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Gas chromatography–mass spectrometry of lipopolysaccharide 3-hydroxy fatty acids: comparison of pentafluorobenzoyl and trimethylsilyl methyl ester derivatives

Zbigniew Mielniczuk*, Srinivas Alugupalli, Eugenia Mielniczuk and Lennart Larsson

Department of Medical Microbiology, University of Lund, Solvegatan 23, 223 62 Lund (Sweden)

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ABSTRACT

3-Hydroxytetradecanoic and 3-hydroxyhexadecanoic acids were used as chemical markers for the determination of lipopolysaccharides by gas chromatography–mass spectrometry of their pentafluorobenzoyl and trimethylsilyl methyl ester derivatives The latter derivatives were simpler to prepare than the former, although both were chemically stable Analysis of pentafluorobenzoyl derivatives (chemical ionization mode with negative ion detection) provided somewhat better sensitivity than analysis of trimethylsilyl derivatives (electron impact mode) 1 pg (injected amount) of pentafluorobenzoyl derivatives was detectable under routine conditions Both types of derivative gave similar values when used to measure lipopolysaccharides in a bacteria-contaminated pharmacological product The described methods are useful for the determination and characterization of lipopolysaccharides in various environments

INTRODUCTION

Lipopolysaccharides (LPSs, endotoxins) are characteristic, toxic outer-membrane constituents of gram-negative bacteria [1], and they can induce several pathophysiological reactions in humans [2] Airborne LPSs are considered to be a major occupational health problem and have been associated with, for example, development of respiratory diseases, headache, fever, and irritation of the eye [3–6]

The main method for the determination of endotoxins is the *Limulus* amebocyte lysate (LAL) test, which is very sensitive and, in the chromogenic version, provides quantitative results Unfortunately, the specificity of the LAL test is limited since it can be activated by many chemical structures other than LPSs [7,8] Hence, in several laboratories, ours among them, the possibility of using gas chromatography–mass spectrometry (GC–MS) for endotoxin determination has been considered Analytes used with GC–MS are LPS-specific 3-hydroxylated fatty acids Both methyl-trimethylsilyl (Me/TMS) derivatives (analysed by electron impact ionization (EI) [9,10]), and methyl-pentafluorobenzoyl (Me/PFBO) derivatives (analysed by chemical ionization with negative ion detection (NICI) [11,12]) have been used However, no data comparing the GC–MS characteristics of these derivatives have been reported Notably, most modern bench-top GC–MS instruments lack facilities for NICI

This paper compares the Me/TMS and Me/PFBO derivatives of LPS-specific 3-hydroxy fatty acids with respect to their ease of preparation, chemical stability, and GC–MS characteristics

Correspondence to Dr L Larsson, Department of Medical Microbiology, University of Lund, Solvegatan 23, 223 62 Lund, Sweden

* Permanent address Child Health Centre, Warszawa-Miedzylesie, Al Dzieci Polskich 20, Poland

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EXPERIMENTAL

Chemicals and glassware

Nonanoic, tridecanoic, tetradecanoic, and hexadecanoic acids hydroxylated in position 3 (3-OH 9 0, 3-OH 13 0, 3-OH 14 0, and 3-OH 16 0, respectively) were purchased from Larodan Lipids (Malmo, Sweden) Bis(trimethylsilyl)trifluoroacetamide (BSTFA, 98%), pentafluorobenzoyl chloride (PFBO-Cl, 98%), dichloromethane (p a , stabilized with 50 ppm amylene), diethyl ether (p a) and acetonitrile (99%) were from Janssen Chimica (Geel, Belgium) Methanol (p a) and *n*-hexane (99%) were from Lab Scan (Dublin, Ireland), acetyl chloride (p a) and pyridine (p a) from Merck (Darmstadt, Germany), and triethylamine (TEA) from Sigma (St Louis, MO, USA) All chemicals were used without further purification The glass test tubes (equipped with PTFE-lined screw-caps) were heated at 350°C overnight before use

