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Comparison of three GC/MS methodologies for the analysis of fatty acids in *Sinorhizobium meliloti*: Development of a micro-scale, one-vial method

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ABSTRACT

Three protocols for fatty acid analysis in *Sinorhizobium meliloti* were improved by the addition of a number of standards/controls and a silylation step which allowed the determination of recoveries, extents of conversion of lipids to fatty acid methyl esters (FAMEs) and extents of side reactions. Basic hydrolysis followed by acid-catalyzed methylation and transmethylation with sodium methoxide, were the best for the analysis of 3-hydroxy- and cyclopropane fatty acids, respectively. A micro-scale, one-vial method that employed sodium methoxide/methanol was equally efficient and on a 1000-fold smaller scale than standard methods. Because this method avoids aqueous extractions, 3-hydroxybutanoic acid was detected as its trimethylsilyloxy methyl ester along with FAMEs.

1. Introduction

Fatty acid analysis in bacteria can be used as an approach to understand metabolic networks and the function of genes involved in fatty acid metabolism. The soil bacterium *Sinorhizobium meliloti* is found in root nodules and fixes nitrogen for plants such as alfalfa. The association between the bacterium *S. meliloti* and alfalfa is considered one of the leading model systems for nitrogen fixation and symbiosis studies [1,2]. This work was driven by the need for simple, comprehensive analytical fatty acid methods that could be applied in functional genomics studies of *S. meliloti*.

Fatty acid analyses are routinely performed by conversion of lipids into fatty acid methyl esters (FAMEs), typically followed by gas chromatographic analysis [3,4], although methods using high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have also been reported [5,6]. FAMEs are normally generated from lipids by either basic hydrolysis (saponification) followed by methylation of the free fatty acids or by acid or base-catalyzed transesterification of lipids [7]. Each of these approaches has their own advantages and disadvantages. First, it is often assumed that the transformation of lipids into FAMEs is quantitative and uniform across lipid classes. Second, recovery and internal standards are rarely used in these methods so there is no way to evaluate the completeness of transformation reactions or losses due to side reactions. Hydrolysis to afford fatty acids is widely considered an important side reaction in the preparation of FAMEs, due to the presence of traces of water, leading to poor recoveries of FAMEs; yet the extent of hydrolysis is rarely measured directly or taken into account [8–11]. Hydrolysis is reported to be the main cause of why well-established methodologies fail when sample size and reagent volumes are scaled down [12]. Third, the protocols for preparing FAMEs from lipids require one or more aqueous extraction steps to isolate FAMEs after methylation or transmethylation reactions [7,13–16]. Moreover, most methods have been developed and validated for applications in the food industry where sample size (10–100 mg) is not a limitation. Although recent pyrolitic methods using μg amounts of lipids have been reported, their application as routine procedures is limited since manual sample loading and special apparatus are required [17,18]. Finally, the analysis of bacterial fatty acids poses additional challenges due to the presence of labile hydroxylated and cyclopropane-containing fatty acids [3,8,19].

Fatty acid compositions of *S. meliloti* and other species of the genus *Rhizobium* have been used to establish taxonomic relationships between species and for the chemotaxonomic identification of unknown strains [20,21]. The fatty acid composition of *S. meliloti* was obtained using GC with flame ionization detection (GC/FID) based on the Sherlock Microbial Identification System (MIS) [21]. Fatty acid identification using this system is based solely on retention times since FID is a non-specific detector [22]. Additionally,

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fatty acid analyses were conducted using *S. meliloti* strain Rm1021, commonly used as wild type [20,21]. However, it has been demonstrated recently that wild type strain Rm1021 carried a single nucleotide C-deletion mutation in the *pstC* gene [23]. Thus, this mutation has been repaired in *S. meliloti* strain Rm1021 and the resulting strain, RmP110, is now used as a wild type [24,25]. Therefore, we used in our study the corrected *S. meliloti* wild type (strain RmP110) for which fatty acid composition has not been reported. We employed GC analysis with mass spectrometric detection (GC/MS) thus structural information of fatty acids in *S. meliloti* can be obtained. GC/MS analysis of fatty acids is preferred over GC/FID since the electron impact mass spectra of FAMEs provide structural information; thus identification can be conducted using retention times and mass spectral information [22,26].

In this study, we investigated the three most commonly used methodologies in the preparation of fatty acid methyl esters: (a) basic hydrolysis of lipids followed by acid-catalyzed methylation (employed by the MIS system), (b) acid-catalyzed transmethylation using sulfuric acid in methanol and (c) base-catalyzed transmethylation using sodium methoxide in methanol. The aims of the current work were: (1) to determine the best method for the analysis of bacterial fatty acids in *S. meliloti*, particularly cyclopropane fatty acids, (2) to determine the relative transformation efficiencies for major lipid classes for each method and (3) to introduce appropriate analytical controls to monitor derivatization efficiencies, recoveries and losses due to side reactions. An important motivation was to develop a small-scale, one-vial method for the analysis of bacterial fatty acids to avoid the need for tedious extraction and sample handling steps. A comparison of these methods was conducted with *S. meliloti* samples grown in the presence and absence of inorganic phosphate (P_i).

2. Experimental

2.1. Reagents and materials

Fatty acid standards ($C_{9:0}$–$C_{25:0}$) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were obtained from Sigma–Aldrich (Saint Louis, MO) and fatty acid methyl esters of saturated fatty acids ($C_{9:0}$–$C_{25:0}$) were obtained from Alltech Associates (Deerfield, IL). All solvents used were HPLC grade (Caledon Labs, Caledon, ON). Phospholipid standards [1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine (PC-19:0/19:0), 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine (PE-17:0/17:0), 1,1′,2,2′-tetramyristoyl cardiolipin (CL-14:0/14:0/14:0/14:0, sodium salt), 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (PG-12:0/12:0, sodium salt) were purchased from Avanti Polar Lipids (Alabaster, AL). *N*-octadecanoylsphingosylphosphorylcholine (SM-18:0/18:0) was obtained from MJS BioLynx (Brockville, ON). Glyceryl tri(hexadecanoate-d_{31}) was purchased from CDN Isotopes Inc. (Pointe-Claire, QC). 3-Hydroxy fatty acids (3-hydroxytetradecanoic acid (3-OH-$C_{14:0}$), 3-hydroxyhexadecanoic acid (3-OH-$C_{16:0}$), 3-hydroxyoctadecanoic acid (3-OH-$C_{18:0}$)) and their fatty acid methyl esters were obtained from MJS BioLynx (Brockville, ON). Cyclopropane-containing fatty acids, *cis*-9,10-methylenehexadecanoic acid (*cis*-9,10-$C_{17:0\ cyclo}$) and *cis*-9,10-methyleneoctadecanoic acid (*cis*-9,10-$C_{19:0\ cyclo}$) and their fatty acid methyl esters were obtained from MJS BioLynx (Brockville, ON) while *cis*-11,12-methylenoctadenoic acid (*cis*-11,12-$C_{19:0\ cyclo}$) was obtained from Cedarlane Laboratories Limited (Hornby, ON). 3-Hydroxybutyric acid and methyl 3-hydroxybutyrate were obtained from Sigma–Aldrich (Saint Louis, MO).

