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Gas chromatography–mass spectrometry methods for the analysis of mycocerosic acids present in *Mycobacterium tuberculosis*

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Abstract

Mycocerosic acids of *Mycobacterium tuberculosis* were studied by gas chromatography–mass spectrometry. Mycobacterial cells were subjected to alkaline hydrolysis and then acidified before being extracted using petroleum ether to collect the acids. Methyl ester, trimethylsilyl and pentafluorobenzyl derivatives of the mycocerosic acids were formed. In the electron impact mode, using selected-ion monitoring, the methyl ester derivative of 2,4,6,8-tetramethyloctacosanoic acid (C_{32} mycocerosic acid) was detectable down to 20 pg (injected amount), whereas in the negative-ion chemical ionization mode, the pentafluorobenzyl derivative was detectable down to 2 pg, provided that the temperature of the ion source was at least 240°C. The described methods can be used to demonstrate and quantify certain species of *Mycobacterium*, including *M. tuberculosis*, in clinical or environmental samples. © 1998 Elsevier Science B.V.

Keywords: Mycocerosic acids; *M. tuberculosis*; Derivative; Mass spectrometry

1. Introduction

Mycobacteria synthesize a range of characteristic lipids that can be used in taxonomic studies and routine species identification [1,2]. Among these lipids are tuberculostearic acid, secondary fatty alcohols, mycolic acid cleavage products, 3-hydroxy fatty acids and mycocerosic acids [3–8], all of which are well suited to analysis by gas chromatography–mass spectrometry (GC–MS). Some of these lipids have proven potential as markers in the direct detection of mycobacteria in clinical and environmental specimens without prior culturing [9–16]. Using GC–MS, tuberculostearic acid and secondary alcohols have been identified in drinking water and sputum samples, with the results found to be in general agreement with culture results for mycobacteria [10,13,14,17–19].

Mycocerosic acids are long-chain multimethyl branched-chain fatty acids found exclusively in the mycobacterial waxes of *Mycobacterium tuberculosis*, *M. bovis*, *M. gastri*, *M. haemophilum*, *M. kansasii*, *M. leprae*, *M. marinum* and *M. ulcerans* [1–3,7]. In the early 1980s, it was shown that methyl esters of mycocerosic acids could be detected in extracts of five-day cultures of sputum samples from tuberculosis patients by using electron ionization (EI) GC–MS [20]. More recently, negative-ion chemical

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ionization (NICI) GC–MS was used to detect pentafluorobenzyl (PFB) esters of mycocerosic acids directly in sputum samples from tuberculosis patients and from skin biopsies from leprosy patients [9,11,12,15,16].

Clearly, to detect mycocerosic acids directly in clinical specimens, the GC–MS system must provide a very high degree of analytical sensitivity and specificity. The aim of the present study was to compare different GC–MS modes of operation and three different chemical derivatives of mycocerosic acids as regards detection sensitivity and specificity in cultured mycobacterial cells and in sputum samples from patients with pulmonary tuberculosis.

2. Materials and methods

2.1. Chemicals

Pentafluorobenzyl (PFB) bromide, *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA), acetyl chloride, acetonitrile, pyridine, tetrabutylammonium hydrogen sulfate (TBA) and heptacosanoic acid (C_{27}:0) were purchased from Sigma (St. Louis, MO, USA); hexane, methanol, methylene chloride and toluene were from Baxter Scientific (Muskegon, MI, USA); petroleum ether was from Fluka (Buchs, Switzerland). All chemicals used were of analytical grade.

2.2. Mycobacteria

M. tuberculosis H37Rv type strain and ten clinical isolates of *M. tuberculosis* were studied. The latter strains had been isolated from sputum received at the Clinical Microbiology Laboratory, Lund Hospital, Lund, Sweden using standard culture techniques. In brief, the sputum specimens were mixed with an aqueous solution of sodium lauryl sulfate (3%, w/v) and sodium hydroxide (1%, w/v), shaken for 20 min and centrifuged (15 min at 3000×*g*). The pellet obtained was neutralized with aqueous sulfuric acid (0.45%, v/v) and incubated on slants of Lowenstein-Jensen medium for up to five weeks. Then, the mycobacteria were subcultivated on Middlebrook 7H10 medium at 37°C for six weeks. After autoclaving (120°C for 30 min), the bacteria were washed twice with distilled water, lyophilized and used for GC–MS analysis.

