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ANALYSIS OF *IN SITU* METHYLATED MICROBIAL FATTY ACID CONSTITUENTS BY CURIE-POINT PYROLYSIS–GAS CHROMATOGRAPHY–MASS SPECTROMETRY

G. HOLZER*, T. F. BOURNE
School of Applied Biology, Georgia Institute of Technology, Atlanta, GA 30332 (U.S.A.)
and
W. BERTSCH
Department of Chemistry, University of Alabama, University, AL 35486 (U.S.A.)

SUMMARY

A newly developed Curie-point pyrolyzer has been used to analyze microbial fatty acid constituents. The pyrolysis was carried out on ferromagnetic wires, which were loaded into the gas chromatographic injector–pyrolyzer via a retractable Pyrex glass tube. Fatty acid methyl esters were obtained by *in situ* methylation of the free and glycerol bound fatty acids with trimethylanilinium hydroxide. The procedure was applied sucessfully to whole microorganisms. There was good agreement between the fatty acid methyl ester profiles from the whole-cell pyrolysis and from the lipid extracts of the corresponding organisms.

INTRODUCTION

Pyrolysis (Py)–gas chromatography (GC) and Py–GC–mass spectrometry (MS) have developed into powerful techniques for the analysis of non-volatile synthetic polymers and complex biological materials[1]. Thermal degradation of these substances generate a large variety of decomposition products, which range from chemically related isomers to compounds that differ in polarity, structure and molecular mass. Such a multitude of fragments is desirable if chromatographic profiles are used to characterize biopolymers or whole microorganisms; however, the diverse chemical nature of the decomposition products demands rigid standards for the analytical system.

Characterization of microorganisms by pyrolysis profiles requires strict control of factors such as culture medium, pH, physiological age of cells, temperature, isolation technique and pyrolysis conditions[2]. However, even standardized procedures often fail to maintain good reproducibility of the pyrolysis patterns of whole microorganisms. Better results have been obtained when the characterization was based on the composition of cellular components, such as the cell wall fraction or membrane constituents. Pyrolysis patterns of these cellular subfractions provide more

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definitive characteristic information for taxonomic comparison. However, the problem with this approach is that the sample preparation prior to analysis is quite extensive.

One of the most popular signature components of microorganisms are cellular fatty acids[3,4]. Fatty acid methyl ester profiles have been used successfully in the classification of microorganisms[4,5], in ecological studies aimed at defining microbial community structures[6], in the typing of depositional environments[7], and as specific markers in tracing the origin of organic materials in deposits[8,9]. The analysis of fatty acids is generally preceded by a sample preparation, which includes the extraction of the lipids with organic solvents, followed by the hydrolysis of the saponifiable glycerides. The free fatty acids are then converted into their corresponding methyl esters[10]. The volatile nature of these esters allows the direct analysis by GC.

The extensive sample preparation necessary for this type of analysis, led us to investigate the release of cellular fatty acids from whole microorganisms by Py–GC techniques. Since chromatographic separation of fatty acid methyl esters is preferred over the chromatography of free acids, a number of methylating reagents for *in situ* derivatization of free and glycerol-bound fatty acids during Py–GC were investigated. We report here the use of trimethylanilinium hydroxide[11] as an additive to whole cells prior to pyrolysis.

A Curie-point pyrolysis unit was selected over the conventional, filament type pyrolyzer. The unit has a low dead volume, which is important for maintaining a high concentration of the derivatizing agent during pyrolysis and ensures a fast removal of the reaction products on to the chromatographic column. The disposable ferromagnetic filaments used in this type of pyrolyzer, are easily loaded with sample and can be heated to final temperatures ranging from 360 to 770°C in less than 0.5 s. Optimum conditions for sample derivatization and transfer on to the column can be fine-tuned by split flow adjustment.

EXPERIMENTAL

Lipid standards were obtained from Sigma (St. Louis, MO, U.S.A.) and from Supelco (Supelco Park, Bellefonte, PA, U.S.A). A 1% solution of trimethylanilinium hydroxide (TMAH) in methanol, (Eastman-Kodak, Rochester, NY, U.S.A.) was used as the reagent for *in situ* methylation. The following microorganisms were grown on tryptic soy agar (Difco Lab., Detroit, MI, U.S.A.) at 37°C: *Escherichia coli*, *Enterobacter aerogenes*, *Micrococcus luteus*, *Pseudomonas aeruginosa*, *Pseudomonas maltophilia* and *Staphylococcus aureus*. Cells were harvested after 48 h by scraping the cells off the agar surface with an inoculating loop. Fatty acid extracts of the microrganisms were prepared according to the procedures described by Moss[3] and Miller[12].

