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# DETERMINATION OF ENVIRONMENTAL LEVELS OF PEPTIDOGLYCAN AND LIPOPOLYSACCHARIDE USING GAS CHROMATOGRAPHY WITH NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRY UTILIZING BACTERIAL AMINO ACIDS AND HYDROXY FATTY ACIDS AS BIOMARKERS

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SUMMARY

D-Alanine and diaminopimelic acid originating from bacterial peptidoglycans and hydroxy fatty acids from lipopolysaccharides (endotoxins) were analysed by gas chromatography using a chiral column (Chirasil-Val as stationary phase) and selected-ion monitoring detection with negative-ion chemical-ionization mass spectrometry. The amino acids were analysed as N-heptafluorobutyryl isobutyl esters after rapid hydrolysis of peptidoglycan followed by isolation of the amino acids with disposable ion-exchange columns. Racemization of amino acid enantiomers was controlled by using deuterium chloride in the hydrolysis. The hydroxy acids were analysed as O-pentafluorobenzoyl methyl esters. Most of the bacteria present in airborne dust from a poultry confinement building were found to be Gram-positive according to the analytical chemical method whereas the *Limulus* amoebocyte lysate test suggested the presence of appreciable amounts of lipopolysaccharides of Gram-negative bacteria. Further studies are required to compare the utility of these two methods for determining endotoxins in complex environments.

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## INTRODUCTION

Airborne lipopolysaccharides (LPS, endotoxins) of Gram-negative bacteria are present in a variety of industrial and agricultural environments, and it has been suggested that they are a major cause of pulmonary disease among exposed workers [1–4]. Often, however, most of the bacteria isolated from the air are Gram-positive [2, 5–7]. Peptidoglycan (PG), the major component of the cell wall in Gram-positive bacteria, has been demonstrated to be capable of inducing chronic inflammatory diseases and eliciting certain "endotoxic" effects [8, 9]. The most widely used method for quantitating LPS, the *Limulus* amoebocyte lysate (LAL) test, is very sensitive; however, it has been shown that PG can activate the test [10]. Thus, LAL tests on samples containing high concentrations of PG may indicate inadequate amounts of LPS. An alternative approach for determining LPS and PG could be based on measuring "biomarkers", i.e., specific compounds characteristic of these macromolecules but not found elsewhere in nature [11, 12].

LPS is present in the outer membrane of most Gram-negative bacteria. The lipid part of LPS, lipid A, is the endotoxic portion of the molecule [13]. Major components in lipid A are 3-hydroxy-substituted fatty acids among which 3-hydroxymyristic acid (3-OH-14:0) is the most abundant [14]. It has been suggested previously that these fatty acids may serve as biomarkers for LPS [15].

PG constitutes the cell wall skeleton of almost all bacteria and contains several useful biomarkers, including muramic acid (Mur), diaminopimelic acid (DAP), D-alanine (D-Ala) and D-glutamic acid (D-Glu). The D-amino acids and Mur are general biomarkers for PG whilst DAP is found predominantly in Gram-negative bacteria [11, 16]. Trace amounts of Mur have been demonstrated in tissues of animals injected with bacterial cell walls [17, 18]. D-Ala and other PG markers have been detected in environmental samples [19, 20].

In this paper we describe a chemical analytical approach for determining LPS and PG in environmental samples using gas chromatography (GC) and mass spectrometry (MS). The hydroxy fatty acids in LPS are analysed as their O-pentafluorobenzoyl methyl (O-PFBO-Me) esters as previously described [21], and the amino acids in PG as N-heptafluorobutyryl isobutyl (N-HFB-isoBu) esters. The amino acid method utilizes a simplified sample preparation technique compared with previously reported methods [19, 20], including hydrolysis in deuterated hydrochloric acid ($^2$HCl) and pre-derivatization clean-up using disposable ion-exchange columns. $^2$HCl is used to label specifically only the D-amino acid molecules generated from racemization during the hydrolysis [22]; the derivatized enantiomers are then separated on a chiral GC column and analysed by MS. By using selected-ion monitoring (SIM) detection with negative-ion chemical-ionization (NICI), this approach allows the trace determination of LPS and PG in the air of poultry confinement buildings.

