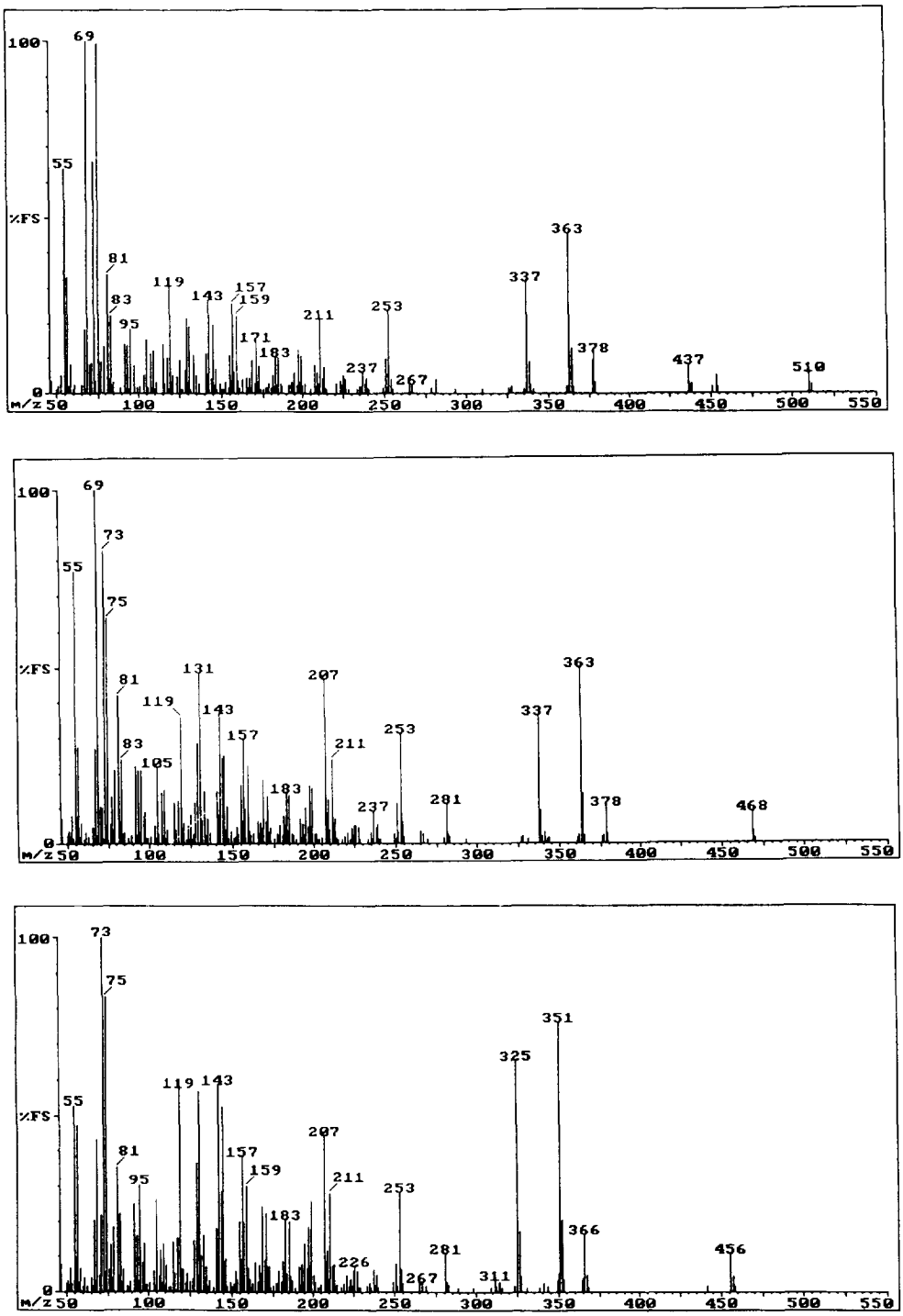

Fig. 1. Mass spectra of the tBDMS derivative of ergosterol (upper), the TMS derivative of ergosterol (center), and the TMS derivative of the internal standard dehydrocholesterol (lower).

