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Direct Quantitation of Fatty Acids Present in Bacteria and Fungi: Stability of the Cyclopropane Ring to Chlorotrimethylsilane

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The stability of the cyclopropane ring and the fatty acid composition of microbial cells were determined using chlorotrimethylsilane as reagent with three different conditions 80 °C for 1 h, 60 °C for 1 h, and 60 °C for 2 h. Chlorotrimethylsilane permits a simultaneous extraction and derivatization of fatty acids. A basic method was used as reference. The bacteria, *Escherichia coli*, *Burkholderia cepacia*, and *Lactobacillus brevis*, and fungi *Aspergillus niger* and *Gibberella fujikuroi* were used. The stability of the cyclopropane ring on acidic conditions was tested using the cyclopropanecarboxylic acid and a commercial mixture of bacteria fatty acid methyl esters (BAME). Fisher's least significant difference test showed significant differences among the methods. The method using chlorotrimethylsilane and 1-pentanol for 1 h at 80 °C gave the best results in cyclopropane, hydroxyl, and total fatty acid recoveries. This procedure allows the fast and easy one-step direct extraction derivatization.

KEYWORDS: Chlorotrimethylsilane; fatty acids; gas chromatography; *Aspergillus niger*; *Burkholderia cepacia*; *Escherichia coli*; *Gibberella fujikuroi*; *Lactobacillus brevis*

INTRODUCTION

The study of the fatty acid compositions of bacteria and fungi is useful for taxonomic studies. The analysis of the lipids present in bacteria, the pattern of fatty acid methyl esters (FAMEs), and the presence of unusual membrane fatty acids have been extensively used to identify Gram-negative and Gram-positive bacteria, to differentiate species, and to differentiate strains in subgroups (*1, 2*). The fatty acid profile also has potential use as a bioprospecting tool to locate fungi capable of producing high value lipids, i.e., gamma-linolenic acid and arachidonic acid (*3*). Moreover, commercially available microbe libraries based on gas−liquid chromatography (GLC) analysis of fatty acid composition have been used extensively in microorganism taxonomy when grown and when harvest conditions are well established (*4–6*).

The analysis of fatty acids also helps to explain the physiological and biological activities of microorganisms. The analysis of endogenous cell fatty acid composition, phospholipids, and other membrane lipids allows the selection of suitable growth factors, to study enzymatic activity, and to establish oxygen tolerance (*7, 8*). Among these fatty acids, those based on cyclopropane have an important role as constituents of membrane phospholipids. The role of these compounds in the acid shock response in *Salmonella enterica* has been studied using gas−liquid chromatography coupled mass spectrometry (GLC-MS) data (*9*). The same method was used to establish the influence of these acids in solvent tolerance and the survival of *Pseudomonas putida* after lyophilization (*10*). Fatty acid composition analysis has also led to the discovery of new fatty acids with possible new applications (*11*). Moreover, the effect of fatty acids biosynthesis inhibitors can be monitored by fatty acid composition studies (*12*).

Because the study of fatty acid composition in bacteria and fungi presents some special features, standard analytical methods have been adapted to accommodate these particular requirements (*13*). To determine the fatty acid composition in acylglycerides, it is usual to perform a lipid extraction. Once extracted, the lipid sample should be derivatized before its characterization. Finally, the fatty acid composition can be determined by GLC or GLC-MS analysis (*14, 15*). Three factors are decisive in obtaining the quantitative recovery of each fatty acid: the extraction method, the derivatizing reagent, and the reaction conditions (*16, 17*). Cyclopropane acids, which are characteristic of some bacteria, are considered to be sensitive to strongly acidic conditions. Consequently, basic catalysis is an alternative method to analyze these acids (*18*). However, basic catalysis is not recommended for the analysis of free fatty acids (*19*). Methanolic HCl seems to satisfy both requirements. Consequently, it has been proposed as a system to derivatize both free fatty acids and cyclopropane acids (*20, 21*).