## EXPERIMENTAL

### *Chemicals and glassware*

Analytical-reagent grade D- and L-enantiomers of Ala, Glu and lysine (Lys) and D-norleucine (Nle) and DAP were obtained from Sigma (St. Louis, MO,

U.S.A.). Stock solutions were prepared in 0.1 *M* hydrochloric acid and stored at 4°C. N,N-Dioctylmethylamine, deuterium chloride ($^2$HCl) (20% in deuterated water, >99.5 atom-% $^2$H), 2-hydroxytetradecanoic acid (2-OH-14:0), heptafluorobutyric anhydride (HFBA) and 2,3,4,5,6-pentafluorobenzoyl chloride (PFBO-Cl) were purchased from Fluka (Buchs, Switzerland), acetyl chloride from Merck (Darmstadt, F.R.G.), 2-hydroxydodecanoic acid (2-OH-12:0) (purity >98%) from Ventron (Karlsruhe, F.R.G.) and the bacterial fatty acid reference mixture (Part No. 4-5436) from Supelco (Bellefonte, PA, U.S.A.). The 3-hydroxy fatty acid standards 3*R*-hydroxynonanoic acid (3-OH-9:0) and *rac*-3-hydroxytetradecanoic acid (3-OH-14:0) were synthesized [23]. Solvents and reagents were of analytical-reagent grade. Disposable cation-exchange columns, benzenesulphonylpropyl (SCX, Bond Elut), were obtained from Analytichem International (Harbor City, CA, U.S.A.).

All glassware was washed with 5% Deconex, rinsed several times with tapwater (hot) and distilled water, soaked in 5 *M* hydrochloric acid overnight and rinsed with water and ethanol (95%) before being heated for 10 h at 400°C. The test-tubes used had PTFE-lined screw-caps.

*Microorganisms*

*Alcaligenes faecalis* (CCUG 710), *Enterobacter agglomerans* (CCUG 539) and *Streptococcus faecalis* (CCUG 9997) were studied. In addition, one strain each of *Acinetobacter calcoaceticus, Pseudomonas aeruginosa, Streptococcus viridans, Streptococcus mutans, Clostridium perfringens, Cryptococcus neoformans, Candida albicans* and *Torulopsis glabrata,* all isolated from clinical or environmental samples, were used. The bacterial species were selected for study because each had been previously found in swine and poultry confinement buildings [2, 24] and the fungi were used as controls in the racemization experiments. The microorganisms were cultured on agar plates, washed with sterile water, autoclaved and lyophilized before being subjected to GC–MS analysis.

*Dust samples*

Airborne dust samples from a poultry confinement farm in southern Sweden were collected on Millipore (Bedford, MA, U.S.A.) cellulose acetate filters (37 mm diameter, 0.8 $\mu$m pore size) by personal sampling in the breathing zone. The air flow-rate was 3 dm$^3$/min and sampling periods were ca. 1 h. The filters were then shaken in 10 ml of pyrogen-free water for 10 min after which the washings were split in two equal volumes; one was subjected to the LAL test (chromogenic version) and the other to chemical analysis after storage at −20°C. The latter samples were divided into two equal volumes and lyophilized. One fraction was subjected to LPS analysis and the other to PG analysis.

*Derivatization of LPS hydroxy fatty acids*

*Hydrolysis.* The sample and the internal standard (3-OH-9:0) were heated in 0.5 ml of 2 *M* methanolic hydrochloric acid under nitrogen at 85°C for 18 h [14]. After cooling, 0.5 ml of water was added and the hydrolysate was extracted twice

with 1 ml of hexane. The combined organic phases were evaporated to dryness under reduced pressure.

*Derivatization.*The sample was redissolved in 60 μl of acetonitrile and 10 μl of PFBO-Cl were added; the mixture was then heated at 150 °C for 1 h. After cooling, 0.5 ml of dichloromethane and 1 ml of 1 *M* phosphate buffer (pH 7.0) were added. The tube was shaken and centrifuged (about 1.000 *g*) and the organic phase evaporated [21]. The sample was made up with heptane prior to analysis.

*Derivatization of PG amino acids*

*Hydrolysis.*The sample was heated in 0.5 ml of 6 *M* $^{2}$HCl under nitrogen at 150 °C for 6 h. After cooling, D-Nle and D-Lys were added as internal standards. The $^{2}$HCl was evaporated in a lyophilizer and the residue dissolved in 0.2 ml of 0.1 *M* hydrochloric acid.

*Cation exchange.*Prior to use, the SCX column (1 ml) was washed with two column volumes (2 ml) of methanol, three volumes of water, three volumes of 4 *M* hydrochloric acid at a flow-rate of about 0.5 ml/min and six volumes of water. The hydrolysate was applied quantitatively to the column and allowed to pass through the column at a flow-rate of about 0.5 ml/min. The resin was washed slowly with two volumes of water, after which the amino acids were eluted with four volumes of 4 *M* hydrochloric acid. Ten samples could be processed simultaneously using the Analytichem Vac-Elut system. The eluates were lyophilized overnight.

*Derivatization.*To the sample, 0.2 ml of dichloromethane was added. Traces of water were evaporated azeotropically in a desiccator under reduced pressure. The carboxyl group was esterified by heating in 50 μl of 3 *M* isobutanolic hydrochloric acid under nitrogen at 120 °C for 20 min [25]. After cooling, excess of reagent was evaporated, 0.2 ml of dichloromethane added and the sample dried. The amino group was acylated with 50 μl of HFBA under nitrogen at 150 °C for 12 min [25]. After cooling, 0.5 ml of dichloromethane and 1 ml of 1 *M* phosphate buffer solution (pH 7.0) were added and the tube was shaken and centrifuged (1000 *g*). The organic phase was transferred into another tube, evaporated and the residue dissolved in ethyl acetate.