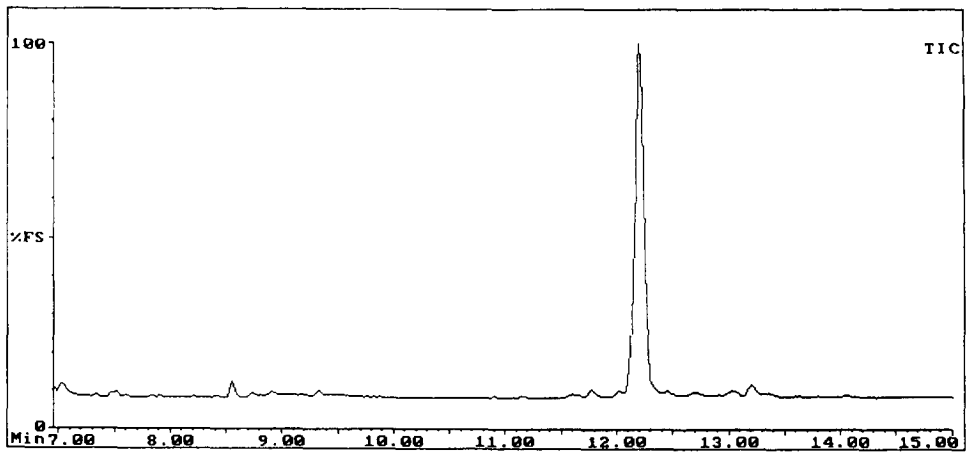

Fig. 2. Total-ion current chromatogram of a derivatized hydrolysate of *Aspergillus flavus* showing that ergosterol (as a TMS derivative) is the predominating sterol (retention time 12.2 min).

has been widely used as a chemical marker for the determination of fungal biomass in ecological microbiology, mainly by using HPLC [5–8]. This technique is simple to apply, since samples can be injected onto the column directly after hydrolysis, i.e. derivatization is not required. Obviously, it should be possible to improve detection performance considerably by applying GC–MS. Use of chemical ionization MS in combination with negative-ion detection (NICI) has been shown to provide a high detection sensitivity e.g. of pentafluorobenzoyl derivatives of hydroxy fatty acids and alcohols [14–16] and pentafluorophenyldimethylsilyl (flophemesyl) derivatives of sterols [17]. However, in our laboratory, attempts to apply GC–NICI-MS for determining ergosterol were unsuccessful because (1) the pentafluorobenzoyl derivative of ergosterol was apparently degraded in the GC injector or in the column, and (2) the flophemesyl derivative gave rise to a NICI mass spectrum in which ions representing the flophemesyl moiety dominated and the detection sensitivity was low (data not shown).

It has previously been reported that the tBDMS derivative of ergosterol possesses higher chemical stability than the TMS derivative does, and that tBDMS derivatives of sterols other than ergosterol provide higher detection sensitivities in GC–MS than TMS derivatives do since abundant ions are formed in the high-mass region [18,19]. However, in the present study, we found that both TMS and tBDMS derivatives of ergosterol were stable for at least 11 days when stored in excess of the reagents, and, because their mass spectra are almost identical, they provided a similar GC–MS detection sensitivity. The TMS derivatives may be preferred for routine application due to a quicker sample preparation procedure. To avoid an unsatisfactory peak shape, we found it necessary to keep the glass liner in the injector port free from carbon deposits and also to cut off the first 10–15 cm of the column regularly. Injection of underivatized ergosterol frequently resulted in severe peak tailing (data not shown). The silica gel column purification step was very efficient in removing non-polar lipids from the sample hydrolysates. The hydrolysis conditions used appeared to be efficient in liberating ergosterol since neither the use of higher temperatures, prolonged time, or higher concentrations of KOH was found to increase the yield (data not shown).

Until now, analytical conditions for determining chemical markers to quantify and character-