The aim of this study was to demonstrate the applicability of chlorotrimethylsilane and 1-pentanol as transesterification

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Table 1. Influence of the Reaction Conditions on the Percentage of Cyclopropanecarboxylic Ester Recovered[a]

	conditions			
	60 °C 1 h	60 °C 2 h	80 °C 1 h	*P* value
methyl ester	82.1 ± 1.8%[a]	77.2 ± 0.6%[b]	83.3 ± 1.0%[a]	0.002
pentyl ester	96.9 ± 4.3%[a]	94.7 ± 1.3%[a]	96.1 ± 0.6%[a]	0.766

[a] Different superscript letters (a,b) in the same row indicate significant differences among methods at the 95% level of significance according to Fisher's least significant difference test.

reagent and extracting solvent to the determination of fatty acid composition in a variety of microorganisms. Moreover, the behavior of cyclopropane fatty acids under these direct derivatization conditions was also studied. Because bacteria and fungi can generate different results, three bacteria and two fungi were chosen for this purpose. A method, extensively used in the analysis of FAC in microorganisms, based on a pretreatment with *N,N*-dimethylformamide (DMF) and methylation with methanolic sodium methoxide (*21*), was used as the reference.

MATERIALS AND METHODS

Bacteria and Fungi Strains. Culture and Harvest. Three bacterial strains, *Escherichia coli* (NCTC 9001, UdLTA 1.107), *Lactobacillus brevis* (CECT 216, UdLTA 1.41), and*Burkholderia cepacia* [*Pseudomonas cepacia*] (CECT 322, UdLTA 1.150), and two fungal strains, *Gibberella fujikuroi* (UdLTA 3.104) and *Aspergillus niger* (UdLTA 3.37), were obtained from the collection of the Microbiology Unit of the Food Technology Department, Lleida University (UdLTA).

The microorganisms were cultured under various conditions to obtain the necessary cell masses for analysis. *Escherichia coli* and *Burkholderia cepacia* were incubated in TSB broth (tryprone, 17 g/L; papaic digest of soybean meal, 3 g/L; glucose, 2.5 g/L; dipotassium phosphate, 2.5 g/L; and sodium chloride, 5 g/L at pH 7.3) for 48 h at 30 °C, *Lactobacillus brevis* in MRS broth (proteose peptone, 10 g/L; meat extract, 8 g/L; yeast extract, 4 g/L; glucose, 20 g/L; sodium acetate, 5 g/L; triammonium citrate, 2 g/L; magnesium sulfate, 0.2 g/L; manganese sulfate, 0.05 g/L; dipotassium phosphate, 2.0 g/L; and polysorbate 80, 1 g/L, pH 5.7) for 48 h at 30 °C, and *Gibberella fujikuroi* and *Aspergillus niger* in ME broth (malt extract, 20 g/L; peptone, 10 g/L; glucose, 20 g/L; pH 5.6) for 5 days at 25 °C, with orbital agitation.

Cell masses of bacteria were collected by centrifugation at 10000*g* for 15 min, and freeze-dried. Fungi were harvested by vacuum filtration using a Büchner funnel and freeze-dried. Bacteria and fungi were stored at −30 °C until lipid analysis.

Esterification of Cyclopropanecarboxylic Acid. A mixture of 1.5 mmol of cyclopropanecarboxylic acid, 50 mmol of either methanol or 1-pentanol, and 3.5 mmol of chlorotrimethylsilane were added to a Teflon screw-cap reaction vial filled with nitrogen. The vials with the reaction mixture were heated and magnetically stirred under three different conditions: (a) at 60 °C for 1 h, (b) at 60 °C for 2 h, or (c) at 80 °C for 1 h. After cooling, the mixtures were diluted with diethyl ether and neutralized with saturated sodium bicarbonate solution. The organic solutions were dried over anhydrous magnesium sulfate, and 2 mL of tridecane solution (1 mg/mL in isooctane) was added as an internal standard (IS). All of the experiments were carried out in triplicate. Compound purity was assessed by thin layer chromatography (TLC) and GLC-MS. Methyl and pentyl cyclopropanecarboxylate yields were calculated by GLC using the IS.

Transesterification with Sodium Methoxide (Method A). The procedure, developed in previous studies (*21, 22*), consists of the pretreatment of samples with *N,N*-dimethylformamide, followed by derivatization with sodium methoxide in methanol. Butylated hydroxytoluene was added as an antioxidant, and all assays were carried out in quintuplicate.