2.3. Sputum specimens

Six sputum samples, collected from patients with known pulmonary tuberculosis, and four sputum samples that were culture-negative for *M. tuberculosis* were studied. The sputum samples were treated with two volumes of dithiothreitol (Sputolysin; Behring Diagnostics, La Jolla, CA, USA), homogenized by shaking for 15 min and centrifuged (15 min at 3000×*g*). The obtained sediments were then divided into two portions, one for culturing as described above and the other for direct GC–MS analyses.

2.4. Hydrolysis and extraction

Freeze-dried cells of *M. tuberculosis* H37Rv type strain (30 mg) were mixed with 30% (w/v) methanolic potassium hydroxide (2 ml) and toluene (2 ml) and heated at 100°C for 1 h. After cooling, the alkaline hydrolysates were extracted with petroleum ether (2 ml) and the petroleum ether (upper layer) layer was discarded. The bottom phase was then acidified with 20% (v/v) aqueous hydrochloric acid (4 ml) before samples were again extracted with petroleum ether (2×2 ml). The petroleum ether layer was transferred into another test tube and evaporated to dryness under a stream of nitrogen. The dried sample, containing free mycocerosic acids, was mixed with 3 ml of petroleum ether and then divided into six different test tubes, each containing 500 μl. These samples were then evaporated to dryness under a stream of nitrogen. Two samples were used for each of the three studied derivatization procedures, i.e. formation of (a) methyl esters, (b) trimethylsilyl (TMS) esters and (c) PFB esters, respectively.

Another set of four samples (in duplicate), each containing cells of *M. tuberculosis* H37Rv type strain (5 mg dry weight), were mixed with 30% (w/v) methanolic KOH (1 ml) and toluene (1 ml) and heated at 100°C for 1, 3, 5, 7 and 18 h, respectively. After cooling, the hydrolysates were

acidified and extracted with petroleum ether. The petroleum ether layer was separated and dried and the mycocerosic acids were subjected to methylation.

All ten sputum samples were subjected to the hydrolysis procedure as described above for 1 h. The mycocerosic acids were extracted, subjected to PFB derivatization and analyzed using NICI–GC–MS.

2.5. Derivatization

Methyl ester derivatives were formed by adding 1 M methanolic hydrochloric acid (1 ml) to the dried extracts, which were then heated at 80°C for 30 min. After cooling, 1 ml each of hexane and water were added to the reaction mixture, which was then vortex-mixed for 2 min and centrifuged at 2000×*g* for 5 min. The hexane layer was then transferred to another test tube. The hexane extraction procedure was repeated twice. The combined hexane layers were evaporated to dryness under a stream of nitrogen and then dissolved in 2 ml of hexane. Immediately before GC–MS analysis, the samples were evaporated to dryness under a stream of nitrogen and mixed with 1 ml of hexane solution containing methyl ester-derivatized C_{27}:0 internal standard (10 pg/μl).

TMS derivatives were formed by adding BSTFA (50 μl) and pyridine (10 μl) to the dried samples, which were then heated at 80°C for 15 min. After cooling, pyridine was removed by using a stream of nitrogen, and hexane (2 ml) containing TMS-derivatized C_{27}:0 internal standard (10 pg/μl) was added.

