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USE OF MASS SPECTROMETRY FOR CHARACTERISING MICROBIAL COMMUNITIES IN BIOAEROSOLS

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Abstract: The use of chemical marker analysis for characterising microbial communities in organic dust samples is exemplified in a comparative study of dusts collected in a home and a swine confinement building, respectively. The chemical markers studied included 3-hydroxy fatty acids (markers of endotoxin), ergosterol (marker of fungal biomass), and muramic acid (marker of peptidoglycan/bacterial biomass). Samples were hydrolysed and subjected to various chemical manipulations for rendering the markers suitable for gas chromatography-tandem mass spectrometry analysis. Considerable differences between the dust samples were revealed. Swine dust contained 46 ng/mg of ergosterol (house dust 2.1 ng/mg), 0.096 nmol/mg of endotoxin (house dust 0.020 nmol/mg), and 483 ng/mg of muramic acid (house dust 366 ng/mg). The 3-hydroxy fatty acid and muramic acid results demonstrated a much higher proportion of Gram-negative bacteria to Gram-positives in swine dust than in house dust, and ergosterol results demonstrated a much higher proportion of fungi. The different distribution of 3-hydroxy fatty acids in the 2 samples illustrated differences in their flora of Gram-negative bacteria. The described method allows accurate determination of markers even when present down to trace levels in chemically complex matrices and should be useful in evaluating the role of microorganisms in the development of occupational lung disease, e.g. in agricultural environments.

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INTRODUCTION

A high prevalence of occupational lung diseases such as bronchitis, asthma, and airway hyperreactivity as a result of exposure to organic dusts in agricultural environments has been documented for several decades [8, 18, 27]. Dusts that become airborne in such environments are complex and originate from a wide variery of sources like feed components, dander, feathers, faecal material, pollen grains, insect parts, microorganisms, and other bioactive materials [9, 11, 15, 28]. Microorganisms are suspected of playing an important role in the development of the diseases mentioned, hence it is important to have access to adequate methods for measuring both bacteria and fungi, as well as the components thereof, in bioaerosols. Unfortunately, none of the methods in current use are satisfactory. **Culturing** is frequently applied although it is widely assumed that only a small fraction (0.1-10%) of the total microbial flora in environmental samples is culturable. **Microscopy** provides little of the microbial characteristics of the collected material; also, particles of microbial and non-microbial origin may be confused, especially when studying samples from highly contaminated environments that contain large amounts of debris of non-microbiological origin [23, 24]. Microscopic methods that involve washing of filters after air dust collection

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followed by staining and microscopic analysis of the resulting suspension are time consuming. **Limulus analysis** is the most commonly used analytical method to determine endotoxin and is based on the Limulus Amebocyte Lysate (LAL) assay. In this test, endotoxin activates a proenzyme and cleavage products of a synthetic substrate are measured. This test is very sensitive but the interlaboratory reproducibility is poor. **The polymerase chain reaction (PCR)** has to date not been extensively used in studies on the microbiology of indoor environments due to difficulties in achieving quantitative results, but this technique is constantly being improved and it is likely that it will be much more commonly applied in the future.

It is obvious that new and more reproducible and quantitative methods are required to further evaluate the role of microorganisms in the development of occupational lung disease, e.g. in agricultural environments. The aim of the present paper is to illustrate the performance of an analytical-chemical method, by applying the so-called chemical marker concept, for characterising and quantifying the microbial population in organic dust samples.

CHEMICAL MARKER ANALYSIS

In the chemical marker concept, the microbial contents of samples such as airborne and settled dust collected from indoor environments are measured by quantifying certain unique monomeric compounds – the chemical markers – by using analytical-chemical methods. Such markers are substances that, since they are unique to bacterial or fungal cells, can be used to quantify and characterise microorganisms *in situ* without any need for culturing. An advantage of this approach in measuring airborne microbiological material is that the filters used for air sampling can be directly subjected to hydrolysis. In other words, in contrast to biological assays, methods for analysing chemical markers do not exhibit inconsistencies due to variations in the efficiency of the procedures used to extract the microbiological materials from the filters. Furthermore, analysis of these markers does not discriminate between species - an unavoidable disadvantage of all culture-based methods. Chemical markers are usually compounds covalently linked to various structures in the cell membranes, thus, prior to analysis, a sample must be hydrolysed to cleave these linkages. Thereafter, the analytes are purified by extractions and, finally, derivatised.