A schematic diagram of the Curie-point pyrolysis apparatus is shown in Fig. 1. The system, which is similar to the prototype used in a study by Meuzelaar *et al.*[13], was provided by Somatogenetics Instruments (Broomfield, CO, U.S.A.). It is equipped with a 75 W/400 kHz radiofrequency (RF) power supply. The samples were deposited on disposable ferromagnetic filaments that have Curie points ranging from 360 to 770°C (Philips Electronics Instruments, Mahwah, NY, U.S.A.). The filament was placed into a 1.5 mm I.D. Pyrex glass tube, which was then inserted into the injection port. During this procedure, the backflush valve was open to prevent air from entering

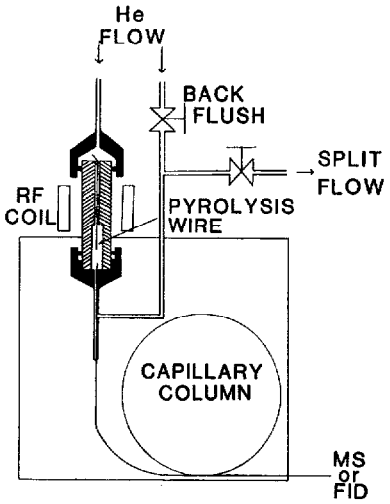

Fig. 1. Schematic diagram of the Curie-point Py–GC system.

the chromatographic system. The valve was closed after 30 s, and the pyrolyzer was fired for 10 s.

The separation was achieved on a 7.2 m × 0.2 mm I.D. capillary column, coated with 0.11 μm of DB5 (J&W Scientific, Folsom, CA, U.S.A.), using a HP 5890 gas chromatograph, equipped with a flame-ionization detector and HP 5970 series mass-selective detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). The mass-selective detector was operated at 70 eV. Electron impact (EI) mass spectra were collected from 40 to 400 a.m.u. at a rate of 0.74 scans per s. The column temperature was programmed from 100 to 250°C at 8°C/min and at 20°C/min to 300°C, using a helium pressure of 90 kPa. The injector was kept at 290°C, and the split ratio was adjusted for optimum chromatographic performance.

RESULTS AND DISCUSSION

One of the requirements for the pyrolytic release of cellular fatty acids from whole microorganisms is the preservation of the structural integrity of the com-

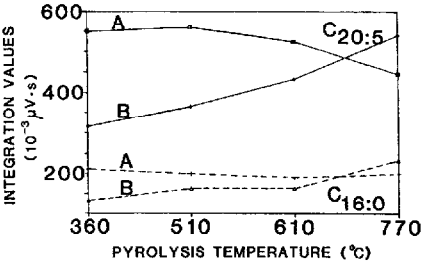

Fig. 2. Desorption efficiency of $C_{16:0}$ and $C_{20:5}$ fatty acid methyl esters at different pyrolysis temperatures. Curve A: power supply operated at full power (75 W), curve B: power supply operated at one-third power. Chromatographic conditions are described in the Experimental section.

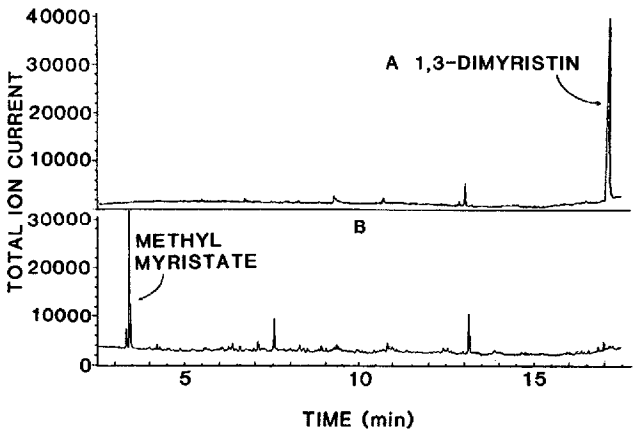

Fig. 3. Py–GC–MS of 1,3 dimyristin. (A) 1,3-dimyristin, (B) 1,3-dimyristin in presence of TMAH. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 610°C.

ponents. In addition, the method requires that the free as well as glyerol-bound fatty acids be liberated from the organisms as their corresponding methyl esters. Thus, desorption rather than thermal degradation of the fatty acid fraction is desired. These conditions could be established with the newly developed pyrolyzer, with its short heat-up rates, low dead volume, and fast removal of the released compounds on to the GC column.