PFB derivatives were formed by adding 1 M aqueous NaOH (500 μl), 0.1 M aqueous TBA (500 μl) and methylene chloride (1 ml) to the dried samples. The samples were vortex-mixed for 2 min and then centrifuged at 2000×*g* for 5 min. The bottom phase (methylene chloride) was removed to a new test tube and 100 μl of 35% (v/v) PFB bromide in acetonitrile was added. The reaction was allowed to proceed at room temperature for 30 min, after which, 1 ml of hexane–water (1:1, v/v) was added and the reaction mixture was shaken for 5 min. The hexane phase was then collected, evaporated, and redissolved in hexane (2 ml) containing PFB-derivatized C_{27}:0 internal standard (10 pg/μl) before analysis.

2.6. Gas chromatography–mass spectrometry

The mass spectra of the three different mycocerosic acid derivatives were recorded and ions suitable for use in selected-ion monitoring (SIM) analysis were selected. Thereafter, samples of the mycobacterial extracts containing the various derivatives of the mycocerosic acids were analysed after serial dilutions, to estimate the lowest detectable amounts. Finally, the PFB-derivatized sputum samples were analyzed in the NICI mode.

A VG Trio-1 GC–MS system was used. The gas chromatograph was a Hewlett-Packard model 5890 equipped with a fused-silica capillary column (30 m×0.25 mm I.D.) containing cross-linked DB-5HT as the stationary phase. Injections were made using a Hewlett-Packard model 7673 autosampler in the splitless mode; the split valve was opened 1.5 min after injection. Helium was used as the carrier gas, at an inlet pressure of 12 p.s.i., and the temperature of the column was programmed from 200 to 320°C, at 15°C/min. Both the injector and the interface (between the gas chromatograph and the mass spectrometer) were held at 310°C. The methyl ester and TMS derivatives were analysed in the EI mode and the PFB derivatives in the NICI mode, with an ion source temperature that was varied between 150 and 240°C. Ammonia, at a pressure of 6 p.s.i., was used as the reagent gas in the NICI mode. Ionization was performed at 70 eV.

3. Results and discussion

Four different mycocerosic acids, ranging from 29 to 32 carbon atoms, were identified in the type strain *M. tuberculosis* H37Rv and in all ten of the clinical isolates studied. The EI mass spectra of methyl ester derivatives of these acids exhibited characteristic fragments of *m/z* 88 (C_2–C_3 cleavage), *m/z* 101 (C_3–C_4 cleavage) and *m/z* (M)+, whereas TMS derivatives showed fragments at *m/z* 146 (C_2–C_3 cleavage), *m/z* 159 (C_3–C_4 cleavage) and *m/z* (M−15). The NICI mass spectra of the PFB-derivatized mycocerosic acids exhibited a molecular-specific fragment at *m/z* (M−181). As an example, the mass spectra of different derivatives of 2,4,6,8-tetra-

methyloctacosanoic acid (C_{32} mycocerosic acid) are shown (Fig. 1).

The ions of m/z 88 (for methyl ester derivatives) and m/z 146 (for TMS derivatives) were monitored in the analyses of the mycocerosates in EI mode, whereas m/z (M−181) (for PFB derivatives) was monitored in NICI mode. As an example, mass chromatograms of all of these derivatives are shown