The most suitable analytical technique for quantifying chemical markers in heterogeneous environments is to use gas chromatography (GC) in combination with mass spectrometry (MS). Modern GC-MS technology allows accurate determination of markers even when present down to trace levels in chemically complex matrices. We used a bench-top ion-trap GC-MS instrument equipped with a standard non-polar GC column and an autosampler that can be run in the electron impact (EI) and chemical ionisation (CI) modes utilising tandem MS (MSMS). The introduction of ion-trap instruments in recent years has made it practical – and economical - to perform GC-MSMS. In MSMS, ions of diagnostic potential are isolated and fragmented by the addition of energy, this results in the production of daughter ions that can be used in monitoring. An unparalleled detection selectivity is achieved since a compound is identified as a specific analyte only if it remains throughout the entire sample preparation procedure and is identical with the analyte as concerns retention time and the production of a diagnostic ion which, in the second fragmentation, yields the specific product ions that are used for monitoring. Consequently, chromatograms produced by GC-MSMS are much more distinct than those obtained by GC-MS run in the selected ion monitoring (SIM) mode.

CASE: DETERMINATION OF SOME FUNGAL AND BACTERIAL CHEMICAL MARKERS IN ORGANIC DUST

In the following, the performance of the described chemical-analytical approach will be illustrated by comparing two dust samples of different origin as regards their contents of markers of lipopolysaccharide (endotoxin), fungal biomass, and peptidoglycan/bacterial biomass. One of the samples was dust collected from a book-shelf in a private home using a vacuum-cleaner and the other was an airborne dust sample collected on a polycarbonate filter in a swine confinement building. Approx. 2–5 mg of dust was used in each analysis.

Endotoxin. Endotoxins (lipopolysaccharides, LPS) are major constituents of the outer membrane of Gram-negative bacteria. LPSs are able to provoke high inflammatory response and can activate the immune system in both humoral and cellular ways. Inhalation exposure to endotoxin can develop progressive airflow obstruction manifested by the decrease of spirometric values and reported symptoms have been strongly related to the endotoxin concentration in organic dusts and bioaerosols [16, 38]. Among symptoms caused by this highly potent and multivalent molecule are respiratory and general problems such as cough, chest tightness, sneezing, fever, chills, and airways irritation. These symptoms are due either to nonspecific stimulation of the immune system or to exacerbation of allergic reactions; both processes may induce cell infiltrations and inflammatory response in the lungs [21, 37]. Illnesses attributed to endotoxin exposure include byssinosis, airway hyperreactivity and organic dust toxic syndrome. The role of endotoxin exposure in modulation of immune system development in early childhood has been recently recognized [10].

Chemical marker analysis of endotoxin makes use of the fact that the typical enteric LPS contains unique 3-hydroxy fatty acids (3-OH FAs) with 10–18 carbon chains linked to the glucosamine disaccharide moiety of