First, the stability of several fatty acid methyl esters was examined under pyrolysis conditions. A mixture of methylpalmitate ($C_{16:0}$), methyleicosapentaeno-

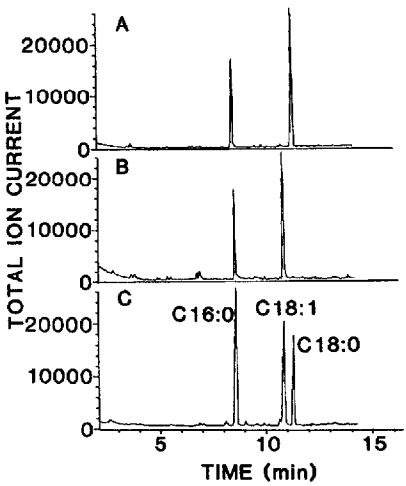

Fig. 4. Py–GC–MS of triglycerides and phosphoglycerides in presence of TMAH. (A) 1,2-distearoyl-3-palmitoyl glycerol–TMAH, (B) 1,3-dioleoyl-2-palmitoyl glycerol–TMAH, (C) phosphatidic acid–TMAH. The chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

ate($C_{20:5}$) and 2-hydroxymethylpalmitate was applied to a 610°C Curie-point wire in amounts ranging from 50 to 200 ng per component. The chromatographic analyses showed no evidence of thermal degradation. Recovery of the fatty acid methyl esters was quantitative, within the margin of error due to the application of the sample solution to the wire. Similar results were observed when the methyl esters of $C_{20:5}$ and $C_{16:0}$ were desorbed at different temperatures (Fig. 2). The higher final pyrolysis temperatures did not result in thermal degradation of the compounds examined. The effects of decreased power, which results in longer heat-up rates, was also examined with the same fatty acid methyl ester mixture. If operated at full power (75 W), the temperature rise time was less than 400 ms. Fig. 2 shows the desorption efficiency of the two fatty acid methyl esters at one-third power, which should result in a *ca.* three-fold decrease of the heating rate. The desorption efficiency at the highest pyrolysis temperature (770°C) was similar for the slow and fast heating rates; however, it dropped somewhat at lower pyrolysis temperatures when the heat-up rates were greater. The 75 W power supply produced consistent desorption efficiencies at different pyrolysis temperatures; however, for some applications a more powerful pyrolyzer may be desirable.

The reactions of TMAH with a number of saturated, unsaturated, and hydroxy fatty acids were investigated at different pyrolysis temperatures. Typically, 1 μl of a chloroform solution, containing the free fatty acids was transferred to the wire. After the solvent had evaporated, 10 μl of a 1% methanolic TMAH solution was applied. In all cases, the reaction with the corresponding methyl esters was complete. This was verified by the disappearance of the underivatized acids from the chromatogram. Hydroxy fatty acids were only methylated at the carboxylic function.

In a different set of experiments, the reaction between diacylglycerides, triacylglycerides, phosphoglycerides and TMAH was examined. The top tracing in Fig. 3 shows 1,3-dimyristin, analyzed by desorption from a 610°C Curie-point wire. The lower tracing was obtained after treating the wire with TMAH. The compound was identified by its mass spectrum as methylmyristate. The reaction was assumed to

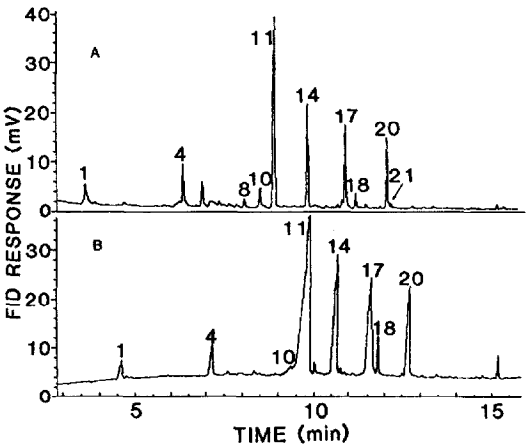

Fig 5. Py–GC–FID of *E. coli* cells. (A) *E. coli* cells in presence of TMAH, (B) *E. coli* cells. The names of the compounds are listed in Table I. The peaks in chromatogram B represent free fatty acids. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

