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Characterization of the microbial community in indoor environments by chemical marker analysis: an update and critical evaluation

Abstract We published recently an integrated procedure for applying chemical marker analysis to characterize the microbiology of indoor environments comprising a scheme for extraction and analysis of markers of endotoxin, peptidoglycan/bacterial biomass, and fungal biomass. In the present paper, we report some significant improvements and also new possibilities of the described approach. We found that while 3-hydroxy fatty acids (3-OH FAs) of 10–14 carbon chain lengths are useful endotoxin markers, longer 3-OH FAs (i.e. with 16 carbon atoms and more) may rather serve as markers of Actinobacteria. We introduced ^{13}C -labeled 3-hydroxytridecanoic acid, from labeled *Pectinatus cerevisiiphilus*, as an internal standard to improve quantification of the 3-OH FAs in the gas chromatography-mass spectrometry analysis. Finally, in experiments aiming to identify a suitable method for collection of house dust for chemical marker analysis, we found that the marker compositions of dusts sedimented on plexiglass plates that were spatially well-distributed in a studied room at different heights above floor level, were undistinguishable. This type of sampling thus appears to be well suited for use, e.g. in epidemiological studies. In summary, the presented work describes important new capabilities of chemical marker analysis in defining human exposure to microorganisms in indoor environments.

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Key words: Chemical markers; Indoor air; Microbiology; Endotoxin; Ergosterol; Muramic acid.

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Practical Implications

We are developing an integrated methodology for characterizing the microbiology of indoor environments where specific microbial monomeric constituents in building materials and inhalable house dust particles are determined by using mass spectrometry-based methods. The methodology should represent a firm basis in research aiming to relate microbial exposure indoors to well-being and health.

Introduction

We have developed a chemical-analytical approach for characterizing the microbial community in indoor environments. The aim of this research is to be able to provide a tool for associating indoor air microbiology with well-being and health. Focus has been laid on endotoxins (lipopolysaccharides, LPS) (Michel, 2001; Wan and Li, 1999), peptidoglycan (Fox et al., 1993), and fungal biomass (Dales et al., 1999; Pepys, 1969). An integrated procedure was presented recently (Sebastian and Larsson, 2003) that included an extraction and analysis scheme for 3-hydroxy fatty acids (3-OH FAs) as markers of LPS, muramic acid (MuAc) as a marker of peptidoglycan/bacterial biomass, branched-chain fatty acid as markers of certain gram-positive bacteria, and

ergosterol as a marker of fungal biomass. These marker compounds are covalently linked to various structures in the cell membranes, and prior to analysis, a sample must therefore be hydrolyzed. Afterwards, the markers are purified by extractions and finally they are derivatized. In our laboratory we analyzed them by using gas chromatography-ion-trap tandem mass spectrometry (GC-MSMS), which provides the high degree of detection selectivity that is required for being able to determine the markers even when present down to nanogram levels in chemically complex matrices.

While the procedure described (Sebastian and Larsson, 2003) constitutes the main frame of the approach there will constantly be room for modifications and improvements. For example, we reported recently a simplification of the sample preparation method used

to determine MuAc (Sebastian et al., 2004). The aim of the present study was to investigate certain additional aspects of the integrated procedure according to the following.

1. Air dust sampling for chemical marker analysis has hitherto mainly included pumping of air through filters. Even when using high capacity pumps (> 20 l/min) sampling periods of 24 h or more are frequently required to collect enough amounts of dust for analysis (Fox et al., 2003) which makes active sampling less attractive for application in epidemiological studies. Sampling of sedimented dust on floors, chairs, etc. is easy but may include dust that has not been airborne and thus may be less relevant for exposure assessment. In the present study, therefore, we have applied and evaluated controlled passive sampling comprising sedimentation of airborne dust on defined, spatially evenly distributed, surfaces in rooms during a controllable period of time.

2. The described procedure (Sebastian and Larsson, 2003) utilizes ^{13}C -labeled 3-OH $\text{C}_{16:0}$ in labeled cyanobacteria as internal standard in the quantification of 3-OH FAs in environmental samples such as house dust. Thus, cyanobacterial hydrolysates are added to sample hydrolysates before further sample preparation procedures. The MSMS spectrum of ^{13}C -labeled 3-OH FAs contains a dominating peak of m/z 133. The same ions however, although less abundant, are also formed from non-labeled 3-OH FAs (representing LPS in the dust), and therefore these ions interfere to some extent with the quantification of dust 3-OH FAs. In this study, we introduce the use of ^{13}C -labeled 3-OH $\text{C}_{13:0}$ (3-OH $\text{C}_{13:0}$ is rarely found in organic dust), in labeled *Pectinatus cerevisiiphilus*, as a new internal standard.