Transesterification with Chlorotrimethylsilane. The samples were derivatized, without previous pretreatment, according to the previously described method introducing few modifications in temperature and time reaction (*23*). The following three different conditions were used for each sample: mixture heated at 80 °C for 1 h (method B); mixture heated at 60 °C for 1 h (method C); mixture heated at 60 °C for 2 h (method D). Butylated hydroxytoluene was added as antioxidant, and all assays were carried out in quintuplicate.

Transesterification of a Commercial Mixture of BAMEs. BAME mix (Supelco (Madrid, Spain) in methyl caproate (800 μL) was transesterified to pentyl esters using method B. The sample was analyzed by GLC-MS.

The results were compared with those obtained with GLC-MS from the remaining commercial mix (0.2 mL).

Free Fatty Acid Analysis (Method E). Triundecanin (1 mL of 500 μg/mL) chloroform solution was added to a previously weighed 15 mL reaction vial fitted with a poly(tetrafluoroethylene) (PTFE)-lined cap. The solvent was evaporated using a dry nitrogen stream. The sample (100 mg), butylated hydroxytoluene (2 mg), and 2 mL of *N,N*-dimethylformamide were then added to each reaction vial. The reaction vials were filled with nitrogen and heated on a stirring block at 100 °C for 30 min. After cooling, 20 mg of sodium chloride and 1 mL of 3.5% hydrochloric acid were added to each vial. The vials were vortexed, extracted with 2 mL of a hexane-diethyl ether (1:1) mixture, and centrifuged at 822*g* for 20 min.

The upper layers were recovered and dried over anhydrous magnesium sulfate. Each sample was analyzed by GLC-flame ionization detection (FID) and GLC-MS, using triundecanin as the IS. All assays were carried out in quintuplicate.

Chromatographic and Statistical Analysis. Quantitative analysis of the methyl and pentyl esters from the bacteria and *Aspergillus niger* samples was carried out using a ThermoQuest Trace 2000 series GC with an FID detector. The analytical column was a 30 m × 0.25 mm fused silica capillary coated with a 0.20 μm film of poly(80% biscyanopropyl−20% cyanopropylphenyl siloxane) (SP-2330; Supelco, Madrid, Spain). The oven temperature was programmed to 100 °C for the first 5 min, then increased to 220 at 5 °C/min, and then held isothermally at 220 °C for 4 min. A 1:20 split injection ratio was used. Helium was used as the carrier gas at a constant flow of 1 mL/min. The injection volume was 1 μL. The injection system and the FID system were held at 270 °C.

The esters from *Gibberella fujikuroi* were analyzed using a Hewlett-Packard 6890 GC with an FID detector and a 105 m × 0.25 mm fused silica capillary column coated with a 0.20 μm film of poly(90% biscyanopropyl−10% cyanopropylphenyl siloxane) (RTX 2330; Restek, Teknokroma, Sant Cugat del Valles, Spain). The oven temperature was set to 100 °C for the first 10 min and was then increased to 240 at 3 °C/min and held for 30 min. The injection parameters were a 1:20 split injection ratio, H_2 as the carrier gas at a constant flow of 2 mL/min, and an injection volume of 1 μL. The injection system and the FID system were held at 270 °C.

Free fatty acids were analyzed using a Hewlett-Packard 6890 with an FID detector and a 30 m × 0.25 mm fused silica capillary column coated with a 0.25 μm film of treated polyethylene glycol (BP-21; SGE, CromLab S.L., Barcelona, Spain). The oven was programmed to increase in temperature from 90 to 240 °C at 12 °C/min and was then held isothermally at 240 °C for 12 min. A 1:20 split injection ratio was used, with helium as the carrier gas at a flow of 1 mL/min. The injection volume was 1 μL. The injection system and the FID system were held at 260 °C.

Qualitative analysis was performed with GLC-MS using an Agilent 6890N GC coupled to a 5973 Mass Selective Detector. The analytical column was an SP-2330, as described above. The chromatographic parameters were identical to those used for the analysis of the bacteria and *Aspergillus niger*.

The response factors of methyl esters of commercially available saturated and unsaturated fatty acids were calculated under the same chromatographic conditions as those indicated above. The response factors of pentyl esters were also calculated from the corresponding esters obtained from 1-pentanol and each commercially available fatty acids (*23*).