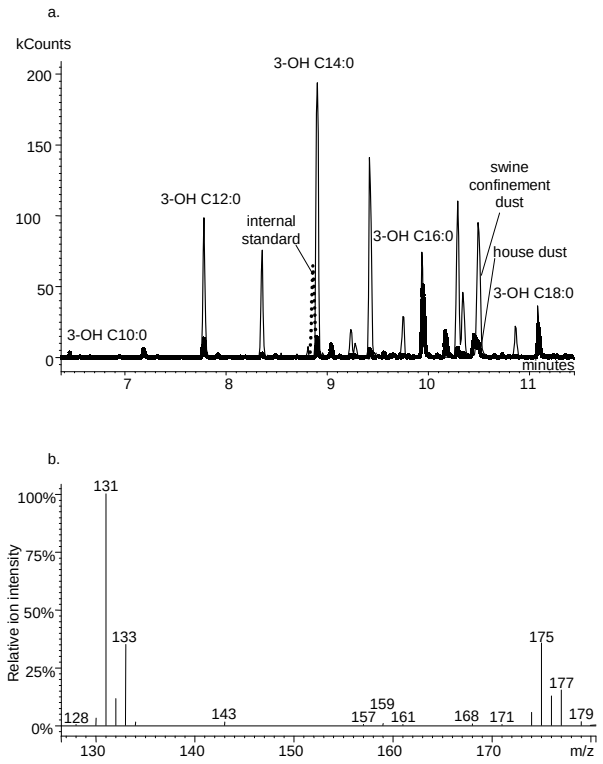

Figure 1. 3-Hydroxy fatty acid determination by GC-MSMS of swine confinement dust and house dust. Chromatogram and 3-OH FA distribution (a) and spectrum (b) of the methyl ester/TMS derivative of 3-OH 16 are shown. Molecular (parent) ion is *m/z* 175, daughter ion is *m/z* 131.

Lipid A (the toxic part of the LPS molecule). GC-MS analysis of these acids represents a chemical-analytical alternative to the *Limulus* test mentioned above. When using GC-MS, the numbers of moles of each 3-OH FA are calculated and the sum of the moles is divided by four to receive the number of moles of LPS in the sample. To analyse the 3-OH FAs, dust samples are heated in 2 M methanolic HCl at 100ºC overnight, after which methyl esters are extracted with n-hexane and water. An internal standard, pentadeuterated methyl 3-hydroxytetradecanoate, is added. The hexane layer is evaporated under a stream of nitrogen, redissolved in hexane:dichloromethane (1:1 v/v), and applied on a disposable silica gel column to separate the methyl esters of hydroxylated from non-hydroxylated acids. The column is washed with diethyl ether and hexane:dichloromethane before use, and hexane:dichloromethane is added after applying the methyl ester preparation. The hydroxy fatty acid methyl esters are eluted with diethyl ether and subsequently evaporated. Trimethylsilyl (TMS) derivatives are prepared for analysis by adding N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and pyridine to the sample and then heating for 15 min at 80ºC. EI mass spectra of the methyl ester/TMS derivatives show abundant ions of *m/z* (M-15), due to loss of a CH_3 group, and *m/z* 175, due to cleavage of C3-C4 linkage. The charge (z) of ions formed is invariably 1 in this kind of MS analysis; therefore the mass-to-charge (m/z) values represent molecular weights (m) of the fragments formed in the instrument's ion source. The derivatised acids are measured by monitoring *m/z* 175 when using conventional GC-MS in the SIM mode [22, 31] and by monitoring *m/z* 131 (a product ion of *m/z* 175) when running GC-MSMS [4].

Figure 1 shows GC-MSMS results of 3-OH FAs monitoring at *m/z* 131. The characteristic MSMS spectrum of the 3-OH FA derivative in an ion trap instrument can be seen on Fig. 1b. Monitoring ions *m/z* 131 relates to its parent ions *m/z* 175 which are isolated in the ion trap and subsequently cleaved into this specific fragment. The result is of high analytical specificity and, as an internal standard is used, quantitation is achieved; the technique can thus be applied for analytical determination of the studied endotoxin markers.