Reagents

Methanolic HCl (1 3 and 3 6 *M*) was prepared by adding acetyl chloride (1 or 3 ml) dropwise to methanol (9 or 7 5 ml) at 0°C, 35% PFBO-Cl solution was prepared by adding 650 μl of acetonitrile to 350 μl of PFBO-Cl, and 2% TEA solution was prepared by mixing 980 μl of acetonitrile with 20 μl TEA All reagents were stored at 4°C in glass tubes with PTFE-lined screw caps, and used within one week of preparation

Standard solutions

Stock solutions of the free hydroxy acids were prepared by dissolving 5 mg of each acid in 5 ml of hexane–diethyl ether (4 1, v/v) LPS stock solutions for the construction of calibration curves were prepared by diluting *Escherichia coli* serotype 055 B5 LPS (Sigma) in pyrogen-free water to concentrations of 1 and 10 ng/μl The solutions were stored at 4 °C

Derivatization of hydroxy acids and construction of calibration curves

The free hydroxy acids 3-OH 14 0 and 3-OH 16 0 (250 μg of each) were heated in 1 ml of 1 3 *M* methanolic HCl at 80 °C for 30 min After the addition of 1 ml of distilled water, the samples were extracted twice with 1 ml of *n*-hexane, and the combined hexane phases were evaporated to dryness under a stream of nitrogen Next, the samples were dissolved in 2 5 ml of hexane and divided into five equal parts, which thus each contained 50 μg of each acid The solvent was then evaporated, and the methyl esters were subjected to TMS or PFBO derivatization as described below

To four of the dried methyl ester samples were added 30 μl of 35% PFBO-Cl and 20 μl of 2% TEA (both in acetonitrile) To determine the influence of the reaction temperature on the yield of the PFBO derivatives, the four samples were heated for 1 h at 80°C, 100°C, 120°C and 150°C, respectively Subsequently, *n*-hexane (1 5 ml) and distilled water (1 ml) were added and after extraction, the hexane phase was evaporated and the sample re-dissolved in 250 μl of *n*-hexane

TMS derivatization was accomplished by adding, to the fifth sample, 50 μl of BSTFA and 5 μl of pyridine The sample was then heated at 80°C for 15 min After evaporation of the pyridine, using a nitrogen stream, *n*-hexane was added to achieve a final volume of 250 μl Both the Me/PFBO and the Me/TMS derivatives were analysed by GC using flame ionization detection (see below)

To construct calibration curves, the pure 3-OH 14 0 and 3-OH 16 0 acids, plus an internal standard (250 μg of each acid), were subjected to methanolysis as above PFBO derivatization (using 3-OH 9 0 as internal standard) was performed at 80°C for 1 h, and TMS derivatization (using 3-OH 13 0 as internal standard) was performed as described above After serial dilution the final preparations were analysed by GC–MS in the EI (for the Me/TMS derivatives), or the NICI (for the Me/PFBO derivatives) mode

Calibration curves for LPS analysis

To known amounts of LPS (in the range 5–1000 ng), 50 ng of either 3-OH 9 0 (for PFBO derivatization) or 3-OH 13 0 (for TMS derivatization) were added The (dry) samples were heated overnight (about 18 h) in 3 6 *M* methanolic HCl, and the methyl esters were extracted with 1 5 ml of hexane and 1 ml of water The hexane layer was evaporated under a stream of nitrogen, and redissolved in 1 ml of hexane–dichloromethane (1 1, v/v) Each sample was then applied to a disposable silica gel column (1 ml Bond-Elut Analytichem, Harbour City, CA,

USA) to separate the methyl esters of hydroxylated acids from those of non-hydroxylated acids Prior to use the column was washed with 1 ml of diethyl ether followed by 1 ml of hexane–dichloromethane (1 1, v/v) After the methyl ester preparations had been applied to the column, 0 6 ml of hexane–dichloromethane (1 1, v/v) was added The hydroxy fatty acid methyl esters were eluted with 1 5 ml of diethyl ether, and the solvent was evaporated Thereafter, PFBO or TMS derivatization was performed as described above

A calibration curve for the Me/PFBO derivative was constructed by plotting the ratios of *m/z* 452 (LPS acid 3-OH 14 0) peak areas to *m/z* 382 (internal standard 3-OH 9 0) peak areas against the amounts of endotoxin added Analysis was performed in selected-ion monitoring (SIM) NICI mode Similarly, for the Me/TMS derivative, the ions *m/z* 315 (LPS acid 3-OH 14 0) and *m/z* 301 (internal standard 3-OH 13 0) were used Analysis was performed in SIM EI mode The final preparations were brought to a volume of 100 μl with *n*-hexane, 1 μl of which was analysed by GC–MS