*Gas chromatography*

A Carlo Erba (Rodano, Italy) Model 4160 gas chromatograph equipped with a flame ionization detector, an all-glass splitless injection system and a fused-silica capillary column (25 m × 0.22 mm I.D.) coated with Chirasil-L-Val (Chrompack, Middelburg, The Netherlands) was used. Helium, at a flow-rate of 0.7 ml/min, served as carrier gas. The temperature of the injector was 250 °C and that of the detector was 290 °C; the column temperature was programmed (starting 1 min after injection) from 70 to 200 °C at 7 °C/min. The split valve was opened 1 min after injection. A Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A electronic integrator was attached to the gas chromatograph.

*Gas chromatography–mass spectrometry*

A Ribermag (Rueil-Malmaison, France) R10-10c quadropole GC–MS data system was used. The gas chromatograph was a Carlo Erba Model 4160 equipped

with an all-glass splitless injector and the chiral column used in the GC analyses (see above). Helium, at an inlet pressure of 0.8 kg/cm$^2$, served as carrier gas. The temperature of the injector was 250°C, that of the interface between the gas chromatograph and the ion source 250°C and that of the ion source 120°C. The amino acid analyses were performed using an initial column temperature of 50°C; after 1 min the temperature was increased to 200°C at 7°C/min. The fatty acid analyses were performed with an initial temperature at 100°C with programming at 10°C/min to 200°C. The split valve was opened 1 min after injection. The methane reagent gas (0.7 Torr, purity >99.95%) was ionized with electrons at an energy of 93 eV. The manual integration facility in the MS system standard software was used for peak integration.

The hydroxy fatty acid and amino acid derivatives were analysed using SIM with combinations of different ion monitoring sets characteristic of the different derivatives. For the analysis of hydroxy fatty acids, the time events and ions were 11 min, $m/z$ 382.30; 2 min, $m/z$ 396.30; 5 min $m/z$ 424.40; 5 min, $m/z$ 452.40; and 4 min, $m/z$ 480.40, which correspond to the O-PFBO-Me derivatives shown in Table I. For the analysis of amino acids the time events and ions were 10 min, $m/z$ 321.30; 5 min, $m/z$ 363.30; 5 min, $m/z$ 435,40; 3 min, $m/z$ 574.50; and 7 min, $m/z$ 674.60, which correspond to the N-HFB-isoBu derivatives shown in Table III. In the racemization studies three ions were measured using the same time events for Ala and Lys, and monitoring at M−1, M and M+1, where M represents the molecular ions.

## RESULTS AND DISCUSSION

### *Sample preparation and analysis*

*Hydroxy fatty acids.* The amide-linked hydroxy fatty acids present in lipid A [26] are efficiently released by methanolysis or hydrolysis using 2 *M* hydrochloric acid [14, 27]. A stronger acid solution (6 *M*) may cause degradation of hydroxy fatty acids [28], and alkaline hydrolysis is not applicable as the amide linkage is relatively alkali-stable [27].

PFBO-Me ester derivatives have previously been reported to be suitable for GC–NICI-MS–SIM detection of 3-hydroxy acids [21]. NICI mass spectral data for some 2- and 3-hydroxy acids are given in Table I. The 3-O-PFBO-Me esters all produced molecular radical ions forming the base peak. The 2-O-PFBO-Me esters were less stable under the conditions used, and prominent ions with the charge on the halogen-containing moiety, e.g., fragments of $m/z$ 211 (pentafluorobenzoate anion), $m/z$ 167 (pentafluorophenyl anion) and $m/z$ 148 (tetrafluorophenyl radical anion), were seen. The base peak corresponded either to the molecular radical anions or to the pentafluorophenyl anions. For long-chain 2-hydroxy fatty acids, i.e., 2-OH-14:0 and 2-hydroxyhexadecanoic acid (2-OH-16:0), pentafluorophenyl anions formed the base peak, whereas for shorter-chain molecules, i.e., 2-hydroxydecanoic acid (2-OH-10:0) and 2-OH-12:0, molecular radical anions formed the base peak (Table I).