Table 2. Amounts of Fatty Acids in Bacterial Samples According to Different Methods

	method[a] (mg fatty acid[b]/g bacterial sample)					
	A	B	C	D	E[c]	*P* value
			Escherichia coli			
C12:0	0.4[a]	0.6[b]	0.2[c]	0.4[d]	0.1	<0.001
C14:0	3.2 ± 0.1[a]	4.4 ± 0.1[b]	2.9 ± 0.2[c]	3.5 ± 0.1[d]	0.2	<0.001
C15:0	0.6[a]	1.1[b]			<0.1	<0.001
C16:0	18.5 ± 0.4[a]	22.6 ± 1.0[b]	17.0 ± 1.0[c]	20.3 ± 0.5[d]	0.3 ± 0.1	<0.001
C16:1[d]	1.0[a]	1.9 ± 0.3[b]	0.6[c]	0.7[c]	<0.1	<0.001
C17:0	0.2[a]	0.4 ± 0.1[b]	0.2[a]	0.3[b]	0.1	<0.001
9.10-C16:0$^{\Delta}$[e]	16.0 ± 0.3[a]	16.0 ± 0.6[a]	14.1 ± 0.8[b]	16.3 ± 0.5[a]		<0.001
C18:0	0.2[a]	0.3 ± 0.1[a]	0.2[b]	0.2[a]	0.1	0.110
C18:1[d]	12.3 ± 0.6[a,b]	12.9 ± 0.5[a]	11.9 ± 0.1[b]	12.4[a,b]	0.2 ± 0.1	0.020
C18:2		<0.1				
9.10-C18:0$^{\Delta}$[f]	11.4 ± 0.7[a]	11.5 ± 0.3[a]	10.0 ± 0.6[b]	11.4 ± 0.3[a]	0.1	<0.001
3-OH-C14:0[g]		1.7 ± 0.1[a]	0.3[b]	1.4[c]	0.1 ± 0.1	<0.001
total fatty acids	63.9 ± 1.0[a]	73.5 ± 1.0[b]	57.4 ± 1.8[c]	67.0 ± 1.4[d]	1.2 ± 0.3	<0.001
			Burkholderia cepacia			
C14:0	0.8 ± 0.1[a]	0.4[b]	1.2 ± 0.1[c]	1.5 ± 0.2[d]		<0.001
C16:0	10.8 ± 0.6[a]	19.1 ± 0.6[b]	14.4 ± 0.2[c]	17.5 ± 0.7[d]	0.1	<0.001
C16:1[d]	7.4 ± 0.2[a]	10.1 ± 0.3[b]	9.4 ± 0.3[c]	11.4 ± 0.5[d]	0.1	<0.001
9.10-C16:0$^{\Delta}$[f]	3.6 ± 0.1[a,b]	3.4 ± 0.2[a]	3.8 ± 0.1[b]	4.5 ± 0.2[c]	1.2 ± 0.3	<0.001
C18:0	0.7[a]	2.0 ± 0.1[b]	0.9 ± 0.4[a]	1.1 ± 0.1[a]		<0.001
C18:1[d]	10.6 ± 0.4[a]	17.5 ± 0.3[b]	13.0 ± 0.2[c]	15.8 ± 0.3[d]	1.7 ± 1.0	<0.001
C18:2	0.1[a]	0.4[b]	0.3[b]	0.4[b]		<0.001
9.10-C18:0$^{\Delta}$[f]	1.2[a]	1.1[a]	1.2 ± 0.1[c]	1.4 ± 0.1[b]	0.4 ± 0.3	<0.001
3-OH-C14:0[g]	<0.1[a]	1.2 ± 0.1[b]	0.4 ± 0.2[c]	1.2 ± 0.1[d]		<0.001
total fatty acids	35.3 ± 0.9[a]	55.2 ± 1.2[b]	44.5 ± 1.2[c]	56.7 ± 1.3[b]	3.5 ± 1.7	<0.001
			Lactobacillus brevis			
C14:0		0.7[a]	0.2[b]	0.3[b]		<0.001
C15:0		0.2[a]	0.1[a]	0.1[a]		0.172
C16:0	3.3[a]	3.8 ± 0.1[b]	3.1[a]	3.5 ± 0.2[c]	0.2	<0.001
C16:1[d]	0.2[a]	0.2[a]	0.2[a]	0.2[a]		0.647
C17:0		<0.1				
9.10-C16:0$^{\Delta}$[f]	0.1[a]	0.1[a]	<0.1[b]	<0.1[a,b]		0.013
C18:0	0.1[a]	0.7 ± 0.1[b]	0.2[c]	0.3[c]	<0.1	<0.001
C18:1[d]	3.3 ± 0.1[a]	4.5 ± 0.1[b]	2.4 ± 0.1[c]	2.9 ± 0.4[c]	0.1	<0.001
9.10-C18:0$^{\Delta}$[f]	11.2 ± 0.2[a]	7.4 ± 0.1[b]	8.6 ± 0.2[c]	9.6 ± 1.4[d]	<0.1	<0.001
total fatty acids	18.2 ± 0.2[a]	17.7 ± 0.4[a]	14.9 ± 0.3[b]	17.0 ± 2.1[a]	0.3 ± 0.1	0.001