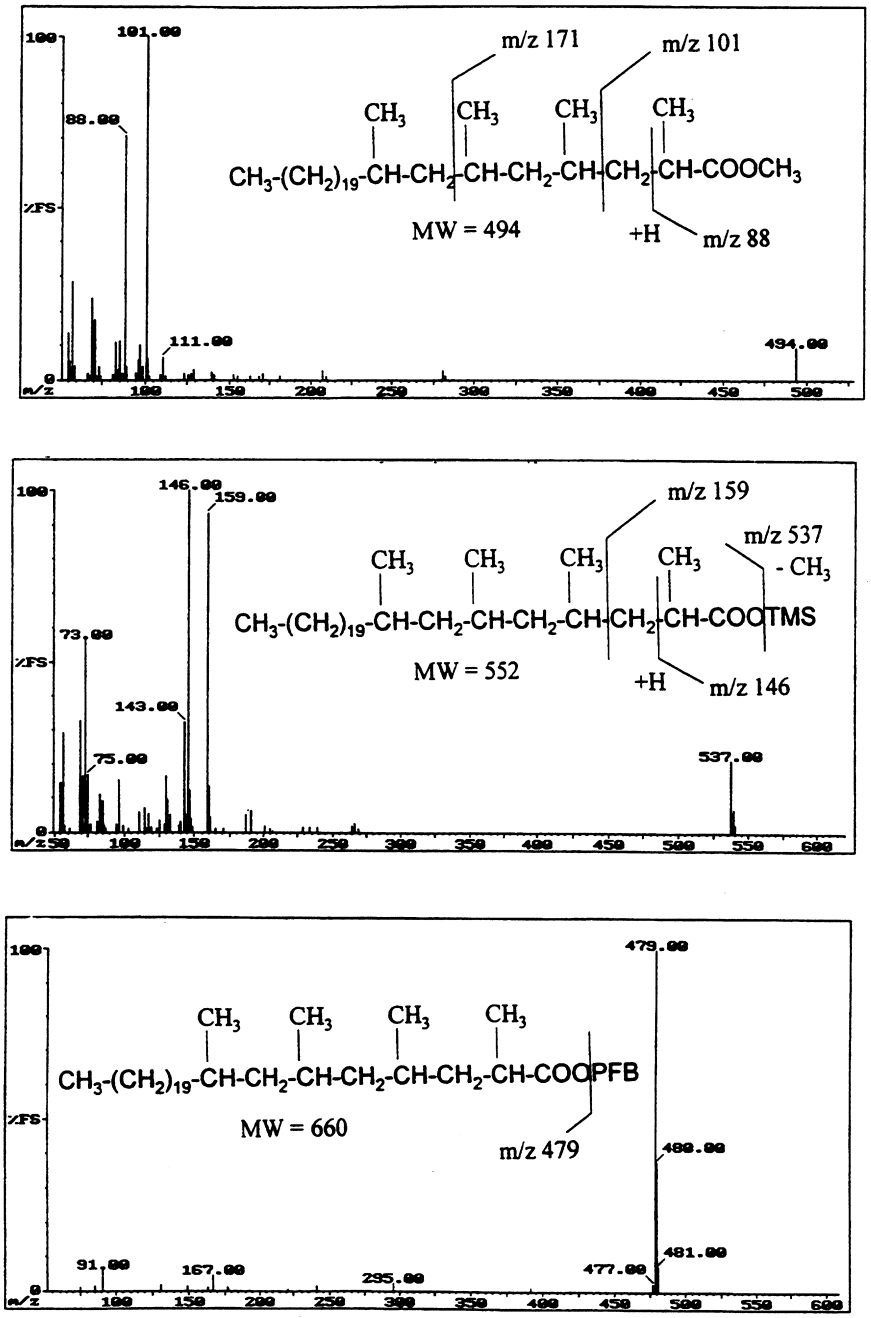

Fig. 1. EI mass spectrum of the methyl ester derivative of C_{32} mycocerosic acid (upper), the TMS derivative (center) and the PFB derivative in NICI mode (lower).

in Fig. 2. When analyzed as methyl ester or TMS derivatives, the C_{31} and C_{32} mycocerosic acids were well separated, whereas the C_{29} and C_{30} mycocerosic acids eluted very close to each other. Although the PFB derivatives of the C_{29} and C_{30} mycoserosic acids analyzed in NICI mode showed similar chro-