Sodium methoxide solutions (0.5 M) were prepared by dissolving a piece of freshly cut sodium in methanol as recommended by Christie [10]; the resulting solutions were stored in glass vials equipped with mininert® valves (Chromatographic Specialties, Brockville, ON) at 4 °C. The use of mininert® valves protected the solutions from atmospheric moisture, facilitating routine work and solution integrity for 2–3 months.

2.2. Quality control standards

Isopropyl tetradecanoate-d_{27} was synthesized from tetradecanoic-d_{27} acid (CDN Isotopes Inc., Pointe-Claire, QC) using sulfuric acid in isopropanol (3%, 2 mL) at 50 °C overnight as described by Christie [7]. Hexane (1 mL) and water (1 mL) were added simultaneously to the reaction mixture. The hexane layer was extracted twice more with water (1 mL); the hexane layers were combined, dried over anhydrous sodium sulfate and evaporated to dryness under a nitrogen stream. Solutions of isopropyl tetradecanoate-d_{27} (10 mg/mL) were prepared in hexane. A hexane solution containing isopropyl tetradecanoate-d_{27} (1000 ng/μL), 9-anthracenemethanol (Sigma–Aldrich, 500 ng/μL), n-eicosane (Sigma–Aldrich, 660 ng/μL), methyl tridecanoate (Sigma–Aldrich, 600 ng/μL) and methyl pentadecanoate (Sigma–Aldrich, 690 ng/μL) was prepared and an aliquot of this solution (typically 10–15 μL) was added to each sample (standards or dried bacterial lipid extracts) prior to analysis.

2.3. Bacterial cultures

Bacterial cultures (*S. meliloti,* RmP110) were grown in MOPs buffered minimal media with glucose (15 mM) as the carbon source in the presence (2 mM) or absence of inorganic phosphate (P_i). Wet cell pellets obtained from 500 to 1000 mL of culture (o.d. 0.4–0.6) by centrifugation were resuspended in 2.5 or 5 mL of medium, respectively, and divided into 250 μL aliquots in Eppendorf tubes. Each tube was centrifuged, the supernatant was discarded and the wet pellets flash frozen in liquid nitrogen and stored at −80 °C. The pellet equivalent to 50 mL of original culture, corresponded to 33.0 ± 4.0 mg wet weight of cells ($n = 15$).

2.4. Lipid extraction

Wet cell pellets were resuspended in distilled water (1 mL) and extracted with a mixture of $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform phase containing the lipids was separated and the aqueous phase was extracted twice more with $CHCl_3$:MeOH (1 mL, 2:1, v/v). The chloroform layers were combined and dried through a small column packed with anhydrous sodium sulphate. The solvent was evaporated using nitrogen gas and the dried lipid residue was either analyzed immediately or stored at −80 °C.

2.5. Methodologies for fatty acid methyl ester analysis

Table 1 summarizes the experimental conditions used in methods A, B, C and one-vial C. In method A, wet cells were spiked with the quality control standards (10 μL) and reacted with KOH in MeOH, followed by acid-catalyzed methylation as described by Jarvis and Tighe [20]. FAMEs were extracted three times using diethyl ether:hexane (1:1, v/v, 2 mL); the organic layers were combined, dried over anhydrous sodium sulphate and evaporated to near dryness under a gentle nitrogen stream. The dried samples were treated with MSTFA (25 μL) and dry pyridine (25 μL) at 37 °C for 30 min. An aliquot of the derivatization solution (5 μL) was diluted either 10-fold or 20-fold with hexane containing ethyl dodecanoate (25 ng/μL) as the internal standard; 1 μL was injected on-column for GC/MS analyses.

Table 1
Experimental conditions of methods A, B, C and C one-vial method

Experimental conditions	Method A	Method B	Method C	One-vial method C
Reactions	(1) Basic hydrolysis (2) Acid-catalyzed methylation (3) Silylation	(1) Acid-catalyzed transmethylation (2) Silylation	(1) Base-catalyzed transmethylation (2) Silylation	(1) Equal to method C (2) Silylation
Sample	Wet cells	Dried lipid extract	Dried lipid extract	Dried lipid extract
Reagents	(1) KOH (0.1 M, 1 mL)/CH_3OH (2) HCl (6 M, 1 mL)/CH_3OH	(1) 3% H_2SO_4 (1 mL)/CH_3OH	(1) $NaOCH_3$ (0.5 M, 1 mL)/CH_3OH	(1) $NaOCH_3$ (0.5 M)/CH_3OH
Reaction conditions	(1) 50 °C, 3 h (2) 80 °C, 15 min	(1) 50 °C, 12 h	(1) 50 °C, 15 min	(1) 25 °C, 15 min
Reagent volumes	1 mL	1 mL	1 mL	25 μL
Liquid/liquid extraction	Yes	Yes	Yes	No

In method B, dried lipid extracts were spiked with the control standards and reacted with 3% sulfuric acid in methanol [27]. The reaction solution was allowed to cool and was extracted three times with hexane (1 mL); the combined hexane layers were dried over anhydrous sodium sulphate and taken to dryness under a gentle nitrogen stream. The residue was silylated and analyzed by GC/MS as described above in method A.

In method C, dried lipid extracts were spiked with the control standards and reacted with sodium methoxide in methanol [27]. Transmethylation was stopped by the addition of glacial acetic acid (100 μL) followed by hexane (1 mL) then water (1 mL); the hexane layer was removed and the hexane extraction repeated (2 × 1 mL). The hexane layers were combined, dried over anhydrous sodium sulphate and taken to dryness carefully using a gentle nitrogen stream. The residue was silylated and analyzed by GC/MS as described above in method A.