The chromatographic peaks of the 3-OH FA derivatives are regular and symmetric, suitable for automatic area count in according to retention times of external standards of syntesized 3-OH FAs. The two types of dusts revealed exclusive patterns (Fig. 1a). 3-OH C14 dominates in the swine dust, whereas 3-OH C16 dominates in the house dust. Also, swine dust seems to be enriched by fatty acids not present in house dust: except fatty acids of 10-, 12-, 14-, 16 and 18-carbon chain lengths, abundant peaks representing 3-OH C15 and 3-OH C17, normal as well as branched, indicate a different microbial community. Certain taxa of Gram-negative bacteria are known for their characteristic features of lipid A composition, such as the presence of 3-OH C14 in *Enterobacteriaceae*, 3-OH C12 in *Neisseria* spp., iso- and anteiso-branched hydroxy fatty acids of odd number of carbons in the alkyl chain in *Bacteroides* spp., etc. Therefore, the described procedure can be used not only to quantitate LPS in environmental samples but also to characterise it. Notably, the internal standard peak (deuterated 3-OH C14) is easily distinguished from the non-labelled 3-OH C14 through their different spectra. This method has revealed positive correlation with endotoxin potency as measured by using the *Limulus* test [28], particularly when considering only the 3-OH FAs of shorter chain lengths. Positive correlations were also found with clinical symptoms (fever reactions, increase of serum IL-6 concentrations, lung function) in a group of volunteers exposed to swine dust [30]. 3-OH FA analysis represents an independent, reproducible, chemical-analytical method for determining endotoxin which should find use in environmental observations as well as in workplace monitoring and studies on the microbiology of indoor environments [25].

Fungal biomass. Numerous health effects have been associated with exposure to indoor air moulds, including irritative symptoms, respiratory infections, and pulmonary allergic diseases [13]. Irritation of respiratory tract, rhinitis, cough, hoarseness, irritation of eyes [5, 7], and lower respiratory symptoms (chest tightness), are common [6]. Several nonspecific symptoms such as fatigue, nausea,

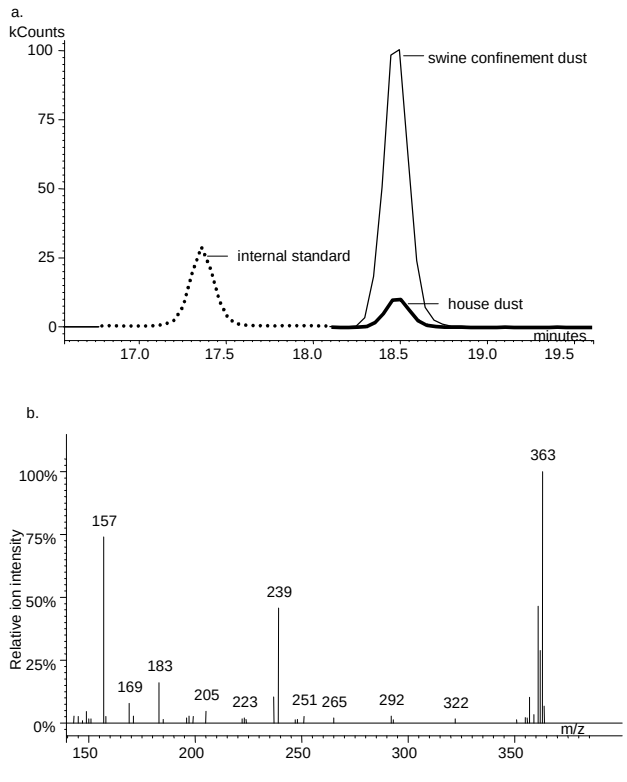

Figure 2. Ergosterol determination by GC-MSMS of swine confinement dust and house dust. Chromatogram (a) and spectrum (b) of TMS derivative of ergosterol are shown. Molecular (parent) ion is *m/z* 363, daughter ion is *m/z* 157.