The calibration graphs obtained were linear over the range 1–1000 ng and followed the equations $y=1.765\cdot10^{-3}x+70.55\cdot10^{-3}$ ($r=0.990$) for 2-OH-12:0 and $y=0.921\cdot10^{-3}x+38.73\cdot10^{-3}$ ($r=0.997$) for 3-OH-14:0. We have previously re-

TABLE I

NEGATIVE-ION CHEMICAL-IONIZATION (METHANE) MASS SPECTRAL DATA FOR O-PENTAFLUOROBENZOYL METHYL ESTER DERIVATIVES OF SOME 2- AND 3-HYDROXY FATTY ACIDS: ION ABUNDANCE

Abbreviations: the fatty acids are designated as the number of carbon atoms with the position of the hydroxyl (OH) group indicated. All 2- and 3-hydroxy fatty acids except 3-OH-9:0 (internal standard) have been reported as constituents of Gram-negative bacteria and LPS [14]. Molecular weights are given in parentheses.

| Ion | 2-Hydroxy fatty acid (%) | | | | 3-Hydroxy fatty acid (%) | | | |
|---|---|---|---|---|---|---|---|---|
| | 2-OH-10:0 (396.30) | 2-OH-12:0 (424.40) | 2-OH-14:0 (452.40) | 2-OH-16:0 (480.40) | 3-OH-9:0 (382.30) | 3-OH-10:0 (396.30) | 3-OH-12:0 (424.40) | 3-OH-14:0 (452.40) |
| $(M)^{-\cdot}$ | 100 | 100 | 56 | 17 | 100 | 100 | 100 | 100 |
| $(C_6F_5CO_2)^-$ ($m/z$ 211) | – | 43 | 22 | 40 | – | – | – | – |
| $(C_6F_5)^-$ ($m/z$ 167) | 1 | 92 | 100 | 100 | 1 | – | – | – |
| $(C_6F_5-F)^{-\cdot}$ ($m/z$ 148) | 2 | 6 | 30 | 5 | 1 | 7 | 1 | 1 |

ported a linear calibration graph over the range 5–1000 ng of *Escherichia coli* LPS when monitoring at $m/z$ 452 of 3-OH-14:0 [21].

*Amino acids.* Current methods (including the N-HFB-isoBu method) for derivatization of amino acids prior to GC analysis are simple [25], although removal of hydrochloric acid after hydrolysis as well as sample clean-up procedures are tedious. However, rapid acid neutralization using an organic base and sample clean-up using disposable hydrophobic and hydrophilic columns have been described for the analysis of carbohydrates [18, 29, 30]. Simplifications based on these approaches were developed for the amino acids in this study.

It has been noted that effective hydrolysis of peptides and proteins can be achieved in 6 h or less at 145–155°C [31, 32] rather than the conventional 16–24 h at 105°C [19, 20]. We found that the former procedure also efficiently hydrolysed the bacterial samples. Except for Glu the amino acids were eluted quantitatively from the ion-exchange columns (Table II). The major part of the Glu was found in the water washing eluates. Therefore, Glu was omitted in quantitative studies. Significantly lower recoveries of Lys and DAP, in particular, were found when less than four column volumes of acid eluent or when an alkaline eluent, viz., 1 *M* aqueous ammonia solution [19], was used.

We also investigated the possibility of using N,N-dioctylmethylamine for neutralizing the samples after hydrolysis. This procedure allowed the samples to be applied to the ion-exchange resin immediately after mild acidification, but the samples became brownish after the HFBA derivatization. Injection of these samples resulted in deterioration of the capillary column and a considerably lower separation efficiency. This procedure was therefore omitted but may be practical for use in combination with column switching for protection of the chiral column.

Racemization induced by hydrolysis of the peptide chain depends on a variety of intrinsic and extrinsic factors. The former include the actual amino acid and

TABLE II

PRECISION OF AMINO ACID ANALYSES AND YIELDS OF INDIVIDUAL AMINO ACIDS FROM THE ION-EXCHANGE COLUMN

Each sample contained ca. 10 μg of each amino acid including the internal standards D-Nle and D-Lys. The samples were analysed by GC with flame ionization detection.

| Amino acid* | Yield** (%) | Coefficient of variation ($n=4$) (%) |
|---|---|---|
| D-/L-Ala | 101 | 2.1 |
| D-/L-Glu | 40 | 12 |
| L-Lys | 102 | 6.0 |
| DAP*** | 98 | 5.0 |

*Abbreviations: Ala, alanine; Nle, norleucine; Glu, glutamic acid; Lys, lysine; DAP, diaminopimelic acid. All amino acids except Nle (internal standard) are constituents of bacterial PG [16].
**Calculated by comparing the average amount ($n=4$) of each amino acid after passage through the column with the average amounts ($n=4$) of the same acids omitting the ion-exchange step.
***Measurements were based on the peak area of the D,D-enantiomer.