With the one-vial method C, dried lipid extracts were spiked with the quality control standards (15 μL) and reacted with sodium methoxide in methanol. In the method development process, various reaction temperatures and reaction times were examined. Transmethylation was stopped by the addition of a solution of acetic acid in dichloromethane (5 M, 10 μL) and the resulting solution dried using a gentle nitrogen stream. The residue was treated with MSTFA (50 μL) and pyridine (15 μL) at 37 °C for 30 min. Aliquots (5 μL) were diluted 10-fold into hexane containing ethyl dodecanoate (internal standard, 25 ng/μL); 1 μL was injected on column for GC/MS analyses.

2.6. Gas chromatography/mass spectrometry

Analyses were performed on a HP 5971A MSD (full scan mode) equipped with HP 5890 Series II GC, a cool on-column injector and a J&W DB-17ht column (50% phenyl/50% methyl silicone, 30 m × 0.25 mm × 0.15 μm film) using helium as the carrier gas. The oven temperature was held at 50 °C for 5 min then programmed at 5 °C/min to 300 °C and held at 300 °C for 5 min. Electron impact ionization (EI^+, 70 eV) was used for all samples.

2.7. Quantitative analyses

Calibration curves and relative response factors (RRFs) were obtained for three classes of fatty acid derivatives: (1) fatty acid methyl esters (FAMEs), (2) trimethylsilyl esters of fatty acids and (3) trimethylsilyl ethers of 3-hydroxy fatty acid methyl esters. Solutions of authentic standards of fatty acid methyl esters (Me-$C_{9:0}$-Me-$C_{25:0}$) were prepared in hexane (1–40 ng/μL). FAMEs of unsaturated fatty acids were prepared by methylation of the unsaturated fatty acids by the diazomethane method [28]. Calibration plots (R^2 = 0.97–0.99) were obtained using peak areas from mass chromatograms and the relative response factors were calculated from the slopes of the calibration plots. Table 2 summarizes the ions used for the identification and quantification of various fatty acid derivatives.

Trimethylsilyl esters were prepared by reaction of authentic standards of fatty acids with MSTFA for 30 min at 37 °C; a mole ratio of total fatty acid:MSTFA of 1:500 was used. Solutions of the silyl derivatives were prepared by dissolving the reaction mixtures in hexane (3–50 ng/μL). Calibration plots ($R^2 \geq 0.99$) were obtained for each fatty acid derivative and the response factors were obtained from the slopes. Trimethylsilyl ethers of methyl esters of 3-hydroxy fatty acids were prepared by reaction of authentic standards of methyl esters of 3-hydroxy fatty acids with MSTFA. Solutions of the trimethylsilyl ethers of methyl esters were prepared by dissolving the reaction mixtures in hexane (3–40 ng/μL) with a 10% MSTFA.

Table 2
m/z values of ions used for the diagnostic and quantification of various fatty acid derivatives and other compounds

Compound	Diagnostic ions	Quantification ions	References
iPr-$C_{14:0}$-d_{27}	255, 297 ($M^{\bullet+}$)	105	
Me-$C_{14:0}$-d_{27}	91, 269 ($M^{\bullet+}$)	77 (74 in FAMEs)	
TMS-$C_{14:0}$-d_{27}	73, 327 ($M^{\bullet+}$)	312 $[M-15]^+$	
9-Anthracenemethanol		208	
TMS ether of 9-Anthracenemethanol	73, 191	280 ($M^{\bullet+}$)	
n-C_{20}	57, 85, 282 ($M^{\bullet+}$)	71	[26]
Et-$C_{12:0}$	101, 228 ($M^{\bullet+}$)	88	
FAMEs of saturated fatty acids	87, $M^{\bullet+}$	74	[22,26,29]
FAMEs of unsaturated fatty acids	74, $M^{\bullet+}$	55	[22,26,29]
FAMEs of cyclopropane fatty acids	55, $[M-32]^+$, $M^{\bullet+}$	69	[26]
TMS ethers of 3-hydroxy-FAMEs	73, 89, $[M-15]^+$	175	[30,31]
TMS esters of fatty acids	73, 75, 117	$[M-15]^+$	[32]

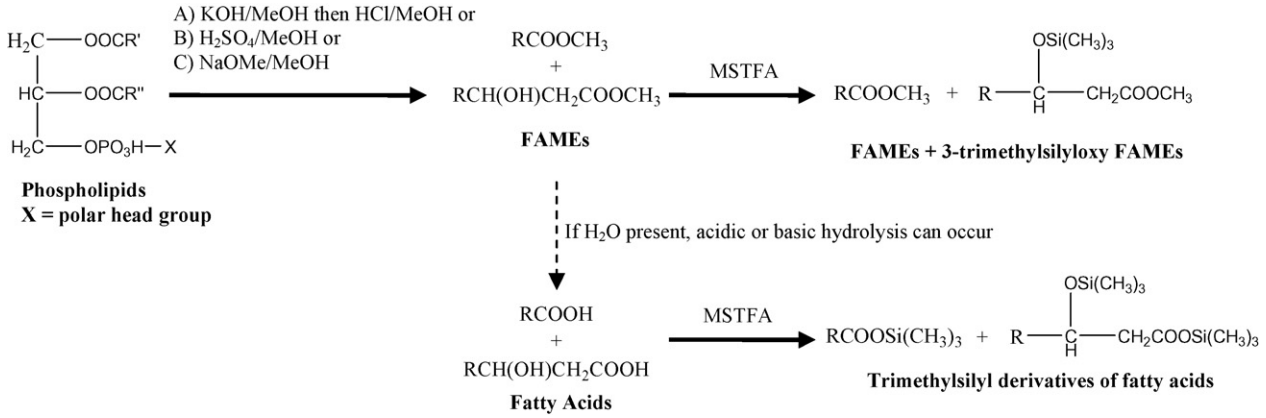

Fig. 1. Schematic of derivatization reactions of phospholipids using methods A, B and C. Other lipid classes react under these conditions.

Calibration curves ($R^2 = 0.99$) were obtained using peak areas from mass chromatograms of the m/z 175 ion.

The total mass of a given fatty acid in a sample was calculated as the sum of the methyl ester and the trimethylsilyl ester using the RRFs for each derivative and normalized by the wet weight of cells (expressed as ng fatty acid/mg wet cells). Relative percentages of fatty acids were calculated by taking the mass of a given fatty acid derivative as a percentage of the total mass of all fatty acids identified in the sample.