3. The specificity of the 3-OH FAs as markers of LPS is not absolute. Instead, 3-OH FAs particularly of 16 and 18 carbon chain lengths have been reported to be present in several actinomycete species of the class Actinobacteria (Zelles, 1997). It is obvious that this may disturb quantification of LPS using 3-OH FA analysis especially as Actinobacteria are frequently found in indoor environments; however, it may also open up an exciting, new possibility of monitoring exposure to these potentially harmful bacteria. In the present study, therefore, we analyzed several actinobacterial strains (both clinical and soil isolates) with regard to 3-OH FA composition. The optical configurations of these acids were also determined.

Materials and methods

Dust sampling

Dust samples were collected from four different locations: a school, a laboratory, an apartment (living-room) and a single-family house (living-room). These locations were chosen randomly and none of

the buildings had been subjected to any known water damage or showed any signs of microbial contamination. The dust was allowed to sediment on plexiglass plates (0.5×0.5 m) hanging from the ceiling. Five plates were used in one room in each location; thus, a total of 20 plates were used. The plates were spatially evenly distributed in each room and positioned at different distances from the floor level. After 5 weeks of sedimentation, dust from each plate was collected by using a rubber scraper and stored in a freezer. One-third of the amounts of dust collected from each plate were used for analysis of fatty acids and MuAc and two-thirds were used for analysis of ergosterol.

Internal standard

Pectinatus cerevisiiphilus ATCC 29359^T was cultured in trypticase soy broth medium containing peptone, NaCl, and K_2HPO_4 . The medium was supplemented with ^{13}C -labeled glucose and sodium acetate (Larodan, Malmö, Sweden) to achieve isotopic labeling. The bacteria were inoculated in 100-ml Erlenmeyer flasks and incubated under anaerobic conditions at 30°C, checked for purity, and autoclaved. Cells were harvested by centrifugation (6000 g, 20 min), washed twice with sterile water, and freeze-dried. About 2 mg (exact weight was recorded) of the dried biomass was subjected to methanolysis (Saraf et al., 1997), evaporated, and diluted in heptane until a 1 mg/ml solution was obtained. The content of ^{13}C -labeled 3-OH $\text{C}_{13:0}$ was determined in 10 μl of *Pectinatus* methanolysate (Sebastian and Larsson, 2003).

3-OH FAs of Actinobacteria

Seventeen species of Actinobacteria comprising both clinical and environmental isolates (Table 1) were studied for 3-OH FA composition. The acids were analyzed as methyl ester/trimethylsilyl (TMS) derivatives (Saraf et al., 1997). In addition, some of the strains with high contents of 3-OH FAs were also subjected to chiral separation for studying the optical configurations of the acids. For this purpose, chiral (S)-phenylethylamide methoxy derivatives of the 3-OH FAs were prepared according to the method described by Gradowska and Larsson (1994) prior to GC-MS analysis.

GC-MS

Samples were analyzed using a Saturn 2000 ion-trap GC-MS instrument (Varian, Palo Alto, CA, USA) equipped with a fused-silica capillary column (CP-Sil 8 CB low bleed, 0.25 μm film thickness, 30 m \times 0.25 mm, i.d.) (Chrompack, Middelburg, the Netherlands). Volumes of 2 μl were injected in the splitless mode with a carrier gas (helium) head