vomiting, backache and joint pain, headache, dizziness and difficulties in concentration have also been described [2]. Certain moulds are known to affect the immune system; immunosuppressive effects on animals in experimental exposure, and strong associations with allergic diseases, have been demonstrated [38, 39]. Fungal allergens are primarily found in the spores but occur also in other structures, e.g. in mycelia [19]. Allergic alveolitis has been reported as the most severe pulmonary disease caused by moulds [26]. The most common form of alveolitis, traditionally reported in occupational exposure situations but seldom in indoor air exposure, is farmer's lung; other types of alveolitis are reported, e.g. among sawmill workers, greenhouse workers and mushroom workers. Further associations have been observed between microbial exposure and high prevalence of chronic bronchitis among nonsmoking farmers exposed to moulds at work [36]. Among adverse health effects suspected of being caused by mycotoxins (toxic secondary fungal metabolites) are disturbances of reproductive health and various neurological symptoms such as tremor, muscle weakness, extreme fatigue and apathy. Severe health effects have been associated with exposure e.g. to trichothecenes [33] which are known to inhibit protein synthesis and damage DNA. Alteration of the immune defence system by fungal toxins may also promote infections by other infective agents. Toxin-producing species seem to be common in farming and houses where long-term moisture accumulation takes place [29], therefore, special precautions should be taken when assessing health disorders and control measures of a mouldy indoor environment [14].

Ergosterol is a fungal membrane lipid that has been widely applied as a marker of fungal biomass. This unique sterol exists both free and ester-linked to other structures and is a factor of membrane fluidity. In aquatic or wet environments ergosterol is a subject of bacterial degradation or removal by fungivorous animals. However, it is stable under conditions that limit microbial growth, i.e. at low humidity. To analyse ergosterol, samples are heated in 10% methanolic KOH at 80°C for 90 min and then extracted with hexane and evaporated to dryness under a stream of nitrogen. The dried samples are subsequently dissolved in 1 ml of dichloromethane:hexane (1:1) and purified using a disposable silica gel column, as described above for 3-OH FA esters. After evaporation of the ethereal solvent, TMS derivatisation is performed by adding BSTFA and pyridine and then heating at 60ºC for 30 min. The preparations are dissolved in hexane prior to analysis. We used dehydrocholesterol as an internal standard. The EI mass spectrum of the ergosterol TMS derivative is dominated by ions of *m/z* 363 (M-105, loss of the trimethylsilanol group and one methyl group), and *m/z* 337 (M-131, loss of the trimethylsilanol group and the C_1-C_3 fragment). The derivative is measured by monitoring both of these ions when performing GC-MS in the SIM mode [3], and by monitoring *m/z* 157 (a product ion of *m/z* 363) when employing GC-MSMS [32].

GC-MSMS results of the two dust samples are illustrated in Figure 2. The MSMS spectrum of the ergosterol derivative in an ion trap instrument is dominated by the isolated parent ion (*m/z* 363) and its main product ion (*m/z* 157) (Fig. 2b). Ergosterol and the internal standard (dehydrocholesterol) appear in the chromatogram at different retention times forming symmetrical peaks without any interference (Fig. 2a). The chromatograms illustrate how rich in ergosterol (and thus fungal biomass) is the swine dust. Home dust vacuumed from shelves revealed a much lower amount, and in an indirect way this may be an indicator of lack of wet conditions in an examined house environment. Damp houses, where several studies have been carried out recently, have been found to present an increase of mould biomass prevalence [1]. No convincing positive correlations between the concentration of airborne ergosterol and health effects have been reported to date.

Peptidoglycan/bacterial biomass. Peptidoglycan, which is the cross-linked macromolecular structure responsible for the rigidity of bacterial cell walls, is sometimes referred to as ”Gram-positive bacterial endotoxin”. It constitutes a major part of the cell wall of Gram-positive bacteria and occurs also in the cell wall of Gram-negative bacteria, but in much smaller amounts. Peptidoglycan and