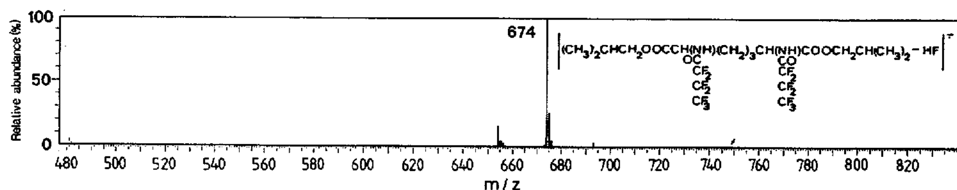


Fig. 1. NICI (methane) mass spectrum of DAP.

TABLE III

NEGATIVE-ION CHEMICAL-IONIZATION (METHANE) MASS SPECTRAL DATA FOR SOME N-HEPTAFLUOROBUTYRYL ISOBUTYL AMINO ACID DERIVATIVES: ION ABUNDANCE

| Ion | Amino acid* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ala | | Nle | | Glu | | Lys | | DAP | |
| | m/z | % | m/z | % | m/z | % | m/z | % | m/z | % |
| $(M-H)^-$ | 340 | 39 | 382 | 31 | 454 | 32 | 593 | 2 | 693 | 3 |
| $(M-2H)^{-\cdot}$ | 339 | 2 | 381 | 4 | 453 | 11 | 592 | 1 | 692 | – |
| $(M-HF)^{-\cdot}$ | 321 | 100 | 363 | 100 | 435 | 100 | 574 | 100 | 674 | 100 |
| $(M-2HF)^{-\cdot}$ | 301 | 4 | 343 | 2 | 415 | 2 | 554 | 12 | 654 | 16 |
| $(M-3HF)^{-\cdot}$ | 281 | – | 323 | – | 395 | 1 | 534 | 3 | 634 | – |
| $(M-OC_4H_9)^-$ | 268 | 6 | 310 | 6 | 382 | 2 | 521 | – | 621 | – |

*For abbreviations, see Table II.

TABLE IV

DETERMINATION OF HYDROXY FATTY ACIDS AS O-PENTAFLUOROBENZOYL METHYL ESTER DERIVATIVES IN SOME DIFFERENT MICROORGANISMS

About 1–3 mg of lyophilized cells were methanolysed in 2 *M* methanolic hydrochloric acid at 85°C for 18 h. The samples were analysed using GC–NICI–MS–SIM.

| Microorganism* | Hydroxy fatty acid** (μg/mg dry weight of cells) | | | |
|---|---|---|---|---|
| | 3-OH-10:0 | 2-OH-12:0 | 3-OH-12:0 | 3-OH-14:0 |
| *A. faecalis* | – | 2.4 | – | 11 |
| *A. calcoaceticus* | – | 0.95 | 3.2 | 1.2 |
| *E. agglomerans* | – | – | – | 13 |
| *P. aeruginosa* | 3.3 | 5.6 | 4.5 | – |

*No hydroxy fatty acids were found in Gram-positive bacteria (*S. faecalis, S. mutans, S. viridans, C. perifringens*) or fungi (*C. neoformans, C. albicans, T. glabrata*).
**For abbreviations, see Table I.

its structural environment. Ala has been shown to exhibit a low relative degree of racemization (ca. 1–2%) whereas aspartic acid has one of the highest (ca. 7%) [22]. Extrinsic factors include the concentration of the acid, the time of hydrolysis, the hydrolysis temperature and the presence of metal ions [22, 33]. Liardon

TABLE V

DETERMINATION OF D-ALANINE AND DIAMINOPIMELIC ACID AS N-HEPTAFLUOROBUTYRYL ISOBUTYL ESTER DERIVATIVES AND THE ENANTIOMERIC RATIO OF D-ALANINE IN SOME DIFFERENT MICROORGANISMS

About 1-3 mg of lyophilized cells were hydrolysed in 6 *M* deuterated hydrochloric acid at 150°C for 6 h. The samples were analysed using GC-NICI-MS-SIM.

| Microorganism* | Amino acid** | | |
|---|---|---|---|
| | D-Ala*** (μg/mg dry weight of cells) | DAP (μg/mg dry (weight of cells) | D-Ala/(D-Ala+L-Ala)*** (%) |
| *A. faecalis* | 0.72 | 1.2 | 6.2 |
| *A. calcoaceticus* | 0.78 | 1.3 | 6.6 |
| *E. agglomerans* | 0.47 | 0.8 | 2.7 |
| *P. aeruginosa* | 0.69 | 1.1 | 5.8 |
| *S. faecalis* | 2.0 | – | 20 |
| *S. mutans* | 2.3 | – | 25 |
| *S. viridans* | 1.8 | – | 16 |
| *C. perfringens* | 1.6 | 2.3 | 15 |

*No D-Ala or DAP were found in fungi (*C. neoformans, C. albicans, T. glabrata*).
**For abbreviations, see Table II.
***After correction for racemization.

et al. [22] showed that deuterated hydrochloric acid can be used to determine the degree of racemization: owing to addition of a deuterium atom to the asymmetric carbon atom, enantiomers produced during the hydrolysis can be recognized and determined by their higher molecular mass (M+1) using MS analysis.