Table 1 3-Hydroxy fatty acids of Actinobacteria

Species/no. of carbon atoms	14	15	16	17	18	19	20	21	22
* <i>Rhodococcus rhodochrous</i> PCM 909			+		+	+	+++	++	+++
<i>Rhodococcus equi</i> PA1 (clinical isolate)		+			+		+++		++
* <i>Gordonia terrae</i> ATCC 25594			+		+++	++	+++	+	+
* <i>Gordonia rubra</i> ATCC 25593			+		+++	++	+++		+
* <i>Mycobacterium rarugoni</i>			++	+	++	+	++		+
<i>Nocardia asteroides</i> PCM 2254 ^T			+		++		++		+
<i>Gordonia bronchialis</i> PCM 2167 ^T			+		++		++		+
<i>Rhodococcus equi</i> ATCC 25729 ^T			+		+++	+	+++		
* <i>Tsakumurella paurometabolum</i> PCM 2413	+		+		+		+		
<i>Corynebacterium hoffmanii</i>	+		+++		+				
* <i>Dietzia maris</i> PCM 2292 ^T	+	+	+	+	++	+	+		
* <i>Saccharopolyspora hirsuta</i> PCM 2279			+	++	+		+		
* <i>Nocardiopsis dassonvillei</i> PCM 2492 ^T			+	+	++	+			
* <i>Streptomyces</i> sp. (soil isolate)			+	+	++	+			
* <i>Oerskovia xantineolytica</i> ATCC27402 ^T	+	b++	+	b+	b+				
<i>Actinomyces</i> sp. (clinical isolate)		b+		b+					
<i>Propionibacterium propionicum</i> ATCC 14157 ^T	+	+++	+						

PCM, Polish Collection of Microorganisms; ATCC, American Type Culture Collection.

*Soil inhabitants; b, branching.

column pressure of 69 kPa using a Combi Pal auto sampler (CTC Analytics AG, Zwingen, Switzerland). The GC-MSMS conditions for analysis of the derivatized MuAc, ergosterol and 3-OH FAs (non-chiral) were those used previously (Sebastian and Larsson, 2003). The chiral (S)-phenylethylamide methoxy 3-OH FA derivatives were analyzed by using a column temperature that was programmed from 90 to 280°C at 20°C/min operating the MS in the selected ion storage mode. The number of moles of LPS was calculated as the sum of moles of C_{10:0}–C_{18:0} 3-OH FAs divided by four.

Statistical analyses

All data were analyzed by SPSS 11.5 (SPSS, Inc., Chicago, IL USA). Total dust and ergosterol data were subjected to log₁₀ transformation before analysis to get near-normal distribution. Group comparisons were carried out by analysis of variance (ANOVA *F*-test) and individual comparisons were further analyzed by Tukey Honest Test. Statistical significance was defined by $P \leq 0.05$.

Results

Dust sampling

The amounts of the collected dust samples (2.7–90.4 mg from plates <1 m above floor level, 4–30.3 mg from plates 1–2 m above floor level, and 3.5–13.5 mg from plates >2 m above floor level) were in all cases sufficient for analysis. The dust concentrations of ergosterol, MuAc and LPS varied between 0.22 and 8.88 ng/mg, 6.90–18.67 ng/mg, and 0.0183–0.0668 nmol/mg respectively. No significant differences were found between the

dust concentrations of any of the markers in relation to how the plates were distributed in a studied room (Table 2). There were, however, clear differences in marker compositions between some of the studied locations indicating heterogeneity in microbial composition especially between the laboratory and the other locations (Table 3).

Internal standard

The dominating 3-OH FA in the studied *Pectinatus* strain was 3-OH C_{13:0}, which is in agreement with results reported previously (Helander and Haikara, 1995; Helander et al., 1994). The mass spectrum of the methyl ester/TMS derivative of this acid (when non-labeled medium was used for culturing) exhibited abundant ions of *m/z* 175 and *m/z* 301 according to fragmentation patterns previously described (Saraf and

Table 2 Dust and markers collected on plexiglass plates at different distances from floor level

Dust, markers	Sampling height			<i>P</i> *
	<1 m (<i>n</i> = 8)	1–2 m (<i>n</i> = 6)	>2 m (<i>n</i> = 6)	
Total dust (mg)	8.974 (2.965)	8.610 (2.075)	4.932 (1.663)	0.392
MuAc (ng)/dust (mg)	12.222 ± 3.419	12.344 ± 2.681	11.656 ± 1.441	0.884
Ergosterol (pg)/dust (mg)	783.4 (3.4)	714.5 (3.4)	977.2 (3.2)	0.899
LPS (nmol)/dust (mg)	0.031 ± 0.011	0.036 ± 0.015	0.034 ± 0.012	0.793
3-OH C _{10:0} (nmol)/dust (mg)	0.013 ± 0.007	0.012 ± 0.009	0.009 ± 0.009	0.649
3-OH C _{12:0} (nmol)/dust (mg)	0.024 ± 0.010	0.027 ± 0.016	0.027 ± 0.014	0.841
3-OH C _{14:0} (nmol)/dust (mg)	0.027 ± 0.012	0.032 ± 0.013	0.032 ± 0.015	0.712
3-OH C _{16:0} (nmol)/dust (mg)	0.062 ± 0.022	0.074 ± 0.036	0.069 ± 0.026	0.746
3-OH C _{18:0} (nmol)/dust (mg)	0.051 ± 0.023	0.061 ± 0.038	0.051 ± 0.023	0.788

Results expressed as mean ± s.d. or geometric mean (s.d.).

n, number of dust samples.