2.8. Statistical analyses

All data are reported as mean values ± S.D.; each sample was analyzed in triplicate. A statistical package (SPSS, version 15.0, SPSS Inc. Chicago, IL, USA) was used for statistical analyses. Analysis of variance (ANOVA) was used for multiple group comparisons ($P \leq 0.05$) while Student's t-test was used occasionally to evaluate two groups of data.

3. Results and discussion

3.1. Quality control standards

Three types of quality control standards were introduced into the methodologies to monitor (1) recoveries due to losses in extractions, handling and side reactions, (2) the efficiencies of transmethylation or hydrolysis/methylation reactions and (3) the efficiency of silylation reactions with MSTFA. An internal standard (ethyl dodecanoate, Et-$C_{12:0}$) was used for quantitation of all analytes.

First, the methyl esters of tridecanoic and pentadecanoic acids (Me-$C_{13:0}$ and Me-$C_{15:0}$) were introduced as recovery standards; these fatty acids which are not present in *S. meliloti* (data not shown) will undergo transformations but should be recovered quantitatively as their methyl esters if no losses or side reactions occurred. These methyl esters have lower molecular masses and are thus more volatile than most bacterial FAMEs so lower recoveries of Me-$C_{13:0}$ relative to Me-$C_{15:0}$ would indicate losses due to volatilization. An alkane (n-eicosane, n-C_{20}) was also added as a recovery standard. This compound does not undergo any transformation reactions under the conditions used, therefore it can be used to measure losses exclusively due to handling and volatilization. Additionally, n-C_{20} can be used to estimate the losses by volatilization of Me-$C_{14:0}$ to Me-$C_{16:0}$ since the normal boiling points of the n-alkane and the FAMEs are in the same range [33,34].

Second, isopropyl tetradecanoate-d_{27} was introduced to determine the efficiency of conversion of a secondary alcohol ester into a methyl ester. Upon transmethylation or hydrolysis/methylation reactions, isopropyl tetradecanoate-d_{27} is transformed to methyl tetradecanoate-d_{27}. Both compounds are readily monitored by GC/MS and their relative amounts provide a measure of the extent of this reaction. Should small amounts of water be present, hydrolysis of the methyl and/or isopropyl esters to tetradecanoic-d_{27} acid could occur; the free fatty acid would likely be undetected using standard analytical procedures. Derivatization with MSTFA would afford the trimethylsilyl ester of tetradecanoic-d_{27} acid, a compound readily identified by GC/MS; thus, the percentage of free fatty acid resulting from hydrolysis can be easily determined. The sum of the methyl, isopropyl and trimethylsilyl esters of tetradecanoic-d_{27} acid should be close to the recoveries of the alkane recovery standard; a sum less than this would indicate that there were losses due to side reactions other than hydrolysis.

Third, 9-anthracenemethanol was introduced to monitor the extent of silylations with MSTFA; since 9-anthracenemethanol and its trimethylsilyl derivative are readily monitored by GC/MS, they provide a direct measure of the efficiency of silylation reactions.

3.2. Derivatization with MSTFA

The silylation of any free fatty acids to their trimethylsilyl esters using MSTFA provided a direct measure of the degree of hydrolysis. This derivatization facilitated the analysis of 3-hydroxy fatty acids by conversion of the hydroxy function into the trimethylsilyl ether, a derivative which afforded narrower chromatographic peaks and better mass spectra. Under EI^+ conditions, 3-hydroxy fatty acid methyl esters produced rather uninformative mass spectra dominated by an ion at m/z 103, while the EI^+ spectra of the corresponding 3-trimethylsilyl ethers provided characteristic ions unique to these derivatives at m/z 89 and m/z 175; the $[M-15]^+$ fragment ion was used to identify individual fatty acid derivatives [30,31]. The injection of pure derivatization mixtures produced total ion chromatograms with high backgrounds and multiple contaminant peaks from the MSTFA and pyridine. Dilution of derivatization mixtures (5–20-fold) produced good quality chromatograms with improved S/N ratios. Since trimethylsilyl derivatives readily hydrolyze in the presence of traces of water, all solutions for GC/MS analyses were diluted using hexane containing 10% MSTFA and analyzed immediately.

3.3. Comparison of methods A, B and C

Mixtures of authentic standards representative of four classes of phospholipids, a triacylglycerol, an amide lipid and the quality control standards were used to evaluate the efficiencies of

Table 3
Recoveries of fatty acid derivatives produced from (a) the quality control standards and (b) the lipid standards using methods A, B and C

Quality control standards	Fatty acid derivatives	Percentages of derivatives recovered from standards, mean ± S.D. (n = 3)		
		Method A	Method B	Method C
(a)				
n-C_{20}	–	94.6 ± 7.2	84.6 ± 5.2	90.0 ± 11.6
Me-$C_{13:0}$	Me-$C_{13:0}$	75.4 ± 7.8	60.0 ± 2.8	65.5 ± 4.9
Me-$C_{15:0}$	Me-$C_{15:0}$	95.1 ± 6.5	81.4 ± 4.6	87.7 ± 5.2
iPr-$C_{14:0}$-d_{27}	iPr-$C_{14:0}$-d_{27}	6.1 ± 0.8	14.6 ± 1.1	18.1 ± 0.9
	Me-$C_{14:0}$-d_{27}	79.7 ± 7.0	73.3 ± 3.3	52.8 ± 3.9[1]
	TMS-$C_{14:0}$-d_{27}	2.8 ± 0.4 [1]	0.1 ± 0.1[2]	6.7 ± 1.4[3]
	Total $C_{14:0}$-d_{27} species	88.7 ± 8.1	88.0 ± 4.5	77.6 ± 5.8

Lipids	Fatty acid derivatives	Percentages of derivatives recovered from lipids, mean ± S.D. (n = 3)		
		Method A	Method B	Method C
b)				
CL-14:0/14:0/14:0/14:0	Me-$C_{14:0}$	87.7 ± 5.8[a]	90.5 ± 4.3[a]	78.8 ± 10.8[a]
	TMS-$C_{14:0}$	3.4 ± 0.3	ND	ND
PE-17:0/17:0	Me-$C_{17:0}$	82.4 ± 3.0[a]	84.1 ± 4.8[a]	68.7 ± 8.1[a]
	TMS-$C_{17:0}$	1.5 ± 0.3	ND	ND
TAG-16:0-d_{31}/16:0-d_{31}/16:0-d_{31}	Me-$C_{16:0}$-d_{31}	67.6 ± 4.7[b]	70.8 ± 3.1[b]	45.4 ± 5.2[1,b]
	TMS-$C_{16:0}$-d_{31}	1.1 ± 0.1	ND	ND
PC-19:0/19:0	Me-$C_{19:0}$	61.4 ± 4.1[b]	60.5 ± 3.7[c]	52.9 ± 8.3[b]
	TMS-$C_{19:0}$	ND	ND	ND
PG-12:0/12:0	Me-$C_{12:0}$	48.6 ± 8.4[c]	37.5 ± 1.2[d]	41.0 ± 4.2[b]
	TMS-$C_{12:0}$	2.7 ± 0.3	ND	ND
SM-18:0/18:0	Me-$C_{18:0}$	24.9 ± 1.9[1,d]	36.4 ± 2.0[2,d]	ND
	TMS-$C_{18:0}$	1.2 ± 0.3	ND	ND