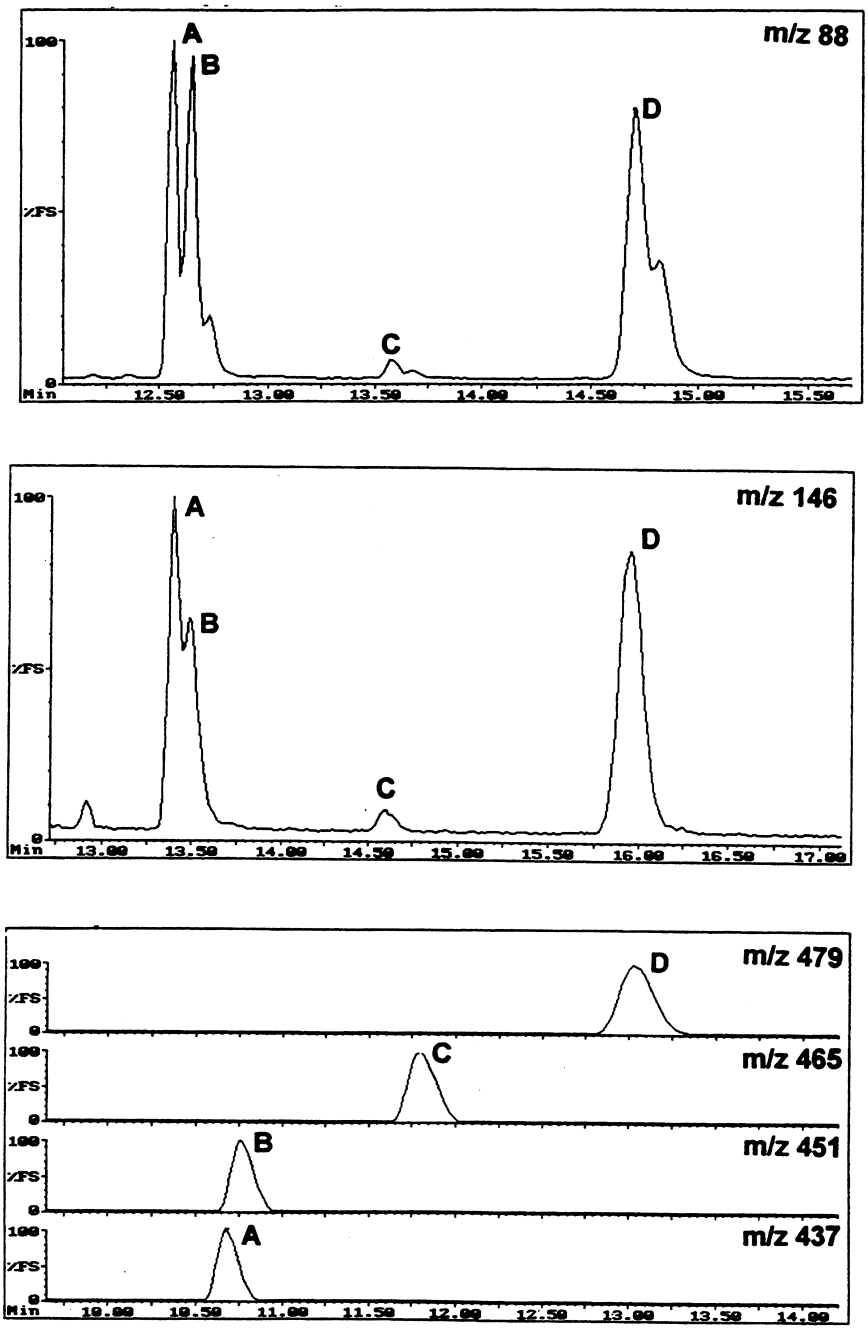

Fig. 2. Mass chromatograms of the methyl ester derivative of mycocerosic acids (upper), the TMS derivative (center) and the PFB derivative in NICI mode (lower). Peak identities: (A) C_{29} mycocerosic acid; (B) C_{30} mycocerosic acid; (C) C_{31} mycocerosic acid and (D) C_{32} mycocerosic acid.

matographic resolution, they were clearly distinguishable due to the specific ions monitored (Fig. 2). Analyses of diluted mycobacterial extracts revealed that the methyl ester derivative of C_{32} mycocerosic acid provided the highest sensitivity in the EI mode and could be detected down to 20 pg (signal-to-noise