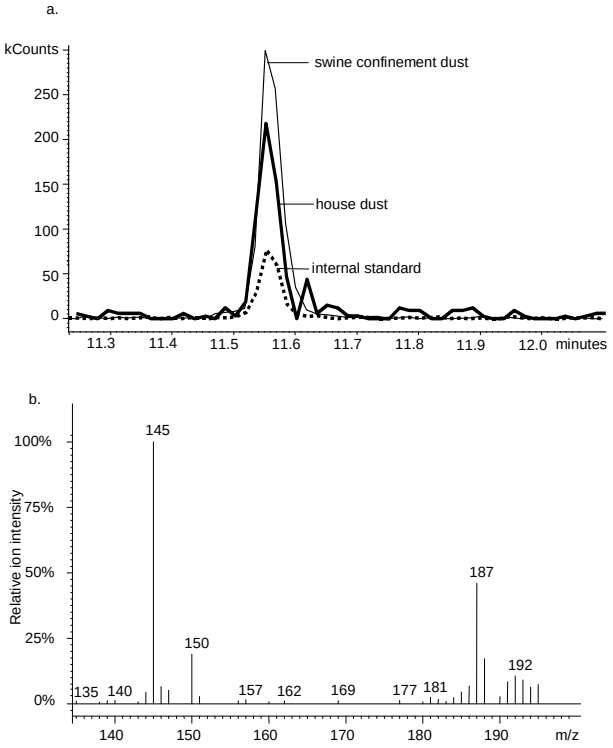

Figure 3. Muramic acid determination by GC-MSMS of swine confinement dust and house dust. Chromatogram (a) and spectrum (b) of methyl ester O-methyl acetate derivative of muramic acid (molecular ion *m/z* 187, daughter ion *m/z* 145) and internal standard (ion *m/z* 150) are shown.

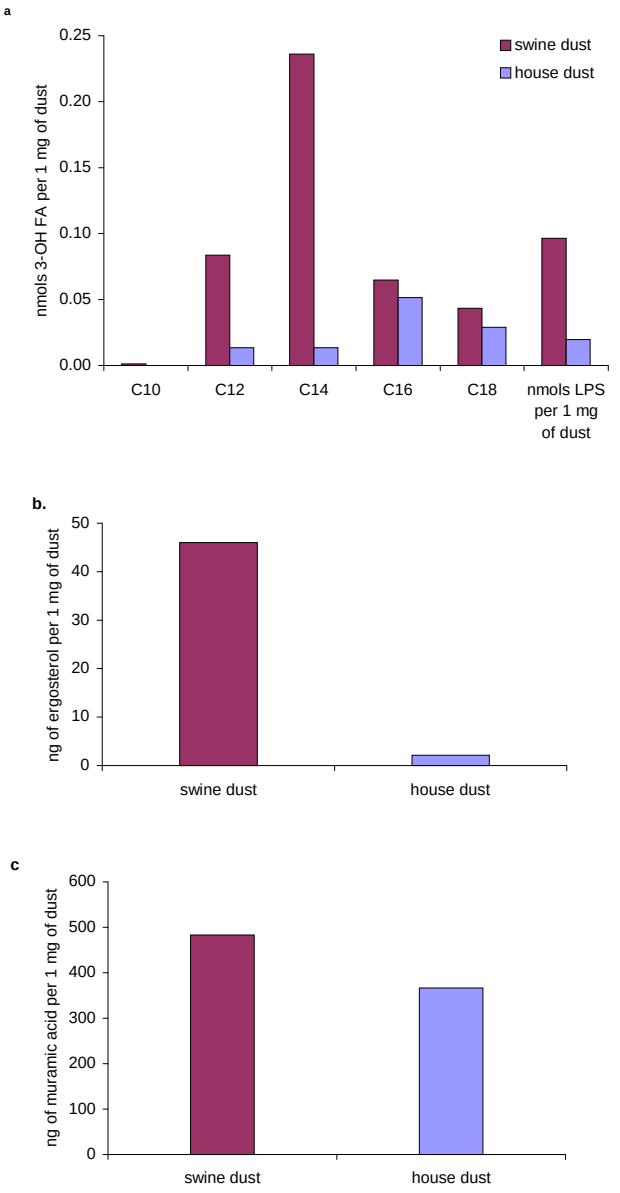

Figure 4. Comparison of swine confinement dust and house dust as regards 3-hydroxy fatty acid composition according to carbon chain length (a), ergosterol (b), and muramic acid (c).

partial structures of peptidoglycan are potent stimulators of the human immune system. They can reproduce most of the major signs and symptoms associated with bacterial infection, such as fever, inflammation, leukocytosis, acute phase responses and lymphocyte activation [12]. The smallest partial structure of peptidoglycan that exhibits full stimulatory capacity for macrophages is muramyl dipeptide, N-acetyl-muramyl-L-alanyl-D-α-glutamic acid. It induces increased phagocytosis, cytostasis or cytotoxicity of several cytokines, prostaglandin synthesis and release, and other activities [20, 35].