[a] Standard deviations not presented are less than 0.1. [b] Expressed as acylglycerides except in method E. [c] Expressed as free fatty acid. [d] Undifferentiated *cis*−*trans* isomers. [e] 9,10-Methylenehexadecanoic acid. [f] 9,10-Methyleneoctadecanoic acid. [g] 3-Hydroxytetradecanoic acid. Different superscript letters (a,b,c,d) in the same row indicate significant differences among methods at the 95% level of significance according to Fisher's least significant difference test.

Table 3. Comparison of Bacterial Acid Pentyl Esters and Commercial BAMEs after the Application of Method B

fatty acid ratio	BAMEs	bacterial acid pentyl esters	% difference
C18:0/9,10-C16:0$^{\Delta}$	1.123	1.221	8.7
C18:0/9,10-C18:0$^{\Delta}$	1.064	1.145	7.6
C18:0/C18 unsat[a]	0.361	0.345	4.4
C18:0/3-OH-C14:0	1.277	1.447	13.1
C18:0/C16:0	1.067	0.976	8.5

[a] C18 unsat: *cis*-9-C18:1, *trans*-9-C18:1, *cis*-9,12-C18:2.

Microsoft Excel 2003 for Windows was used to process the data.

RESULTS AND DISCUSSION

To determine the stability of the cyclopropane ring when chlorotrimethylsilane was used, commercially available cyclopropanecarboxylic acid was esterified to methyl and pentyl esters. The reaction was carried out using the methods B, C, and D.

Table 1 shows that all three methods allowed the esterification of carboxylic acid with both alcohols. However, the recovery of the methyl ester was poorer than the recoveries of the pentyl ester. These differences could be explained by the higher polarity and lower boiling point of the methyl ester. These parameters could affect the recovery of the esters in the neutralization step (*24*). However, the high recoveries found in all cases show that the cyclopropane ring is stable under all of the conditions tested.

Once the stability of the cyclopropane acids was established, the total fat and fatty acid composition of the bacterial samples were determined using each method. Pretreatment and derivatization with the sodium methoxide (method A) was selected as the reference method because it has shown good performance in the recovery of cyclopropane fatty acid (*21*).

Table 2 shows the results obtained after the application of each method to the three bacteria studied. The amount of acylglycerides was measured as milligrams of total acylglycerides per gram of sample for each bacterium. Fisher's Least Significant Difference test showed significant differences among the methods at the 95% level of significance. Considering that method B did not differ significantly from methods A and D in *L. brevis* with this test, it is reasonable to assert that method B gave the highest fatty acid concentrations with the three bacteria assayed. Method C gave the lowest fatty acid concentrations. The results of reference method A were not significantly different from those of method B for *L. brevis*. However, the fatty acid composition determined by this method was significantly lower than the value obtained with method B for *E. coli* and *B. cepacia*. These differences among the methods could be caused by the conversion of free fatty acids to sodium salts