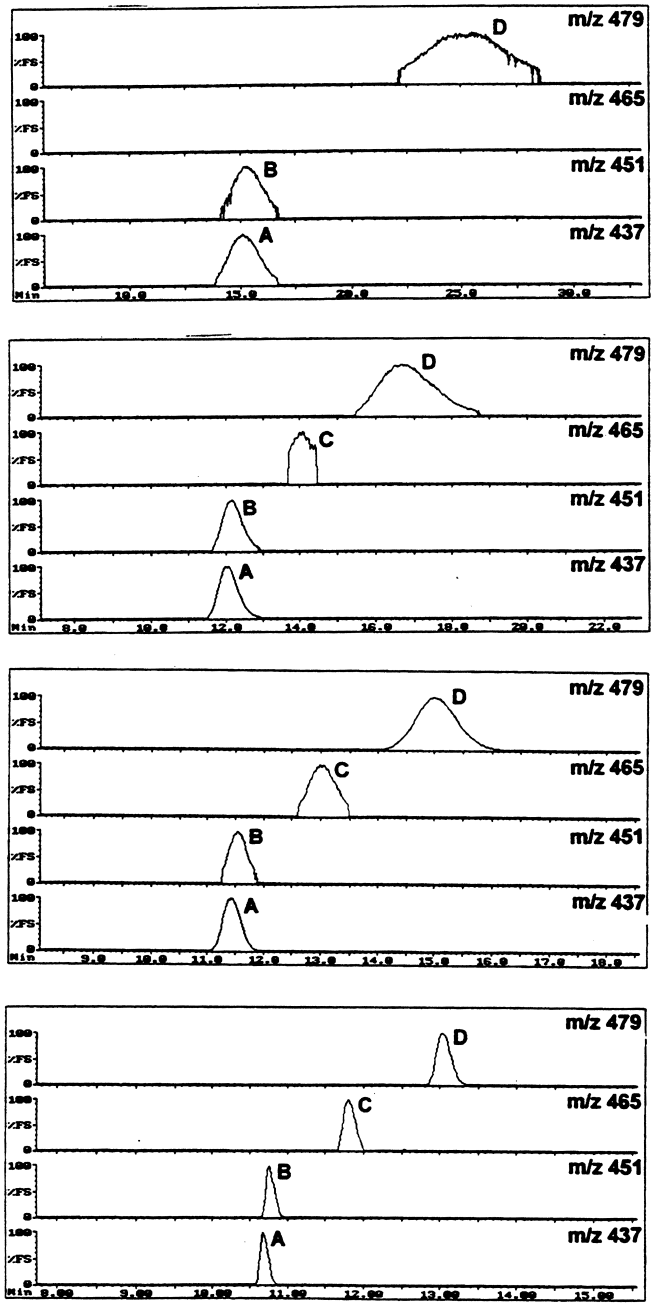

Fig. 3. Mass chromatograms of the PFB derivative of mycocerosic acids at 150°C (upper), at 180 and 200°C (center) and at 240°C (lower). Peak identities: (A) C_{29} mycocerosic acid; (B) C_{30} mycocerosic acid; (C) C_{31} mycocerosic acid and (D) C_{32} mycocerosic acid.

ratio of four) by monitoring at m/z 88. However, the PFB derivative was superior as regards detection sensitivity in the NICI (ammonia) mode, rendering the C_{32} mycocerosic acid detectable when 2 pg were injected (signal-to-noise ratio of four) by monitoring the molecular specific ion at m/z 479, using an ion source temperature of 240°C. Both the methyl ester and PFB derivatives of the acids were found to be chemically stable because no degradation (i.e. no changes in the chromatograms) was observed after two weeks of storage at 4°C.

Variations in ion source temperature did not affect the detection of methyl ester or TMS derivatives of the mycocerosic acids when using the EI mode. In contrast, when using NICI, detection of the PFB derivatives was noticeably affected. Thus, at an ion source temperature of 150°C, the integrity of the peaks was greatly affected, thus causing problems in the elution of mycocerosic acids. However, as the temperature increased from 150 to 240°C, peak integrity and the detection of mycocerosic acids improved significantly (Fig. 3). An ion source temperature of 240°C was the highest tested.