Amino acid enantiomers can be separated using either a chiral stationary phase [34–36] or, after conversion of the enantiomers into diastereomers by derivatization with a chiral reagent, using a conventional non-chiral phase of low polarity [20]. For trace analysis the former procedure is preferable, since "optical" impurities in the chiral reagent, possible differences in reaction rate of the chiral reagent with the D- and L-enantiomers [36] as well as racemization of the chiral reagent induced by other reagents [37] may lead to erroneous results. With a fused-silica capillary column coated with Chirasil-Val, complete separation of all protein amino acid enantiomers is achieved [34, 35]. In our study the enantiomers were separated with the following resolution numbers (average values for four injections of the same sample): 3.75 (Ala), 2.08 (Glu) and 2.33 (Lys). DAP, the last eluting amino acid, had a retention time of ca. 26 min. As the DAP used was a racemate, three GC peaks were obtained (D,D-, *meso*- and L,L-DAP).

The mass spectrum of DAP is shown in Fig. 1, and NICI data on the derivatized amino acids are given in Table III. The radical ions $(M-HF)^{-\cdot}$ form the base peak for all the derivatives. The second largest peak corresponds to the $(M-H)^{-}$ ions for Ala, Nle and Glu and to the $(M-2HF)^{-\cdot}$ radical ions for Lys and DAP. Similar results were obtained by Low and Duffield [38] using the N-pentafluoropropionyl-*n*-butyl derivatives.

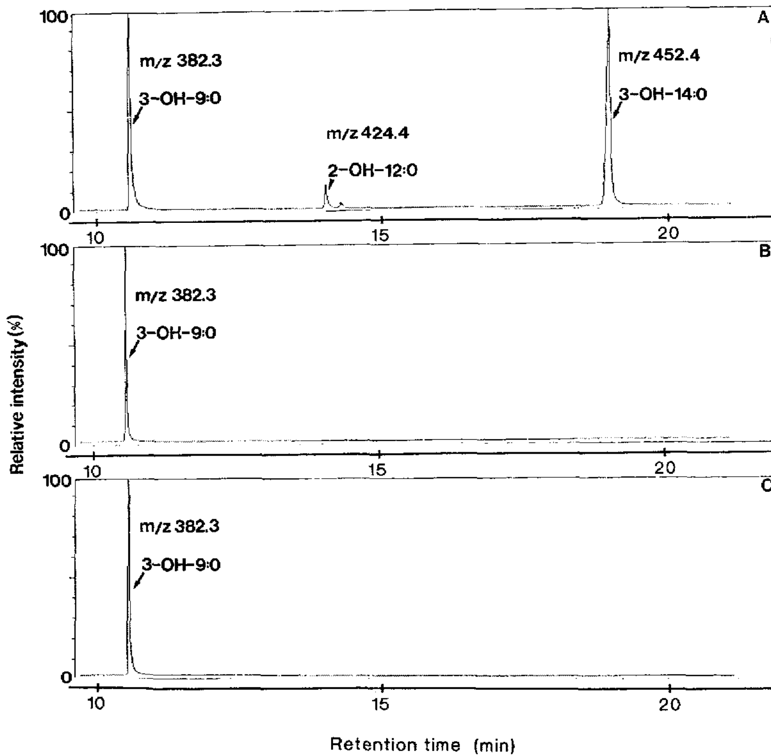


Fig. 2. Mass fragmentograms using NICI (methane) of O-PFBO-Me ester derivatives of hydroxy fatty acids in (A) *A. faecalis*, (B) *S. faecalis* and (C) *C. neoformans*. 3-OH-9:0 was used as an internal standard.

Calibration graphs were linear over the range 1–1000 ng using NICI–SIM, and followed the equations $y=0.023x+1.531$ $(r=0.994)$ for D-Ala and $y=5.649\cdot10^{-3}x+25.83\cdot10^{-3}$ $(r=0.999)$ for DAP (calculated using the total peak area of D,D-, *meso*- and L,L-DAP). D-Nle was used as an internal standard for Ala and Glu, and D-Lys for the diamino acids L-Lys and DAP. D-Lys has only been found as a constituent of the interpeptide chain in *Butyribacterium rettgeri* [39]. The detection limit for D-Ala was in the low femtomole range.

*Microorganisms.* The hydroxy fatty acids and the D-Ala and DAP compositions of the bacteria and fungi are given in Tables IV and V. Representative mass fragmentograms are shown in Figs. 2 and 3.