Before each set of SIM analyses, a standard solution (containing *ca* 500 pg of each derivatized acid) was injected and analysed in scan mode for determination of retention time and *m/z* values for monitoring Injection (after the entire analytical procedure) of blanks of solvents and reagents was performed regularly to detect possible interfering compounds

Application

Internal standards (3-OH 9 0 and 3-OH 13 0, 100 ng of each) and 1 ml of 3 6 *M* methanolic HCl were added to a dry pharmacological product suspected (by the LAL test) to contain LPSs, the sample was then heated at 100°C for 18 h After this treatment the preparation was purified using the Bond Elut column (see above) and divided into two equal parts, one for TMS derivatization and one for PFBO derivatization (see above) These two parts were then analysed by GC–MS in the EI and the NICI mode, respectively

Gas chromatography

A Varian Model 3500 (Los Altos, CA, USA) gas chromatograph was used, equipped with a split/splitless injector, a flame ionization detector, and a fused-silica capillary column (25 m × 0 25 mm I D) coated with OV-1 (SGE, Ringwood, Australia) The column temperature was programmed to rise at 8°C/min from 120°C to 260°C The nitrogen carrier gas flow-rate through the column was 2 ml/min The temperature of the injector was 250°C and that of the detector 270°C The data were evaluated by using a Chrompack control and integration system with an IBM PS/2 Model 30 and a Chrompack BD 70 printer/plotter

Gas chromatography–mass spectrometry

A VG Trio-1 S GC–MS system (Manchester, UK) was used The gas chromatograph was a Hewlett-Packard Model 5890 (Avondale, PA, USA) equipped with a fused-silica capillary column (25 m × 0 25 mm I D) containing cross-linked OV-1 as the stationary phase Injections were made using a Hewlett-Packard Model 7673 autosampler in the splitless mode, the split valve was opened 1 min after the injection Helium was used as the carrier gas, at an inlet pressure of 7 p s i , and the temperature of the column was programmed from 120°C to 260°C at 20°C/min Both the injector and the interface (between GC and MS) were kept at 260°C The Me/TMS derivatives were analysed in the EI mode (ion source temperature 220°C) and the Me/PFBO derivatives in the NICI mode (ion source temperature 150°C), using both scanning and SIM Isobutane at a pressure of 10 p s i was used as reagent gas Ionization was performed at 70 eV

RESULTS

Derivatization of hydroxy acids

The influence of reaction temperature on the yield of the PFBO and TMS derivatives of the hydroxy acid methyl esters was studied by GC TMS derivatives were formed quantitatively after 15 min at 80°C, therefore longer heating did not improve the reaction yield PFBO derivatization was complete after heating at 80°C for 1 h (Fig 1) At a reaction temperature of 150°C, considerable decomposition of the Me/PFBO derivative was observed in the GC profile (Fig 2), the same phenomenon was observed on GC–MS analysis (data not shown) Both the Me/TMS and Me/PFBO derivatives were chemically stable, as no decomposition was observed after several weeks of storage at 4°C

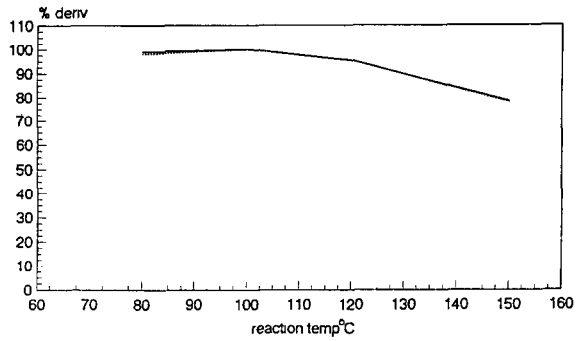

Fig 1 Yield of PFBO methyl ester derivatives of 3-OH 14 0 (solid line) and 3-OH 16 0 (dotted line) as a function of reaction temperature (heating duration 1 h)