In order to obtain optimal sensitivity, the mycocerosic acids in sputum samples were analysed as PFB derivatives on GC–MS in NICI mode. The molecular ions at m/z 437, 451, 466 and 479, corresponding to C_{29}, C_{30}, C_{31} and C_{32} mycocerosic acids, were monitored in the analysis of sputum samples. As in cultures, all mycocerosic acids ranging from 29 to 32 carbon atoms were detected in the sputum samples. No mycocerosic acids were de-

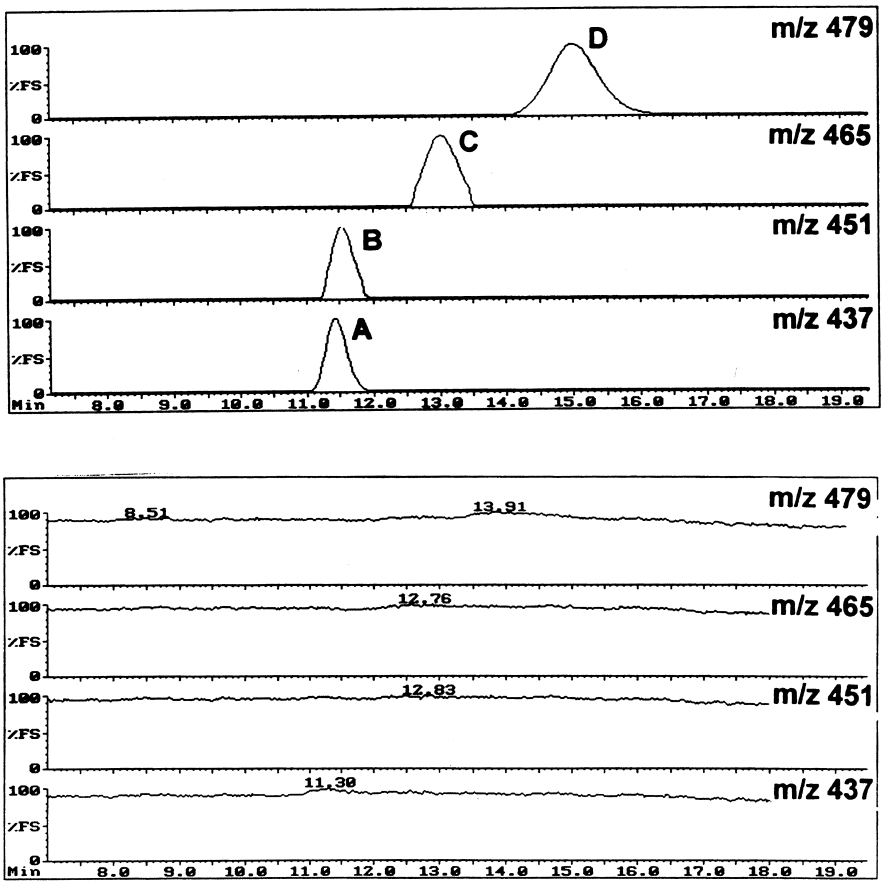

Fig. 4. Mass chromatogram of the PFB derivative of mycocerosic acids found in a culture-positive sputum sample (upper) and in a culture-negative for *M. tuberculosis* (lower).

tected in any of the culture-negative sputum samples (Fig. 4).

From our studies, it is clear that PFB derivatives should be preferred for the trace detection of mycobacterial mycocerosic acids by GC–MS, due to the high sensitivity that can be obtained in the NICI mode, at a high ion source temperature. As an hydrolysis time of 1 h was suitable for the complete release of all of the mycocerosic acids studied, the method is also rapid. The large scale evaluation of this method over a wide number of sputum specimens from patients with known or suspected pulmonary tuberculosis is currently in progress.

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