The 2- and/or 3-hydroxy fatty acids are found in lipid A of LPS in almost all Gram-negative bacteria and usually constitute 5–15 weight-% of the total cellular fatty acids [14]. The composition of hydroxy acids found in the Gram-negative bacteria (Table IV) agrees with that reported in previous studies [14]. No hydroxy fatty acids were detected in any of the Gram-positive species or in the fungi. PG comprises 50–80% of the dry weight of Gram-positive cell walls but less than 10% of Gram-negative cell walls [8]. Consequently, the largest amounts of D-Ala were found in the Gram-positive species (Table V). DAP is present in virtually all Gram-negative bacteria and is also present in a few Gram-positive species,

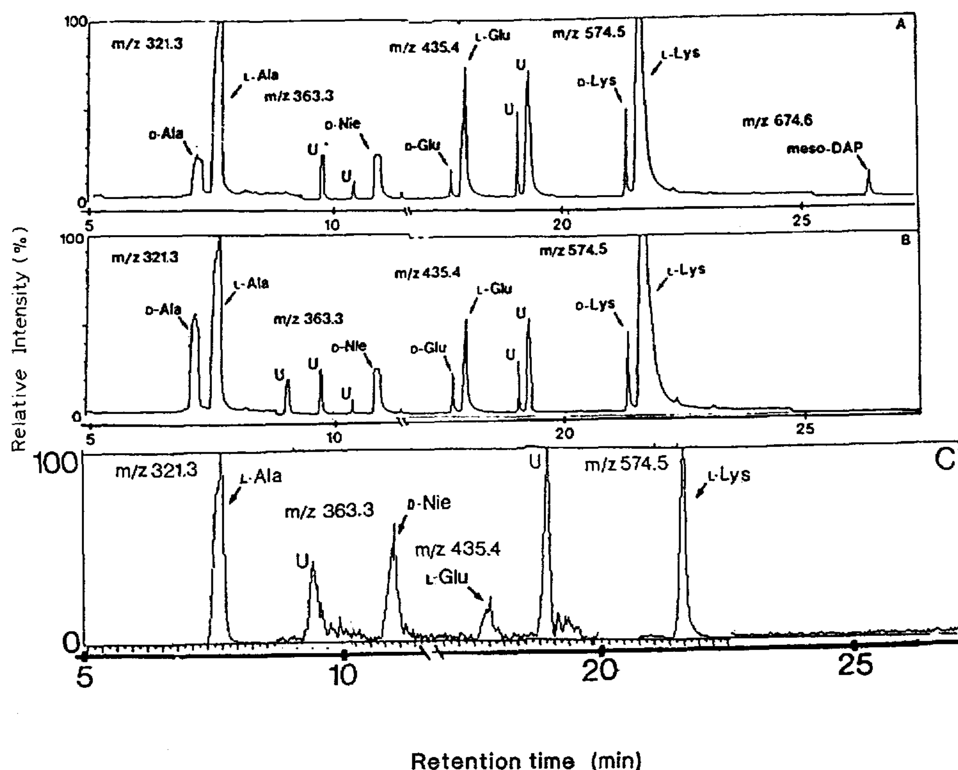


Fig. 3. Mass fragmentograms using NICI (methane) of N-HFB-isoBu ester derivatives of Ala, Glu, Lys and DAP in hydrolysates of (A) *A. faecalis*, (B) *S. faecalis* and (C) *C. neoformans*. D-Nle and D-Lys were used as internal standards (no D-Lys was added to the *C. neoformans* hydrolysate). U = unidentified.

e.g., *Clostridium* and *Corynebacterium* [16]. Notably, the fungi contain no D-amino acids or DAP.

The degrees of racemization for the different peptide-bound and free amino acids were calculated using the method by Liardon et al. [22]. Significant differences in racemization were found between bound and free amino acids. In hydrolysis of the different microorganisms, values between 3.2 and 7.6% for Ala and 0.9 and 6.9% for Lys were obtained, whereas for the free amino acids the racemization was ca. 2% for Ala and ca. 1% for Lys. The values were slightly higher than those reported in earlier studies [19, 22], probably owing to the use of a higher hydrolysis temperature. The D-Ala peak with $m/z$ 322.30 in Fig. 4 originates from racemization of L-Ala during the hydrolysis of *C. neoformans* cells. Liardon et al. [22] reported non-linear results at D-/L-isomer ratios lower than 0.4%, possibly owing to interference from ions [for the NICI (methane) technique, $(M-21)^{-\cdot}$] produced from the deuterated D-isomer. Caution should therefore be exercised in interpreting results from trace analysis of D-amino acids in an extremely high protein background, even when using $^{2}$HCl in the hydrolysis and MS in the detection.