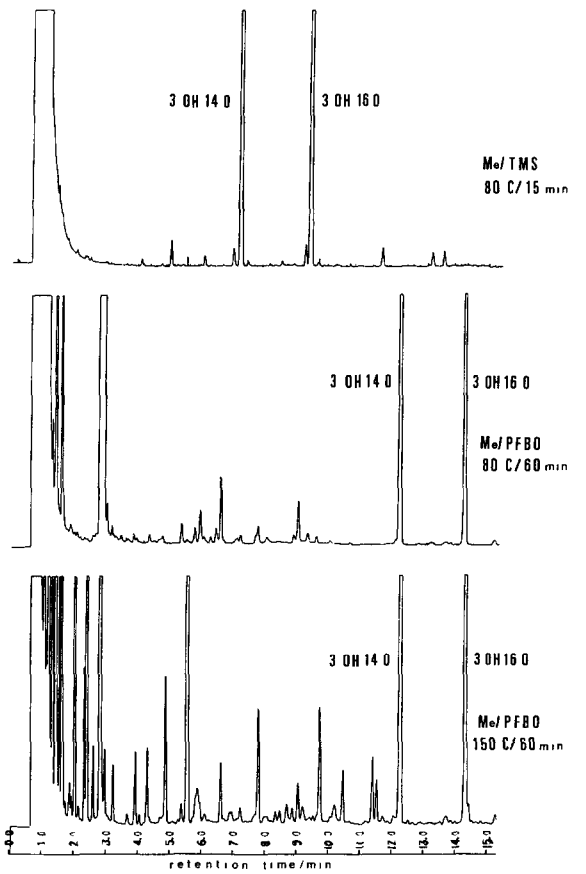

Fig 2 Gas chromatographic analysis of TMS (upper) and PFBO (center, lower) methyl ester derivatives of 3-OH 14 0 and 3-OH 16 0 under different reaction conditions

Mass spectra

Mass spectra of Me/TMS and Me/PFBO derivatives of 3-OH 14 0 analysed in the EI and NICI modes, respectively, are shown in Fig 3 The spectrum of the Me/TMS derivative was characterized by two abundant peaks with m/z 315 (M − 15, loss of a CH_3 group) and m/z 175 (M − 155, cleavage of C_3–C_4 linkage), in the high-mass region, m/z 257 (M − 73, loss of a $(CH_3)_3$ Si group) was also seen The PFBO derivative produced a mass spectrum dominated by m/z 452 (M) The Me/TMS and Me/PFBO derivatives of the other 3-hydroxy fatty acids studied produced analogous mass spectra

Sensitivity and calibration curves

Calibration curves for the Me/TMS and Me/PFBO derivatives of the individual hydroxy acids are shown in Fig 4 The lowest detectable injected amounts of the TMS and PFBO derivatives were, respectively, *ca* 3 and 1 pg (signal-to noise ratio 4), these values were obtained under routine analytical conditions, using SIM focusing on m/z M − 15 (TMS derivatives) and m/z M (PFBO derivatives) When using TMS derivatization, 3-OH 13 0 was used as the internal standard, because the volatility of the 3-OH 9 0 Me/TMS derivative led to its partial evaporation, together with the pyridine, after reaction

Calibration curves for the LPSs, obtained using 3-OH 14 0 as the analyte, are also shown in Fig 4 Detection of a few nanograms of LPSs (starting material) was easily accomplished, when using both Me/TMS and Me/PFBO derivatization, following injection of 1 μl of a final total sample volume of 100 μl The two curves fitted the equations $y = (4\,7x - 54\,4)10^{-3}$ (Me/TMS) and $y = (3\,9x - 50\,6)10^{-3}$ (Me/PFBO), the correlation coefficient was 0 9937 in both cases

Application

Fig 5 illustrates mass chromatograms of the freeze-dried pharmacological product after Me/TMS and Me/PFBO derivatization of hydroxy acids The sample contained several 3-hydroxylated fatty acids, the identities of which (3-OH 12 0, 3-OH 14 0, 3-OH 16 0 and 3-OH 18 0) were established by GC–MS analysis in scan mode The amount of LPSs in the sample was calculated by comparing the peak areas of the hydroxy acids of LPSs with