Table 4. Total Amount of Fatty Acids in Fungal Samples

	method (mg fatty acid[a]/g fungal sample)		
	A	B	*P* value
	Gibberella fujikuroi		
C14:0	0.8 ± 0.1^a	1.4 ± 0.1^b	<0.001
C16:0	29.3 ± 2.9^a	43.8 ± 2.9^b	<0.001
C18:0	10.5 ± 1.1^a	15.7 ± 1.0^b	<0.001
C18:1[b]	34.4 ± 3.5^a	43.9 ± 3.4^b	0.002
C18:2[b]	33.2 ± 3.2^a	41.5 ± 3.2^b	0.003
C18:3	1.3 ± 0.1^a	1.5 ± 0.2^a	0.062
C20:0	0.7 ± 0.1^a	1.1 ± 0.1^b	<0.001
C22:0	0.6 ± 0.1^a	1.0 ± 0.1^b	<0.001
C24:0	0.9 ± 0.1^a	1.8 ± 0.1^b	<0.001
total fatty acids	111.7 ± 11.1^a	151.7 ± 11.0^b	<0.001
	Aspergillus niger		
C15:0	0.1^a	0.3^b	<0.001
C16:0	3.5 ± 0.2^a	6.0^b	<0.001
C17:0	0.1^a	0.4^b	<0.001
9.10-C16:0$^{\Delta}$[c]	0.1^a	0.4^b	<0.001
C18:0	1.6 ± 0.1^a	2.4^b	<0.001
C18:1[b]	7.7 ± 0.2^a	6.6 ± 0.3^b	<0.001
C18:2[b]	8.6 ± 0.2^a	7.1 ± 0.3^b	<0.001
C18:3 + C20:0[d]	1.4^a	0.9^b	<0.001
total fatty acids	23.2 ± 0.7^a	24.1 ± 0.6^a	0.056

[a] Expressed as total acylglycerides. [b] Undifferentiated *cis*−*trans* isomers. [c] 9,10-Methylenehexadecanoic acid. [d] Undifferentiated. Different superscript letters (a,b) in the same row indicate significant differences among methods at the 95% level of significance according to Fisher's least significant difference test.

when the sodium methoxide method was used. However, when the total free fatty acids were determined (**Table 2**), very low amounts of free fatty acid were found in *E. coli* (1.2 ± 0.4 mg/g sample) and *L. brevis*. (0.3 ± 0.07 mg/g sample), whereas *B. cepacia* had a higher amount (3.5 ± 0.1 mg/g sample). Nevertheless, this amount represented less than 1% of the whole amount of the acylglycerides present in *B. cepacia.* These results indicate that free fatty acids are irrelevant in determining the total acylglycerides in these samples. Therefore, it seems that differences among the derivatization methods cannot be attributed to the presence of free fatty acid in the microorganisms studied but to the method used. It is more likely that these differences result from the diversity of fatty acids present in each microorganism and the conditions of each method tested.

The amount of each individual fatty acid was expressed as milligrams of theorycal acylglyceride per gram of sample for each bacterium (**Table 2**). Method B again produced the best recovery of most of the saturated and unsaturated fatty acids, whereas methods A and C gave the poorest recoveries. Nevertheless, the percentages of 9,10-methylenehexadecanoic acid and 9,10-methyleneoctadecanoic acid present in *E. coli* and *B. cepacia* showed no significant difference among methods A and B at the 95% level. For *L. brevis*, method A showed the best recovery for 9,10-methyleneoctadecanoic acid. Method C generally showed the poorest recovery. These results could be attributable to the fact that method C is the shortest procedure, considering its derivation period. Moreover, it is performed at the lowest temperature. It seems that either a higher temperature or extra time were required to efficiently extract and transform the acylglycerides present in this bacteria.

Certain basic catalyses, used in several studies, are commonly considered the best methods for bacterial fatty acid analysis (*12*). It has been assumed that cyclopropane is more stable under basic conditions than under acidic conditions (*25*). However, the three chlorotrimethylsilane methods assayed allowed the determination of cyclopropane acids. The concentrations determined for these acids were equivalent to or higher than those determined using the reference basic method. Moreover, 3-hydroxytetradecanoic acid was determined using the chlorotrimethylsilane methods, whereas the results of basic catalysis with methanolic sodium methoxide were poor.