n-C_{20}: n-eicosane; Me-$C_{13:0}$: methyl tridecanoate; Me-$C_{15:0}$: methyl pentadecanoate; iPr-$C_{14:0}$-d_{27}, Me-$C_{14:0}$-d_{27}, TMS-$C_{14:0}$-d_{27}: isopropyl, methyl and trimethylsilyl esters of tetradecanoic-d_{27} acid, respectively; CL-14:0/14:0/14:0/14:0: 1,1′,2,2′-tetramyristoyl cardiolipin; PE-17:0/17:0: 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine; TAG-16:0-d_{31}/16:0-d_{31}/16:0-d_{31}: Glyceryl tri(hexadecanoate-d_{31})); PC-19:0/19:0: 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine; PG-12:0/12:0: 1,2-dilauroyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol); SM-18:0/18:0: *N*-octadecanoyl-sphingosylphosphorylcholine; ND: not detected.
Mean values are significantly different (1,2,3) between columns ($P \leq 0.05$).
Mean values are significantly different (a,b,c,d) within columns ($P \leq 0.05$). Means with the same letter were not significantly different within columns ($P \geq 0.05$).

three standard fatty acid analysis methods (A, B and C). The quality control standards were added to each analysis to determine the recoveries, transmethylation/methylation efficiencies and the extents of hydrolysis and side reactions for each lipid class. Fig. 1 summarizes the transformation reactions and the derivatives produced. Each lipid produced derivatives (a FAME and possibly a trimethylsilyl ester) corresponding to the single fatty acid in each lipid standard; thus the reaction progress of each lipid could be monitored and compared to the progress of other lipids. Masses of individual lipids in these reactions were in the 10–40 μg range.

Table 3 shows the means and standard deviations obtained using the three methods for the quality control standards (Table 3a) and the lipid standards (Table 3b). The recoveries of the C_{20} alkane (about 90%) and the C_{15} FAME (Me-$C_{15:0}$, 81–95%) were identical for the three methods (Table 3a), indicating that losses due to handling and any side reactions of FAMEs were negligible. However, recoveries of the C_{13} FAME standard (Me-$C_{13:0}$, 60–75%) were somewhat lower than the C_{15} FAME standard, probably the result of losses due to the increased volatility of Me-$C_{13:0}$. The recoveries of the three tetradecanoic-d_{27} acid derivatives (78–89%) paralleled the recoveries of the C_{15} FAME and the C_{20} alkane, consistent with the above results. However, the recoveries of methyl tetradecanoate-d_{27} and trimethylsilyl tetradecanoate-d_{27} showed differences between methods indicating there were different degrees of conversion of isopropyl ester to the methyl ester and different extents of hydrolysis, respectively. The overall transmethylation efficiencies were good for methods A and B (73–79%) but were lower for method C (53%) as measured by the recoveries of methyl tetradecanoate-d_{27}. The extents of hydrolysis (measured by the recoveries of trimethylsilyl tetradecanoate-d_{27}) while low (0.1–7%) were statistically different for the three methods ($P \geq 0.05$); method B had the lowest hydrolysis (0.1%) while method C had the highest (7%).

The recoveries of fatty acids produced from lipid standards are listed in decreasing order in Table 3b. Overall, cardiolipin (CL) and phosphatidylethanolamine (PE) showed good to adequate conversions to FAMEs (69–91%) while the conversions of the triacylglycerol (TAG), phosphatidylglycerol (PG) and phosphatidylcholine (PC) standards were poor to adequate (38–71%). Not surprisingly, conversions of sphingomyelin (SM, an amide lipid) were poor (25–36%); conversions of sphingolipids are known to require vigorous acidic conditions [10,35]. SM was introduced since 3-hydroxy fatty acids in bacterial lipids are found in lipopolysaccharides attached via amide bonds. Conversions of a given lipid to FAMEs using different methods were statistically identical with the exception of the TAG and SM ($P \leq 0.05$); recovery of the $C_{16:0}$ FAME from the TAG standard was particularly low for method C (45%) compared to methods A and B (68–71%).

Overall, methods A and B had similar transmethylation efficiencies for the phospholipid standards (73–80%). The conversions of lipids into FAMEs were statistically identical within lipid classes using the three methods; the sole exception was the triacylglycerol which showed poorer recoveries using method C ($P \leq 0.05$). Recoveries were adequate for the three methods (80–90%) and the extents of competing hydrolysis reactions were small (≤7%). However, the recoveries of fatty acid derivatives from the different lipid classes were strikingly dissimilar, indicating that there must be side reactions occurring which led to differential losses of

Table 4
Relative percentage compositions of fatty acids in *S. meliloti* using methods A, B and C

Fatty acid classes	Fatty acids[a]	Relative percentage composition of *S. meliloti*, mean ± S.D. ($n = 5$)		
		Method A	Method B	Method C
Saturated fatty acids	$C_{14:0}$	0.28 ± 0.04	0.27 ± 0.07	0.13 ± 0.01[1]
	$C_{16:0}$	13.9 ± 0.5[1]	9.4 ± 0.9	8.4 ± 0.4
	$C_{17:0}$	0.16 ± 0.02[1]	0.13 ± 0.02	0.10 ± 0.01
	$C_{18:0}$	6.1 ± 0.7 [1]	4.1 ± 0.4	3.6 ± 0.6
Unsaturated fatty acids	*cis*-9-$C_{16:1}$	1.1 ± 0.3	0.9 ± 0.2	0.8 ± 0.2
	cis-11-$C_{18:1}$	71.3 ± 2.2	69.6 ± 1.9	54.9 ± 1.6[1]
	cis-10-$C_{19:1}$	1.2 ± 0.2[1]	0.5 ± 0.1[2]	ND
3-Hydroxy fatty acids	3-OH-$C_{12:0}$	ND	ND	ND
	3-OH-$C_{14:0}$	2.9 ± 0.6	ND	ND
	3-OH-$C_{16:0}$	0.09 ± 0.03	ND	ND
	3-OH-$C_{18:0}$	0.4 ± 0.2[1]	0.09 ± 0.03[2]	ND
Cyclopropane fatty acids	*cis*-9,10-$C_{17:0cyclo}$	ND	0.47 ± 0.03[1]	0.97 ± 0.08[2]
	cis-11,12-$C_{19:0cyclo}$	2.5 ± 0.7[1]	14.6 ± 0.9[2]	31.0 ± 0.7[3]
Total (%)		100	100	100

Mean values are significantly different (1,2,3) between columns ($P \leq 0.05$). ND: not detected.
[a] Sum of all fatty acid derivatives observed (FAMEs and trimethylsilyl derivatives).