TABLE I
PEAK IDENTIFICATION OF FATTY ACID METHYL ESTERS IN FIGS. 5–12

Peak No.	*Fatty acid methyl ester*	*Peak No.*	*Fatty acid methyl ester*
1	$C_{12:0}$	12	*iso*-$C_{17:0}$
2	2-OH-$C_{12:0}$	13	*anteiso*-$C_{17:0}$
3	3-OH-$C_{12:0}$	14	cyclopropyl-C_{17}
4	$C_{14:0}$	15	$C_{17:0}$
5	*iso*-$C_{15:0}$	16	*iso*-$C_{18:0}$
6	*anteiso*-$C_{15:0}$	17	$C_{18:1}$
7	$C_{15:0}$	18	$C_{18:0}$
8	3-OH-$C_{14:0}$	19	*iso*-$C_{19:0}$
9	*iso*-$C_{16:0}$	20	isopropyl-C_{19}
10	$C_{16:1}$	21	$C_{19:0}$
11	$C_{16:0}$	22	$C_{20:0}$

be quantitative, since the diacylglyceride was absent in the chromatogram. Similar experiments with distearoyl-palmitoyl glycerol, dioleoyl-palmitoyl glycerol and phosphatidic acid were carried out. The chromatograms in Fig. 4 show that the glycerides, pyrolyzed in the presence TMAH produced fatty acid methyl esters in ratios which corresponded to the actual distribution of these residues in the original molecules.

The flame ionization detection (FID) chromatograms in Figs. 5–11 are the fatty acid methyl ester profiles of a select group of microorganisms. The analyses were repeated using mass spectra detection, to confirm the identity of the fatty acid methyl esters. Fig. 5 shows the composition of the pyrolysis pattern of *E. coli* before and after the addition of TMAH. Previous experiments with acylglycerides showed no evidence

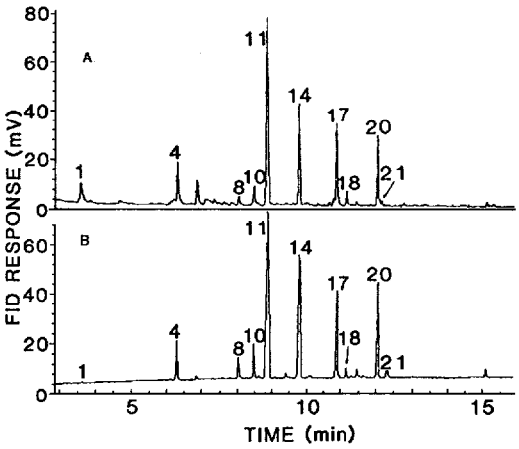

Fig. 6. Fatty acid methyl ester profiles of *E. coli* by Py–GC–FID. (A) *E. coli* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

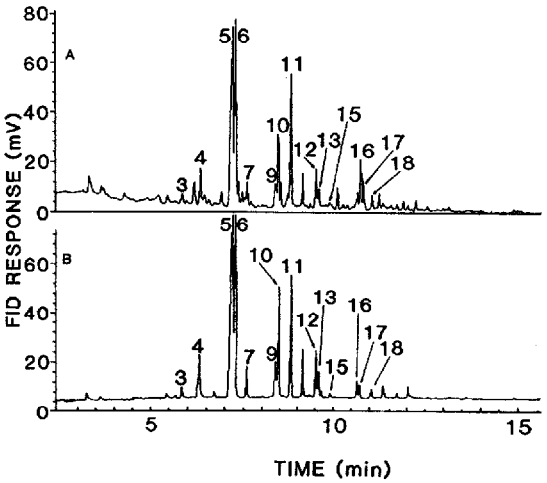

Fig. 7. Fatty acid methyl ester profiles of *P. maltophilia* by Py–GC–FID. (A) *P. maltophilia* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

of thermal degradation of these compounds to fatty acids. Thus, the free fatty acids, which are the major peaks in the bottom chromatogram, are actually part of the lipid content of the organism. The chromatogram above demonstrates the completeness of the reaction with TMAH to the corresponding methyl esters. The mixing of the reagent with the cells is important if consistent results are to be obtained. In a typical experiment, 1–5 mg of the cells are scraped from the agar plate. The cells were then suspended in 500 μl water and 5 μl of the suspension was transferred to the wire. During application, the wire was turned mechanically. Then, approximately 10 μl of