TABLE VI

AIRBORNE DUST IN A POULTRY CONFINEMENT UNIT: ANALYSIS OF SPECIFIC BACTERIAL AMINO ACIDS AND HYDROXY ACIDS

| Sample[a] | DAP (ng/mg) | LPS | | D-Ala[d] (ng/mg) | Surplus D-Ala[d,e] (ng/mg) | Dust[f] ($10^6$ ng/m$^3$) |
|---|---|---|---|---|---|---|
| | | Chemical[b] ($10^3$ ng/mg) | LAL test[c] (ng/mg) | | | |
| 1 | 26 | 1.1 -3.2 | 33 | 260 | 240 | 16.3 |
| 2 | 9.3 | 0.38-1.1 | 25 | 850 | 840 | 13.8 |
| 3 | 3.8 | 0.16-0.47 | 17 | 480 | 480 | 20.0 |
| 4 | 8.3 | 0.34-1.0 | 21 | 210 | 200 | 3.6 |
| 5 | 5.4 | 0.22-0.66 | 27 | 5.2 | 1.2 | 1.6 |

[a]Samples 1 and 4 were collected by the same person, as were samples 2 and 3. Sample 5 was sampled stationary.
[b]Concentration of lipopolysaccharides (LPS) calculated from the concentration of diaminopimelic acid (DAP). Gram-negative bacteria contain ca. 0.1 weight-% of DAP [8, 11] and ca. 5–15 weight-% of LPS [14].
[c]Concentration determined with *Limulus* amoebocyte lysate (LAL) test, chromogenic version.
[d]After correction for racemization.
[e]Concentration of D-alanine (Ala) after subtracting the calculated amount of D-Ala in Gram-negative cells (DAP). Dry weight cells of Gram-negative bacteria contain ca. 0.09 weight-% of D-Ala [8, 11].
[f]The amount of airborne dust was determined by weighing the filters.

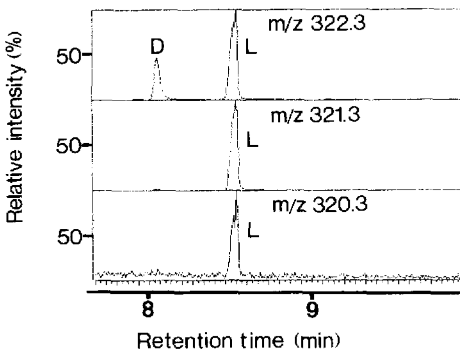


Fig. 4. Mass fragmentograms using NICI (methane) of the N-HFB-isoBu ester derivatives of D- and L-Ala in a hydrolysate of *C. neoformans* cells monitoring at $m/z$ 320.30, 321.30 and 322.30.

*Determination of LPS and PG in airborne dust*

Airborne dust in swine and poultry confinement buildings has been shown to contain Gram-positive and Gram-negative bacteria as well as to free LPS [1, 2, 5]. In previously reported studies for determining airborne microorganisms and LPS, the total bacterial counts were determined after sampling on agar plates, incubation and numbering of the bacterial cells [2, 5–7]. LPS is measured with the LAL test in water extracts of dust samples collected on filters [2, 5, 6]. However, it has been shown that PG may activate the gelation although relatively high concentrations are needed ($10^3$–$10^5$ times larger than for LPS) [10]. Thus,

in samples where most of the bacterial cells are Gram-positive and the amounts are micrograms or larger, the possibility exists of obtaining erroneous results using the LAL test. Analysis of bacterial biomarkers by GC or GC–MS may be a more specific method for measuring LPS [40]. The latter techniques also provide information on the relative proportions of Gram-negative and Gram-positive bacteria in a sample: the levels of D-Ala and DAP indicate the amounts of Gram-positive relative to Gram-negative bacteria, whereas the levels of hydroxy fatty acids and DAP provide information on the levels of free LPS to Gram-negative cell walls.

The results of the filter sample analyses are shown in Table VI. Most of the bacteria collected on the filters were Gram-positive, as judged by the relative amounts of D-Ala and DAP (see surplus D-Ala, Table VI). Similar results have been reported previously [2, 5–7]. The order of precedence for the concentrations of LPS when using DAP measurements was the same as when using the LAL test, but the former method indicated considerably higher levels of LPS. No hydroxy fatty acids were detected. The analytical results indicated the presence of Gram-positive bacteria, some of them containing DAP, in the filters. Analysis of larger numbers of samples will be necessary for evaluating the correlation between the LAL test and the GC–MS method for determining LPS in complex environments. Such studies are in progress in our laboratories.

## CONCLUSIONS

The analysis of specific microbial constituents as halogenated derivatives using NICI–SIM constitutes a highly sensitive and selective technique for determining bacterial cells and/or debris. By combined analysis of amino acids and hydroxy fatty acids it is possible to estimate Gram-positive and Gram-negative bacteria and free LPS in the same sample. Carbohydrate biomarkers, e.g., Mur for PG and heptoses and 2-keto-3-deoxyoctonic acid for LPS, can also be included, thereby providing more information about the bacterial composition of a sample. The use of several biomarkers for determining a bacterial community in environmental and clinical samples has great research and diagnostic potential.

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