FAME analytes from different lipid classes. For those who routinely report percentage fatty acid compositions in lipid mixtures based on these methods, these results are a cautionary tale because the fatty acid composition is clearly related to the relative efficiency of methylation or transmethylation reactions for each lipid class. The addition of the suite of standards used in this protocol allows one to determine the relative degrees of conversions of lipids to FAMEs accurately in each reaction.

In reactions of crude lipid extracts of *S. meliloti* recoveries were good (total derivatives of $C_{14:0}$-d_{27}, 83–103%) while losses due to hydrolysis to the free acid were minimal (TMS-$C_{14:0}$-d_{27}, <1%). Method A yielded the greatest number of fatty acid derivatives (17); fourteen were positively identified as saturated, unsaturated, 3-hydroxy and cyclopropane-containing fatty acids based on comparisons of their retention times and mass spectral fragmentation patterns with authentic standards (Table 4).

All three methods gave essentially identical absolute levels of saturated and unsaturated fatty acids; however, there were significant differences in the levels of 3-hydroxy and cyclopropane-containing fatty acids (Table 4). Only method A released 3-hydroxy fatty acids from bacterial lipopolysaccharides (LPSs) in reasonable yields while method C gave the highest yields of cyclopropane fatty acids. These differences in fatty acid levels resulted in statistically significant differences in the percentage composition data between the methods (Table 4).

For the analysis of cyclopropane-containing fatty acids, method C is the best choice while method A is most suitable for 3-hydroxy fatty acids. These results are not surprising since cyclopropane-containing fatty acids are labile to acidic conditions (e.g., methods A and B). Acidic conditions are reported to be the best for the release of amide-bound fatty acids from lipids, including hydroxy fatty acids [36]; however, method B has been reported to produce low recover-

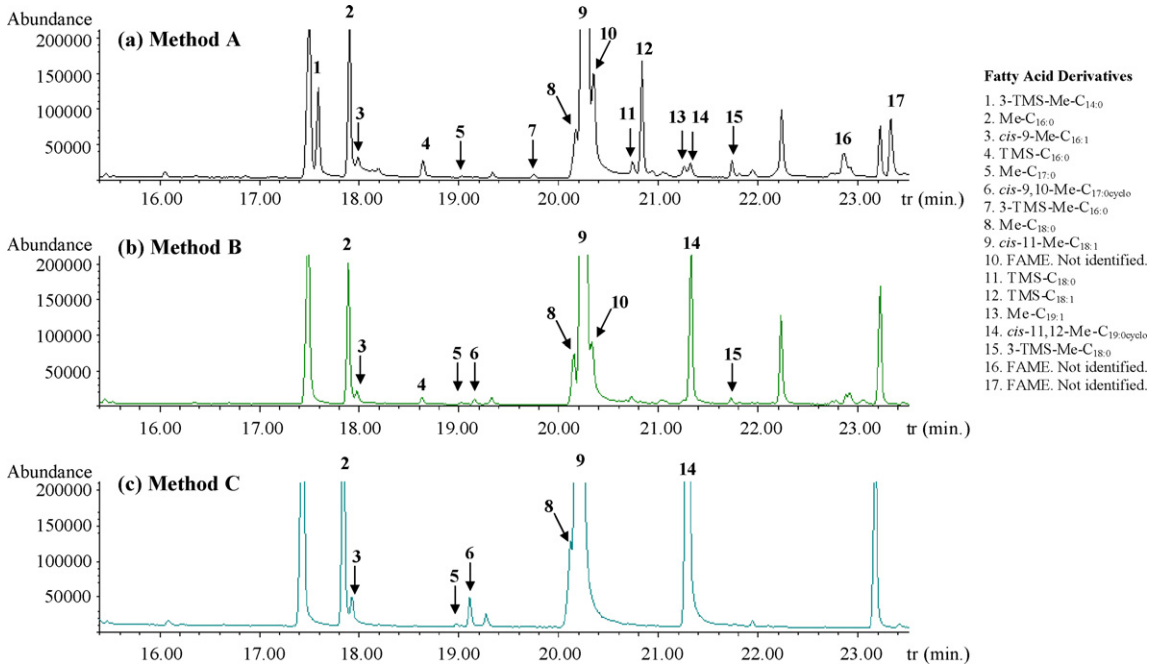

Fig. 2. Representative total ion chromatograms of fatty acid derivatives produced from *S. meliloti* using methods A, B and C.

Table 5
Optimization of transmethylation reaction parameters for one-vial method C