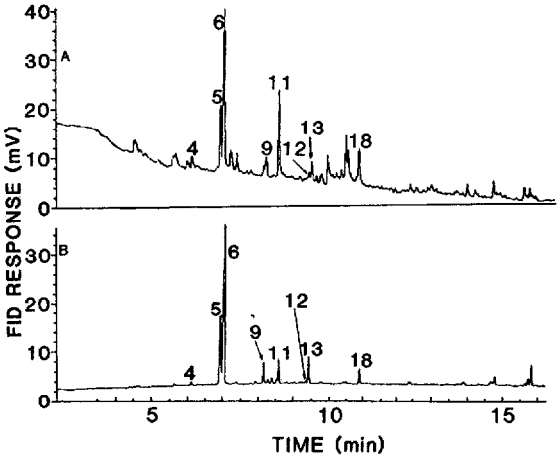

Fig. 8. Fatty acid methyl ester profiles of *M. luteus* by Py–GC–FID. (A) *M. luteus* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

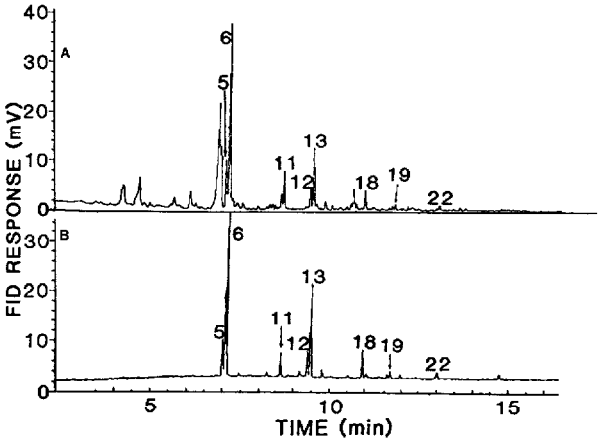

Fig. 9. Fatty acid methyl ester profiles of *S. aureus* by Py–GC–FID. (A) *S. aureus* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

the 1% methanolic TMAH solution was added to the turning wire and the aqueous methanol phase was removed by evaporation over a period of 5 min. This procedure deposited a uniform, thin film of cells on the wire and resulted in highly reproducible pyrograms. Mixing the reagent with water has no apparent effect on its reactivity with the complex lipids. The minimum number of cells necessary to obtain a well defined fatty acid methyl ester profile was determined from dry cell weights, as well as turbitity measurements. Pyrolysis of approximatey $5 \cdot 10^6$ cells produced a total ion chromatogram, which allowed mass spectral interpretation of components present at levels greater than 1% of the total amount of fatty acid methyl esters.

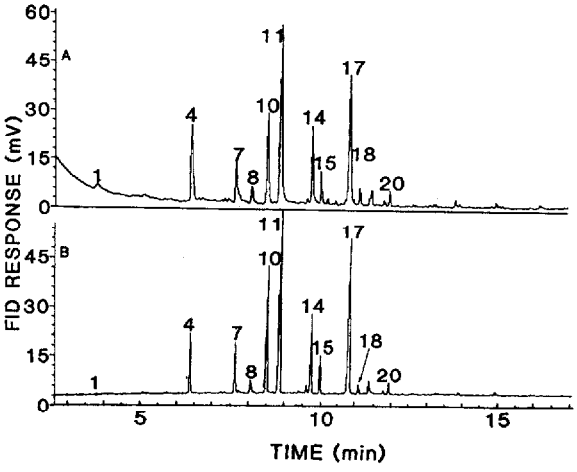

Fig. 10. Fatty acid methyl ester profiles of *E. aerogenes* by Py–GC–FID. (A) *E. aerogenes* cells/TMAH, (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