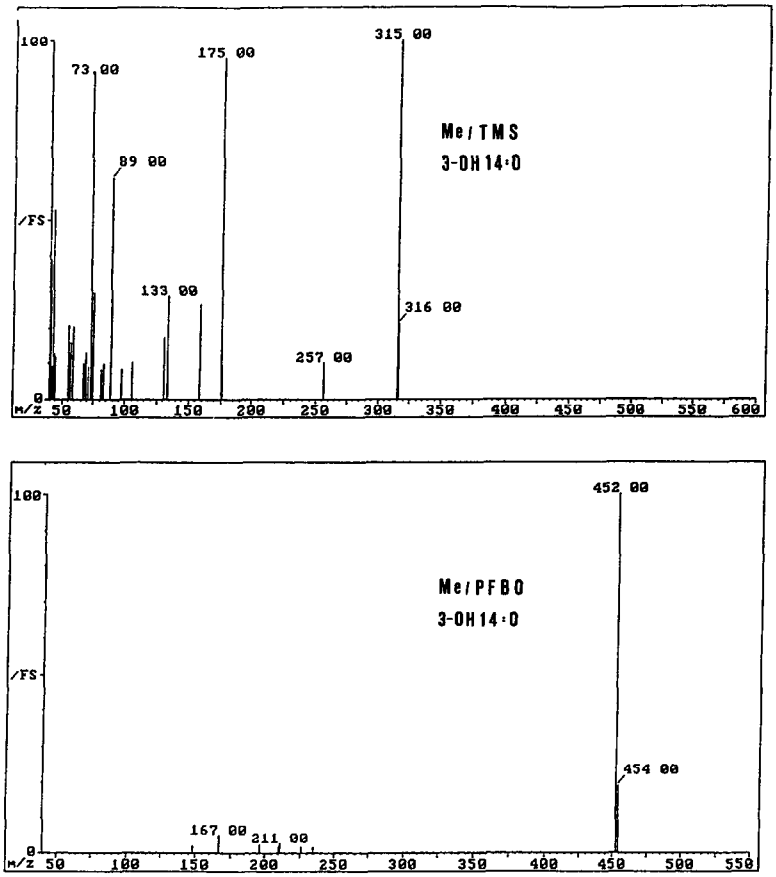

Fig 3 Mass spectra of TMS (upper) and PFBO (lower) derivatized 3-OH 14 0 methyl ester analysed in the EI and the NICI mode, respectively

those of the internal standards The number of moles of the LPS-3-hydroxy acid derivatives was then calculated and subsequently divided by four, since one mole of lipid A generally contains four moles of 3-hydroxy acids The value obtained in this manner was multiplied by an arbitrarily chosen LPS molecular mass, in this case 8000, and the amount of LPSs was thereby estimated to be 576 ng, using the Me/PFBO derivative, and 776 ng, using the Me/TMS derivative (Table I)

DISCUSSION

GC–MS analysis of 3-hydroxylated fatty acids allows accurate quantitative measurement of endotoxins, even in complex environments These acids are present in lipid A, the part of the LPS molecule that is responsible for endotoxic effects [13] In addition, as the distribution of the 3-hydroxylated fatty acids varies between different Gram-negative bacteria, GC–MS analysis can also supply information on the origin of LPS For example, 3-OH 14 0 is the predominant 3-hydroxy acid in *Enterobacteriaceaea*, whereas 3-OH 10 0, 3-OH 12 0 and 3-OH 16 0 are indicative of *Pseudomonadaceae*, and several odd-numbered 3-hydroxy acids have been identified in a variety of bacterial species [14] This information can, for instance, be useful for determining the origin of contamination caused by Gram-negative bacteria

In the present work, we compared TMS and PFBO derivatization of 3-hydroxy fatty methyl es-