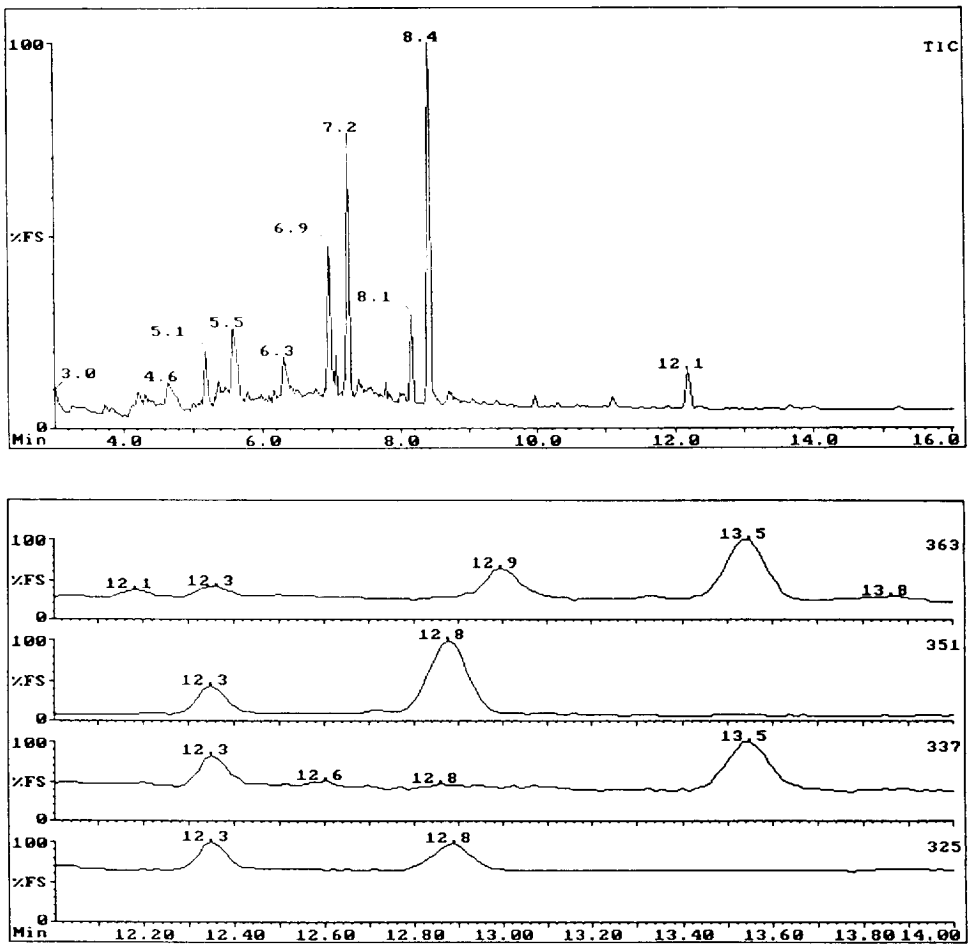

Fig. 3. Total-ion current chromatogram of a TMS-derivatized hydrolysate of air-borne material collected from a pig barn (upper), and selected-ion monitoring tracings of the same sample showing TMS derivatives of ergosterol (retention time 13.5 min) and dehydrocholesterol (retention time 12.8 min) (lower).

ize the microbiological composition of organic dust have been given only for bacterial markers, e.g. 3-hydroxy fatty acids [1], muramic acid [3], and diaminopimelic acid [20]. The GC–MS method for determination of ergosterol presented here shows that it is also possible to measure air-borne fungal biomass. The amounts of ergosterol found in house dust (6–45 μg/g) are slightly higher than previously reported values [21]. Further studies are in progress in our laboratory to relate levels of ergosterol in organic dust to results from other methods of measuring fungi, such as microscopy, culturing, and determination of β(1–3)glucan.

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References

[1] Z. Mielniczuk, E. Mielniczuk and L. Larsson, *J. Microbiol. Methods*, 17 (1993) 91.
[2] M. Walters, D. Milton, L. Larsson and T. Ford, *Appl. Environ. Microbiol.*, 60 (1994) 996.
[3] A. Fox, R. Rosario and L. Larsson, *Appl. Environ. Microbiol.*, 59 (1993) 4354.
[4] A. Fox and R. Rosario, *Indoor Air* 93, 4 (1994) 267.
[5] L. Seitz, D. Sauer, R. Burroughs, H. Mohr and J. Hubbard, *Phytopathology*, 69 (1979) 1202.
[6] J. Schnürer, *Appl. Environ. Microbiol.*, 59 (1993) 552.
[7] K. Suberkropp, M. Gessner and E. Chauvet, *Appl. Environ. Microbiol.*, 59 (1993) 3367.
[8] M. Gessner and E. Chauvet, *Appl. Environ. Microbiol.*, 59 (1993) 502.
[9] R. Rylander, *Am. J. Ind. Med.*, 10 (1986) 221.
[10] R. Rylander, H. Goto and B. Marchat, *Cotton Dust 13th*, (1989) 145.
[11] M. Burr, J. Mullins, T. Merret and N. Scott, *Thorax*, 40 (1985) 688.
[12] M. Burr, J. Mullins, T. Merret and N. Scott, *J. Roy. Soc. Health*, 3 (1988) 99.
[13] S. Platt, C. Martin, S. Hunt and C. Lewis, *Br. Med. J.*, 298 (1989) 1673.
[14] Z. Mielniczuk, S. Alugupalli, E. Mielniczuk and L. Larsson, *J. Chromatogr.*, 623 (1992) 115.
[15] S. Alugupalli, Z. Mielniczuk and L. Larsson, *J. Microbiol. Methods*, 15 (1992) 229.
[16] L. Larsson, *APMIS*, 102 (1994) 161.
[17] P. Robinson, M. Jones and J. Maddock, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 6.
[18] S. Howell, M. Moore, A. Mallet and W. Noble, *J. Gen. Microbiol.*, 136 (1990) 241.
[19] C.J.W. Brooks, E.C. Horning and J.S. Young, *Lipids*, 3 (1968) 391.
[20] A. Sonesson, L. Larsson, A. Fox, G. Westerdahl and G. Odham, *J. Chromatogr.*, 431 (1988)1.
[21] J. Miller, A. Laflamme, Y. Sobol, P. Lafontaine and H. Greenhalgh, *Int. Biodeterior.*, 24 (1988) 103.