Quality control standards	Fatty acid derivatives	Percentages recoveries from standards, mean ± S.D. (n = 3)				
		25 °C, 15 min	25 °C, 30 min	25 °C, 60 min	50 °C, 15 min	60 °C, 15 min
(a)						
iPr-$C_{14:0}$-d_{27}	iPr-$C_{14:0}$-d_{27}	63.7 ± 7.4^{1}	38.6 ± 4.7^{2}	15.7 ± 0.5^{3}	0.0	0.0
	Me-$C_{14:0}$-d_{27}	34.2 ± 5.2^{1}	57.1 ± 5.9^{2}	78.8 ± 6.8^{3}	58.9 ± 5.1^{2}	34.1 ± 13.5^{1}
	TMS-$C_{14:0}$-d_{27}	ND	ND	ND	0.3 ± 0.2	2.4 ± 0.9
	Total $C_{14:0}$-d_{27} species	97.9 ± 12.0^{1}	95.7 ± 8.8^{1}	94.5 ± 6.4^{1}	59.2 ± 5.2^{2}	36.5 ± 14.4^{2}
Lipids	Fatty acid derivatives	Percentages recoveries from lipids, mean ± S.D. (n = 3)				
		25 °C, 15 min	25 °C, 30 min	25 °C, 60 min	50 °C, 15 min	60 °C, 15 min
(b)						
CL-14:0/14:0/14:0/14:0	Me-$C_{14:0}$	120.8 ± 9.8^{a}	108.0 ± 6.7^{a}	99.8 ± 7.5^{a}	$83.7 \pm 6.8^{1,a}$	61.0 ± 18.5^{1}
	TMS-$C_{14:0}$	ND	ND	ND	ND	0.22 ± 0.26
PE-17:0/17:0	Me-$C_{17:0}$	$101.2 \pm 13.9^{a,b}$	$97.4 \pm 7.7^{a,b}$	$95.1 \pm 7.9^{a,b}$	$75.3 \pm 5.9^{a,}$	53.4 ± 14.3^{1}
	TMS-$C_{17:0}$	ND	ND	ND	0.5 ± 0.5	3.5 ± 1.1
TAG-16:0-d_{31}/16:0-d_{31}/16:0-d_{31}	Me-$C_{16:0}$-d_{31}	$80.6 \pm 12.3^{b,c}$	$85.1 \pm 8.7^{b,c}$	86.1 ± 7.3^{a}	$68.3 \pm 5.3^{1,a,b}$	48.7 ± 17.2^{1}
	TMS-$C_{16:0}$-d_{31}	ND	ND	ND	1.1 ± 0.8	6.1 ± 1.4
PC-19:0/19:0	Me-$C_{19:0}$	$89.2 \pm 3.9^{b,c}$	$77.8 \pm 8.7^{c,d}$	76.6 ± 11.4^{b}	$46.0 \pm 6.9^{1,c}$	28.2 ± 10.0
	TMS-$C_{19:0}$	ND	ND	ND	ND	0.3 ± 0.2
PG-12:0/12:0	Me-$C_{12:0}$	69.6 ± 2.8^{c}	63.4 ± 2.2^{d}	54.1 ± 4.2^{c}	$53.6 \pm 7.6^{b,c}$	39.1 ± 14.3^{1}
	TMS-$C_{12:0}$	ND	ND	ND	ND	0.04 ± 0.03

iPr-$C_{14:0}$-d_{27}, Me-$C_{14:0}$-d_{27}, TMS-$C_{14:0}$-d_{27}: isopropyl, methyl and trimethylsilyl esters of tetradecanoic-d_{27} acid, respectively; PE-17:0/17:0: 1,2-diheptadecanoyl-*sn*-glycero-3-phosphoethanolamine; CL-14:0/14:0/14:0/14:0: 1,1′,2,2′-tetramyristoyl cardiolipin; PG-12:0/12:0: 1,2-dilauroyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol); TAG-16:0-d_{31}/16:0-d_{31}/16:0-d_{31}: Glyceryl tri(hexadecanoate-d_{31})); PC-19:0/19:0: 1,2-dinonadecanoyl-*sn*-glycero-3-phosphocholine; Me-: fatty acid methyl ester; TMS: trimethylsilyl ester of fatty acids; ND: not detected.
Mean values are significantly different (1,2,3) between columns ($P \leq 0.05$). Means with the same number were not significantly different between columns ($P \geq 0.05$).
Mean values are significantly different (a,b,c,d) within columns ($P \leq 0.05$). Means with the same letter were not significantly different within columns ($P \geq 0.05$).

ies of hydroxy fatty acids from bacterial lipids [3,10]. In the current work 3-hydroxytetradecanoate (3-OH-$C_{14:0}$), the most abundant bacterial hydroxy fatty acid, was not observed using method B while 3-hydroxyoctadecanoate, a minor component at <0.1% of total fatty acids, was detected. The extraction of LPSs from cells requires especial extraction procedures [37,38]; thus lipid extraction using $CHCl_3$:CH_3OH (methods B and C) while effective for most lipid classes, is inefficient for large molecules of various polarities such as LPSs. Thus, inadequate extraction of LPSs from cells might be the reason why 3-OH-$C_{14:0}$ was not detected using methods B and C. In summary, relative percentage compositions of bacterial fatty acids are highly dependent on the method used; no single method provides a comprehensive fatty acid profile due to different labilities of 3-hydroxy and cyclopropane-containing fatty acids [10].

Two cyclopropane-containing fatty acids were positively identified as *cis*-9,10-methylene-hexadecanoic acid (*cis*-9,10-$C_{17:0cyclo}$) and *cis*-11,12-methylene-octadecanoic acid (*cis*-11,12-$C_{19:0cyclo}$). Their relative percentage compositions were statistically different using the three methods (Table 4, $P \leq 0.05$). The mass spectra of two isomeric methyl esters, *cis*-10-nonadecenoic and *cis*-11,12-methyleneoctadecanoic acids, were virtually identical; differentiation between them was only possible by chromatographic separation (using a DB-17ht column, a 50% phenyl, 50% methyl polysiloxane phase, peaks 13 and 14 in Fig. 2a). Derivatives such as picolinyl esters which have been reported to be useful in localizing cyclopropane rings or double bonds in fatty acids [27] were not used in this work.

3.4. Development of a one-vial method for FAME analyses

Fatty acid analysis methods are laborious procedures requiring multiple extraction steps and drying/evaporation steps prior to GC or GC/MS analysis. Method C which gave the highest recoveries of cyclopropane fatty acids was selected for development of a simplified and micro-scale version of the method. A goal of our research program is the study of genes involved in the biosynthesis of lipids, including the cyclopropanation of lipids. The scales of traditional fatty acid methods would require bacterial growth in large volumes of media. We sought to develop a micro-scale version of method C which would be amenable to small volumes of bacterial cultures, would require minimal sample handling and would be amenable to the analysis of large numbers of samples.

The one-vial method incorporated all of the quality control, recovery standards and internal standards introduced previously. A standard GC/MS vial with a 200 μL insert was used as the "one-vial" reaction vessel; the amounts of lipid introduced into the vial ranged between 10 and 100 μg, corresponding to a 100–1000-fold reduction in the amount of lipid needed compared to conventional methods (10–100 mg lipid per analysis). Reagent volumes were reduced 40–80-fold relative to conventional procedures. In a typical micro-scale analysis lipids equivalent to 30–40 μg of wet cells were injected onto the chromatographic column, making this protocol comparable in sensitivity to recently reported thermochemolysis methods [17]. We believe our procedure is more practical for most researchers than reported pyrolitic methods since a standard GC/MS instrument can be used without the need for additional apparatus or instrument modifications.