**P* value of ANOVA.

Table 3 Dust and markers collected on plexiglass plates at different locations

Dust, markers	Sampling location				P*
	School (n = 5)	Apartment (n = 5)	Single-family house (n = 5)	Laboratory (n = 5)	
Total dust (mg)	6.095 (1.589) ¹	23.605 (2.296) ^{4,5}	4.853 (1.199)	4.285 (1.476)	0.000
MuAc (ng)/dust (mg)	13.200 ± 1.460	10.889 ± 2.611	13.212 ± 3.790	11.006 ± 1.678	0.308
Ergosterol (pg)/dust (mg)	255.9 (1.1) ^{2,3}	544.5 (1.4) ⁵	701.5 (1.8) ⁶	4477.1 (1.8)	0.000
LPS (nmol)/dust (mg)	0.029 ± 0.005 ³	0.029 ± 0.005 ⁵	0.030 ± 0.010 ⁶	0.050 ± 0.011	0.001
3-OH C _{10:0} (nmol)/dust (mg)	0.018 ± 0.009 ^{1,3}	0.005 ± 0.003	0.015 ± 0.006	0.007 ± 0.005	0.009
3-OH C _{12:0} (nmol)/dust (mg)	0.023 ± 0.005 ³	0.015 ± 0.004 ⁵	0.022 ± 0.010 ⁶	0.043 ± 0.010	0.000
3-OH C _{14:0} (nmol)/dust (mg)	0.028 ± 0.004 ³	0.022 ± 0.002 ⁵	0.022 ± 0.006 ⁶	0.048 ± 0.010	0.000
3-OH C _{16:0} (nmol)/dust (mg)	0.047 ± 0.004 ³	0.061 ± 0.010 ⁵	0.060 ± 0.019 ⁶	0.102 ± 0.028	0.001
3-OH C _{18:0} (nmol)/dust (mg)	0.034 ± 0.007 ³	0.053 ± 0.007 ⁵	0.038 ± 0.010 ⁶	0.092 ± 0.026	0.000

Results expressed as mean ± s.d. or geometric mean (s.d.).

n, number of dust samples.

P ≤ 0.05 by ANOVA Tukey Honest Test comparing school and apartment (1), school and single-family house (2), school and laboratory (3), apartment and single-family house (4), apartment and laboratory (5), single family-house and laboratory (6).

*P-value of ANOVA and Tukey Honest Test.

Larsson, 1996). Cultivating *Pectinatus* in medium containing the labeled substrates yielded 125.3 mg of dry biomass that contained 194.8 ng of ¹³C-labeled 3-OH C_{13:0}/mg. The 3-OH FA pattern was dominated by 3-OH C_{13:0}, the retention time of which was virtually identical with that of non-labeled 3-OH C_{13:0}. The mass spectrum of the ¹³C-labeled 3-OH C_{13:0} contained abundant ions of m/z 311 and m/z 178 indicating that the labeling was complete up to at least C₃ but incomplete thereafter as no abundant ions higher than m/z 311 were observed (Figure 1); notably, m/z 314 (301 + 13) should have been observed in a fully labeled molecule.

3-OH FAs of Actinobacteria

The 17 studied actinobacterial strains contained 3-OH FAs that ranged from 14 to 22 carbon chain lengths

(Figure 2, Table 1). Most of the strains contained 3-OH FAs with 16 or more carbon atoms; the shortest 3-OH FA detected, 3-OH C_{14:0}, was found only in five strains, and in comparatively small amounts. The identities of the detected 3-OH FAs were confirmed by the uptake of complete spectra and by their retention times in comparison with commercially available external standards. Additionally, chiral separation of 3-OH FAs of *Rhodococcus rhodochrous*, *Gordonia terrae*, *Nocardioopsis dassonvillei*, and *Streptomyces* sp. revealed that all of them were of (R)-configuration (data not shown).