Our results are consistent with those of several similar studies using acid catalysis. Lewis et al. (*16*) showed that direct derivatization with dry HCl/methanol was better in terms of total fatty acid present than a combination of extraction and HCl/methanol derivatization. Similar results were described when seven different methods were compared to analyze fatty acid composition in lyophilized lactic acid bacteria. In this case, the best method was the direct transesterification with a dry HCl/methanol solution (*21*). The same authors also used a method consisting of pretreatment with *N,N*-dimethylformamide followed by sodium methoxide/methanol transesterification. However, this method gave poorer results than the direct HCl/methanol method. Nikkila et al. (*26*) carried out a study similar to our own work. They compared the usefulness of sulfuric acid as the only catalyst in a direct derivatization in front of the combined basic−acid method as reference. Time and temperature were optimized. They reported that recoveries of cyclopropane acids were best when sulfuric acid was used under optimized conditions.

To confirm the applicability of the selected method, it was applied to the derivatization of a commercial BAME mixture of fatty esters from bacteria. A comparative assay was performed by splitting the commercial sample.

Table 3 shows the octadecanoic acid (C18:0):fatty acid ratios of the commercial BAMEs and bacterial acid pentyl esters prepared from this bacterial fatty acid mix using method B. Fatty acids were chosen according to their putative sensitivity to the derivatization conditions. Differences in percentages are also shown for each derivatized fatty acid.

After derivatization, small differences (<10%), calculated as percentages, were observed for 9,10-C16:0$^{\Delta}$, 9,10-C18:0$^{\Delta}$, C16:0, and the combined *cis*-9,12-octadecadienoate, *cis*-9-octadecenoate, and *trans*-9-octadecenoate acids. 3-Hydroxytetradecanoic acid showed a slightly higher difference (13.1%). This assay confirmed the stability of cyclopropane and hydroxyl fatty acids when method B was used.

Finally, although the analysis of fatty acid composition is more difficult in fungi than in bacteria (*27*), the applicability of method B to fungi was studied. *Gibberella fujikuroi* and *Aspergillus niger* were used, and method A was used as the reference method. **Table 4** shows the results obtained for the fungi using methods A and B. The individual concentrations of each fatty acid were expressed as milligrams of individual fatty acid per gram of fungal sample. Again, Fisher's least significant difference test showed that the total amounts of acylglycerides determined by method B, expressed as milligrams of total acylglycerides per gram of fungal sample, were either equivalent to or significantly higher than the values determined using reference method A.

As for most of the bacteria assayed, total fatty acids in *G. fujikuroi* was higher with the chlorotrimethylsilane method than with the sodium methoxide one. Both methods gave closer results when applied to *A. niger*, with C16:0 acid being the only exception. The significant difference between the two methods in *G. fujikoroi* could be attributable to the higher levels of saturated fatty acids in this fungus. In all cases, the recovery of saturated fatty acids was greater when method B was used. The recoveries of monounsaturated fatty acids and polyunsaturated fatty acids were similar.

We have demonstrated that chlorotrimethylsilane is a suitable reagent for the direct analysis of the acylglycerides present in

microorganisms. No extraction or pretreatment step is required, and the recoveries are either similar to, or higher than, those obtained with the sodium methoxide method. Method B involves fewer steps than the sodium methoxide method. Consequently, it allows the fast analysis of the total and individual fatty acids present in bacteria and fungi. The whole period analysis could be shortened using a rapid GC-FID method as recently proposed (*6*). Moreover, the method developed here can be used to identify and quantify the cyclopropane and hydroxy fatty acids present in the microorganisms studied.

Considering some of our previous results (*28, 29*), several alcohols can be used as the derivatizing agents. Consequently, the method could be used with trichloroalcohols to determine the presence of very low amounts of fatty acids in some microorganisms (*30*). It is worth remembering that using alcohols other than methanol improves the recovery of low-molecular-weight fatty acids (*26*).

Supporting Information Available: Stoichiometry correction factors and response factors relative to C11:0 for the chromatographic assayed conditions. Examples of chromatograms of fatty acids obtained with a ThermoQuest Trace 2000 series GC and Hewlett-Packard 6890 GC. This material is available free of charge via the Internet at http://pubs.acs.org.

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