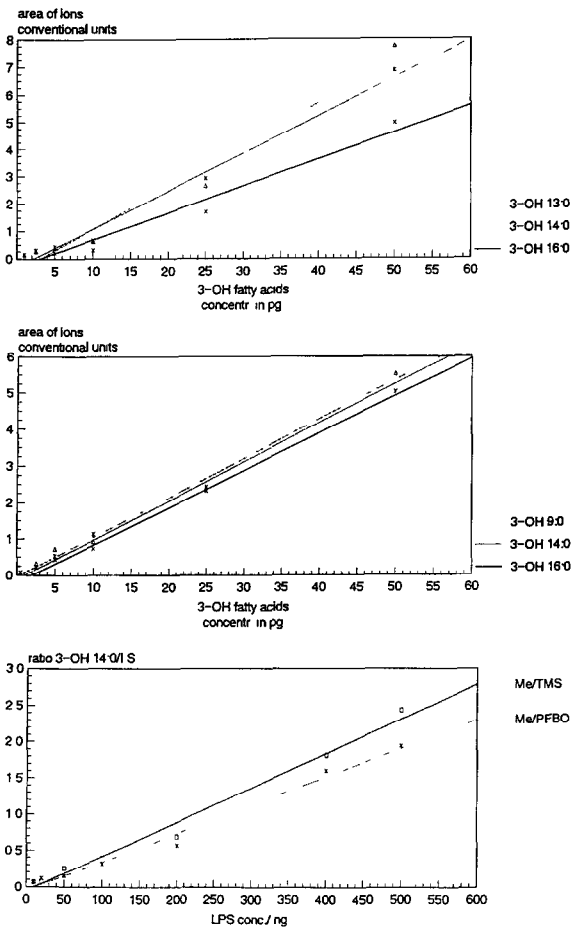

Fig 4 Calibration curves for TMS (upper) and PFBO (centre) derivatized 3-OH acid methyl esters, and for *Escherichia coli* LPS (lower) For the latter, 3-OH 14 0 was used as an analyte, after methylation and TMS or PFBO derivatization

ters for use in GC–MS analysis of endotoxins TMS derivatization was also used in early studies, a minimum detectable amount (using the EI mode) of *ca* 100 ng of LPSs per millilitre of sample was reported [9] Recently, PFBO derivatization was found to provide superior sensitivity (using the NICI mode) an amount of only 1 ng of LPSs was detectable [11] The present detailed comparison of the two types of derivative for LPS analysis was prompted by the fact that several of the compact, low-cost modern GC–MS instruments lack facilities for NICI analysis, and the fact that the performance of GC–MS instruments has been dramatically improved during recent years

The sensitivity of NICI analysis of Me/PFBO derivatives was essentially the same as previously reported [11] *i e* the presence of a nanogram amount of LPSs in a sample was detectable under routine working conditions The difference in sensitivity between the Me/PFBO and Me/TMS derivatives of 3-OH 14 0 was lower than expected The reason for this is unclear, but the adsorption of subpicogram amounts of the analyte in the GC–MS system might be one explanation In our experience, to achieve optimal sensitivity, the following are necessary (1) PTFE-lined screw caps and clean glassware with a non-active surface must be used, (2) contamination from detergents, etc , must be carefully avoided during sample preparation, (3) the glass liner in the injection port of the chromatograph must be changed or cleaned regularly, (4) a short (1–2 m) interchangeable fused-silica pre-column should be used or 1-m lengths of the inlet part of the analytical column should be periodically removed to avoid

TABLE I

LIPOPOLYSACCHARIDE IN A PHARMACOLOGICAL SAMPLE

Values were calculated according to amounts of 3-hydroxylated fatty acids (12 0, 14 0 16 0, and 18 0) analysed as Me/PFBO and Me/TMS derivatives "Relative amount" refers to an internal standard

3-Hydroxy acid	Relative amount	Mol ($\times 10^{-10}$)	Sum (mol)	Mol LPS	LPSs (ng)
Me/PFBO derivatives					
12 0	0 46	1 07	2 86 10^{-10}	0 72 10^{-10}	576
14 0	0 73	1 50			
16 0	0 12	0 22			
18 0	0 04	0 07			
Me/TMS derivatives					
12 0	0 40	0 93	3 89 10^{-10}	0 97 10^{-10}	776
14 0	0 75	1 53			
16 0	0 30	0 55			
18 0	0 53	0 88			