Discussion

Chemical marker analysis can be used to characterize microbial communities in indoor environments. We have noted differences in marker compositions in dusts

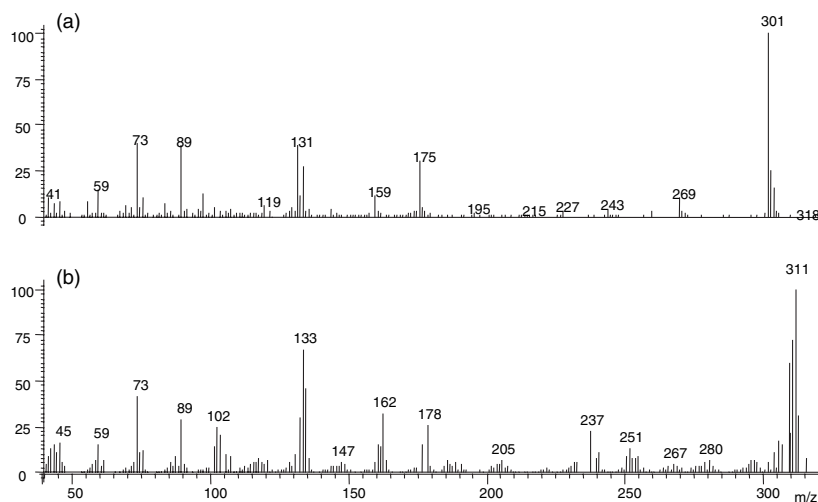


Fig. 1 Electron impact mass spectrum of 3-OH C_{13:0} in *Pectinatus cerevisiiphilus* cultivated in trypticase soy broth medium without (a) and with (b) ¹³C-glucose and sodium acetate added as labeling agents

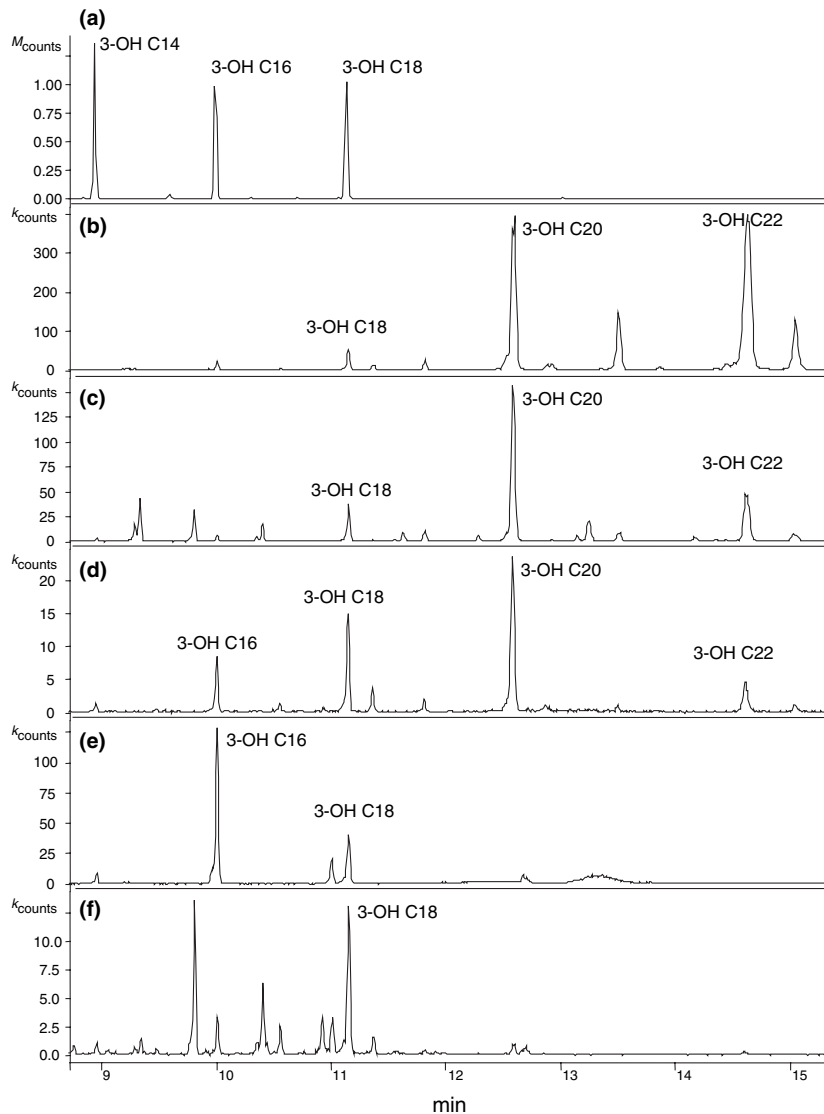


Fig. 2 3-Hydroxy fatty acids in an external standard mixture containing 3-OH C_{14:0}, 3-OH C_{16:0}, and 3-OH C_{18:0} (a) and in *Rhodococcus rhodochrous* 54 (b), *Rhodococcus equi* PA1 (c), *Nocardia asteroides* (d), *Corynebacterium hoffmani* (e), and *Nocardioopsis dassonvillei* (f)