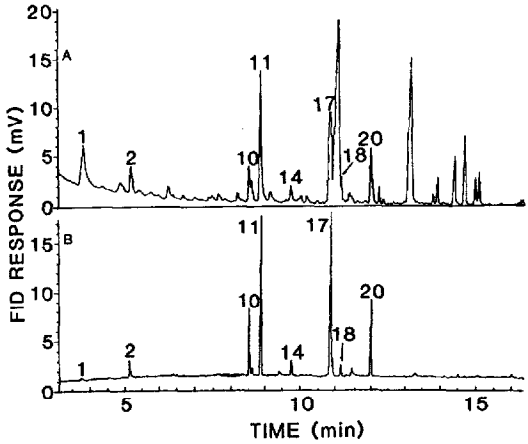

Fig. 11. Fatty acid methyl ester profiles of *P. aeruginosa* by Py–GC–FID. (A) *P. aeruginosa* cells/TMAH. (B) fatty acid methyl ester extract. The peaks are identified in Table I. Chromatographic conditions are described in the Experimental section; pyrolysis temperature 510°C.

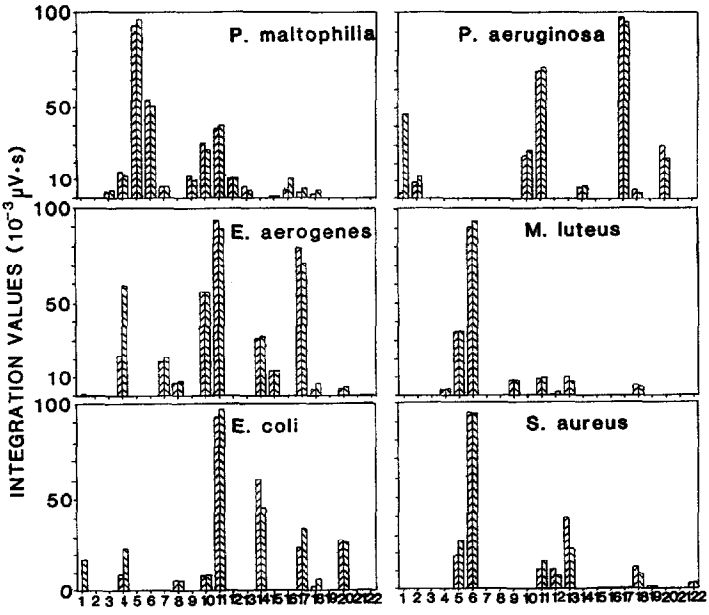

Fig. 12. Comparison of microbial fatty acid methyl ester profiles from whole cells and lipids extracts. Right-hand columns: whole cell pyrolysis in presence of TMAH; left-hand columns: fatty acid methyl ester extracts from the corresponding microorganisms. The original integration values have been multiplied by scaling factors for comparative representation. The fatty acids, identified by peak numbers, are listed in Table I.

Figs. 6–11 illustrate the potential of the method to generate characteristic fatty acid methyl ester profiles of microorganisms without prior extraction of the lipids. The top chromatogram in each figure was obtained by pyrolysis of whole microorganisms in the presence of TMAH. The lower profile represents the fatty acid methyl ester distribution of the hydrolyzed and extracted lipids of the same organism. Extraction and derivatization were performed as outlined in the Experimental section. The fatty acid methyl ester profiles obtained for all microorganisms by the two different methods appear very similar. Closer inspection shows that all of the fatty acids detected in the extract were also present in the pyrolyzates of the cells, including the cyclopropyl and hydroxy fatty acids. A peak-by-peak comparison of the fatty acid profiles from the whole-cell pyrolysis and from the lipid extract is shown in Fig. 12. The overall agreement between the two methods is very good, with the exception of the low-molecular-weight fatty acids, which are recovered at much higher yields during pyrolysis. These compounds may be lost during the sample preparation of the extracted lipids in a step that requires the complete evaporation of the solvent. The ratios of the remaining normal, branched and unsaturated fatty acids are in relatively good agreement.

Some variation between the two methods may be introduced by thermal degradation products which are eluted near a fatty acid methyl ester. Such peaks can be seen in the chromatograms of *P. maltophilia*, *P. aeruginosa* and *M. luteus*. Even though there is not much interference in these particular cases, mass spectral detection with single-ion displays, a longer column, and a stationary phase more suitable for this type of separation will most certainly improve the analysis.

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