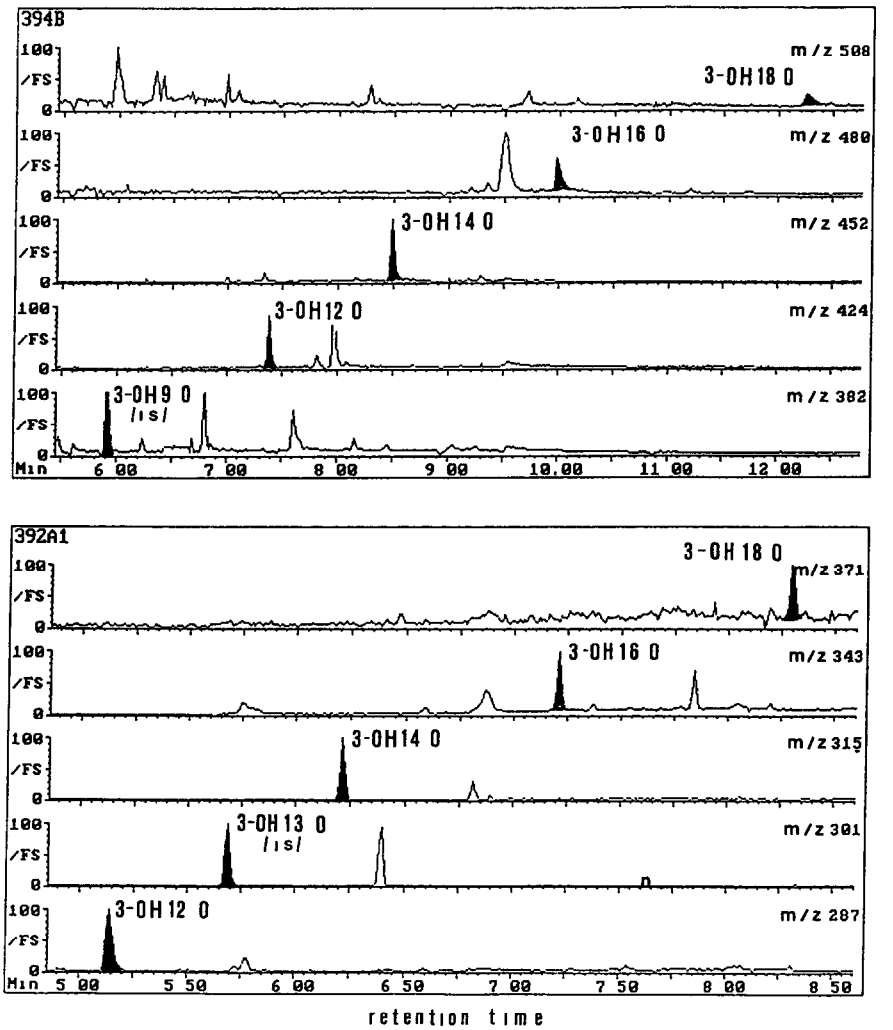

Fig 5 Mass chromatograms of a bacteria-contaminated pharmacological sample obtained by analysing (scan mode) PFBO (upper) and TMS (lower) methyl ester derivatives of 3-hydroxylated fatty acids For abbreviations, see text

sample adsorption and/or decomposition on active sites formed by carbonized material, (5) the ion source should be frequently cleaned

The amount of LPSs found in the studied pharmacological sample when using TMS derivatization differed somewhat from the amount found when using PFBO derivatization (Table I) This difference was almost exclusively represented by a much higher relative amount of 3-OH 18 0 in the former case, the adsorption of PFBO derivatives in the GC–MS system cannot be excluded

TMS derivatization is simpler to perform, *i e* the reaction is quicker and requires one less extraction step than PFBO derivatization Furthermore, the background noise was usually very low in Me/TMS mass chromatograms, even when small amounts of LPSs were analysed Both types of derivative are chemically stable A general advantage that EI analysis has over CI analysis is a longer filament lifetime, but for optimal sensitivity, NICI analysis of Me/PFBO derivatives is recommended

ACKNOWLEDGEMENT

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NOTE ADDED IN PROOF

The results presented in this paper were achieved when the GC–MS instrument had been in use for 18 months Thereafter the quadrupole pre-filter was cleaned This resulted in a considerable increase in detection sensitivity of the 3-hydroxy fatty acid Me/TMS derivatives, especially in SIM mode analysis (data not shown) Thus, not only the condition of the gas chromatograph and the ion source, but also of the quadrupole pre-filter, is vital for high sensitivity

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