from damp vs. non-damp buildings (Nilsson et al., 2004), bed vs. shelf vs. basement in the same house (Sebastian and Larsson, 2003), unoccupied vs. occupied classrooms (Fox et al., 2003), aircraft cabins vs. offices/homes (Hines et al., 2003), and between schools in different geographic regions (Wady et al., 2004). This approach was also applied to study the possible presence of microbiological material in lunar dust collected at the Apollo 11 mission in 1969 (Kozar et al., 2001).

We have found repeatedly that when relating dust 3-OH FAs to endotoxin bioactivity including the longer 3-OH FAs (of 16 and 18 carbon chain lengths) in calculating LPS lowers the correlation between 3-OH FAs and the *Limulus* test (Saraf et al., 1997). It is tempting to speculate that these 3-OH FAs may to an appreciable extent originate from Actinobacteria

rather than from Gram-negative bacterial LPS, which rarely contains for example 3-OH C_{18:0} (Zelles, 1997). It is also possible that several recorded peaks in the region between 3-OH C_{16:0} and 3-OH C_{18:0} that we have observed repeatedly over the years (e.g. Saraf and Larsson, 1996) may represent actinobacterial branched-chain 3-OH FAs. Taken altogether, these results suggest that only the shorter 3-OH FAs should be used as LPS markers and that the longer 3-OH FAs rather may represent indicators of Actinobacteria. Notably, Laitinen et al. (2001) found that among the different 3-OH FAs tested it was 3-OH C_{14:0} that best related to health effects in several studied occupational settings. Interestingly, in the present study the longest 3-OH FAs (3-OH C_{20:0} and 3-OH C_{22:0}) were found in mycolic acid-containing actinobacterial species.

Our decision to produce ^{13}C -labeled *P. cerevisiiphilus* biomass was prompted by observations that the m/z 133 ions of the cyanobacterial ^{13}C -labeled 3-OH FA used as internal standard in the described integrated approach (Sebastian and Larsson, 2003) to a small but noticeable extent also are produced by the sample 3-OH FAs. Helander et al. (1994) and Helander and Haikara (1995) reported that 3-OH $\text{C}_{13:0}$, a compound very rarely found in environmental samples, is the most abundant 3-OH FA in *P. cerevisiiphilus*. Therefore, particularly if labeled, 3-OH $\text{C}_{13:0}$ should constitute an almost ideal internal standard as it would also control for the methanolysis step in the sample preparation. We found that our preparation, although not fully incorporating ^{13}C from the culture medium, was labeled up to at least C_3 producing the fragment of m/z 178 (corresponding to m/z 175 for the non-labeled acids) with no signs of m/z 175. At our laboratory, when using GC-MSMS, the 3-OH FA derivatives are routinely measured by monitoring m/z 131 (a product ion of m/z 175) and therefore the ^{13}C -labeled preparation should be a very useful internal standard.

Sampling methods for marker analysis of house dust have not been evaluated thoroughly. In a recent school study dust was collected by pumping air through filters (36.5 l/min) for several days following analysis of MuAc and 3-OH FAs on the filters. Interestingly, we

found that presence of pupils in the classrooms considerably affected the marker compositions of the airborne dust samples (Fox et al., 2003). Norbäck D, Mi Y-H, Larsson L, Wady L, Tao J and Mi Y-L (unpublished data), in the first study so far on relationship between chemical markers and health, found significant correlations between respiratory symptoms and marker concentrations in dust collected from chairs and floors in schools in Shanghai. However, it is clear that parts of floor dust may never have been airborne or subjected to inhalation. Wady et al. (2004) used dust sedimented during 4 weeks on the same type of plexiglass plates as in the present study to reveal a microbiological heterogeneity, in terms of marker composition, of air in schools in Jordan, Poland and Sweden. This type of controlled dust sedimentation represents a convenient way of sampling that does not depend upon the spatial distribution of the plates in a given room.

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