Muramic acid (MuAc), an amino sugar, is a unique marker of peptidoglycan. This chemical parameter measures total viable and non-viable bacteria as well as bacterial cell wall remnants, whereas culture assesses only the viable portion of the bacterial population. Muramic acid analysis has the potential to become one of standard tests needed in determination of the bacteria consortia in airborne dust and as a marker of indoor air quality [17].

To analyse MuAc, samples are heated in 2 M methanolic HCl at 85°C overnight, after which the internal standard (C^{13}-labelled MuAc in a methanolysate of C^{13}-labelled algal cells) is added. The mixture is extracted with hexane, and the lower phase is evaporated to dryness under nitrogen and further dried under vacuum in a desiccator (1 h). The samples are then acetylated by heating in a mixture of pyridine and acetic anhydride at 60°C for 1 h. The reaction mixture is evaporated and dissolved in dichloromethane, and subsequently washed, first with a 0.05 M HCl solution and thereafter with water. The samples are then evaporated to dryness, dissolved in chloroform, and analysed. Sample preparation results in methylation of the carboxyl and anomeric hydroxyl groups and acetylation of the three remaining hydroxyl groups. The EI fragmentation scheme is described in detail elsewhere [4]. Fragmentation of *m/z* 187 leads to high intensity of the product ion *m/z* 145, which is therefore monitored in GC-MSMS.

The muramic acid MSMS spectrum consists of abundant parent ions (*m/z* 187) and its main product ions (*m/z* 145); ions of *m/z* 150 relate to the internal standard

(Fig. 3b). The latter compound appears in chromatogram at nearly the same retention time as the non-labelled muramic acid derivative, although producing different ions, and represents therefore an ideal internal standard (Fig. 3a). In the samples originating from house and swine confinement environments, regardless of the differences in absolute amounts, the peak shape is symmetrical, and quantitative analysis easy to perform.

Little is known about possible health effects following inhalation of peptidoglycan. Following human exposure to swine dust, the amounts of muramic acid in the dust inhaled correlated significantly to fever reactions [30]. More research is required to elucidate possible inhalation health effects of this interesting macromolecule.

CONCLUSIONS

The potential of the chemical marker approach for characterising the microbial flora of two different indoor environments is illustrated. By using this approach, considerable differences in the microflora of the two different dusts were revealed (Fig. 4). The swine dust sample contained higher concentrations of all studied markers than the house dust sample. The amount of ergosterol was 46 ng/mg swine dust but only 2.1 ng/mg house dust. A similar trend was found for the bacterial markers (LPS concentrations were 0.096 nmol/mg swine dust and 0.020 nmol/mg house dust; muramic acid amounts were 483 ng/mg swine dust and 366 ng/mg house dust). The 3-OH FA and muramic acid results of the two samples indicate a much higher proportion of Gram-negative bacteria to Gram-positives in swine dust than in house dust, and ergosterol results indicate a much higher proportion of fungi. The differences in the distribution of 3-OH FAs between the two samples also illustrate differences in their flora of Gram-negative bacteria.

To summarize, work at our laboratory has led to the establishment of mass spectrometry-based analytical methods to characterise the microbial flora of indoor environments, including settled and airborne organic dust as well as building materials [34]. Further applications of these methods are vital in research aiming to evaluate the microbial contribution to the development of allergy and asthma amongst exposed individuals in indoor environments, and also to improve the design of worksites and homes for ill or risk group individuals.

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