Method development data obtained using standards is reported in Table 5 while application of the protocol to the analysis of *S. meliloti* samples is reported in Table 6. Given the dramatic reduction in scale using the one-vial method, it was important from the outset to determine whether hydrolysis would be a major side reaction and whether transmethylation conditions would result in decreased reaction yields. Christie has cautioned analysts that well-established methods fail when sample size and reagent volumes are scaled down due to hydrolysis caused by the presence of traces of water in the glassware and reagents [12]. To address these issues five transmethylation reaction conditions were compared using a suite of five lipid standards (i.e., those listed in Table 3 except SM). Three temperatures (25, 50 and 60 °C) were compared with a fixed

Table 6
Relative percentage compositions of fatty acids in *S. meliloti* determined using the one-vial method

Fatty acid classes	Fatty acids	Relative percentage composition (%)	
		mean ± S.D. (n = 9)	RSD (%)
Saturated fatty acids	$C_{14:0}$	0.4 ± 0.1	27
	$C_{16:0}$	12.8 ± 2.2	17
	$C_{17:0}$	0.2 ± 0.1	38
	$C_{18:0}$	3.4 ± 0.7	19
Unsaturated fatty acids	*cis*-9-$C_{16:1}$	1.2 ± 0.7	60
	cis-11-$C_{18:1}$	60.4 ± 3.3	5
Cyclopropane fatty acids	*cis*-9,10-$C_{17:0cyclo}$	1.7 ± 0.2	13
	cis-11,12-$C_{19:0cyclo}$	19.9 ± 3.8	19
Total		100.0%	

reaction time of 15 min; the remaining reactions were performed for 30 and 60 min at 25 °C. The results are summarized in Table 5.

The results of these experiments were truly dramatic. Recoveries of the total $C_{14:0}$-d_{27} species were quantitative for the three reaction times at 25 °C but decreased significantly at 50 °C and 60 °C (60% and 37%, respectively, Table 5a). The losses due to hydrolysis, as determined by the $C_{14:0}$-d_{27} TMS ester levels, were insignificant to very small. The poor recoveries of FAMEs are likely the result of side reactions, probably base-catalyzed Claisen-type condensations, resulting in the formation of higher molecular mass products that are not detected by the current GC/MS method. Similar trends were observed in the FAMEs data from reactions of lipid standards; reactions at 50 and 60 °C led to statistically lower FAMEs yields that at 25 °C (Table 5b). There were no significant differences among the three reaction times at 25 °C.

These data lead to two important conclusions for fatty acid analysis: first, reaction temperatures of 50 and 60 °C result in significant losses of the target analytes (FAMEs) presumably due to unknown side reactions and not due to hydrolysis reactions; second, yields of FAMEs vary significantly with phospholipid type. Since most fatty acid analysis methods in the literature use reaction temperatures of 50 °C or greater with reaction times longer than 15 min, the yields of FAMEs will be likely compromised and the reported percentage composition data may not accurately reflect the real compositions. Furthermore, fatty acid data from the analyses of phospholipid mixtures may be compromised due to the differences in relative yields from different phospholipid classes. The protocols described in this work could be readily used or adapted to any fatty acid analysis protocol to allow researchers to assess the relative efficiencies of conversion, degrees of hydrolysis and extents of unwanted side reactions in the protocols used in their laboratories.

It is worth mentioning that the recoveries of FAMEs from different fatty acid classes (saturated, 3-hydroxy and cyclopropane fatty acids) were studied using the same transmethylation conditions (temperatures and times). In general, the recoveries of FAMEs decreased with heating; however, this effect was more accentuated for 3-hydroxy and cyclopropane fatty acids which showed decreases of 56–74% compared to 20–45% decreases in yields for saturated fatty acids (data not shown).

The relative percentage compositions of fatty acids in *S. meliloti* determined using the one-vial method are shown in Table 6. Three sets of bacterial cultures, grown over a period of 6 months, were examined with each culture analyzed in triplicate. The one-vial method provided reproducible results with RSDs between

Table 7
Fatty acid relative percentage composition of *S. meliloti* in the presence (control) and absence of phosphate (stressed) with the one-vial method C (a)

Fatty acid class	Fatty acid	Relative % composition of *S. meliloti*, mean ± S.D. (n = 3)	
		Control	Stressed
(a)			
Saturated fatty acids	$C_{14:0}$	0.4 ± 0.1[a]	0.1 ± 0.0[a]
	$C_{16:0}$	13.8 ± 3.7[a]	2.4 ± 1.0[a]
	$C_{17:0}$	0.3 ± 0.1[a]	0.1 ± 0.0[a]
	$C_{18:0}$	2.7 ± 0.6[a]	0.5 ± 0.1[a]
Unsaturated fatty acids	*cis*-9-$C_{16:1}$	2.0 ± 0.5[a]	0.3 ± 0.0[a]
	cis-11-$C_{18:1}$	61.9 ± 3.6[a]	13.4 ± 2.5[a]
	cis-10-$C_{19:1}$	ND	ND
3-Hydroxy fatty acids	3-OH-$C_{4:0}$ (PHB)	1.2 ± 1.4[a]	74.6 ± 4.8[a]
	3-OH-$C_{12:0}$	ND	ND
	3-OH-$C_{14:0}$	0.1 ± 0.1	0.007 ± 0.002
	3-OH-$C_{16:0}$	ND	ND
	3-OH-$C_{18:0}$	ND	ND
Cyclopropane fatty acids	*cis*-9,10-$C_{17:0cyclo}$	1.7 ± 0.3[a]	0.5 ± 0.1[a]
	cis-11,12-$C_{19:0cyclo}$	15.8 ± 2.0[a]	8.1 ± 1.1[a]
Total		100%	100%
(b)			
Saturated fatty acids	$C_{14:0}$	0.4 ± 0.1	0.3 ± 0.1
	$C_{16:0}$	14.0 ± 3.6	9.3 ± 2.1
	$C_{17:0}$	0.3 ± 0.1	0.2 ± 0.1
	$C_{18:0}$	2.7 ± 0.6[a]	1.9 ± 0.2
Unsaturated fatty acids	*cis*-9-$C_{16:1}$	2.0 ± 0.5[a]	1.4 ± 0.1
	cis-11-$C_{18:1}$	62.8 ± 3.6[a]	52.9 ± 0.2[a]
Cyclopropane fatty acids	*cis*-9,10-$C_{17:0cyclo}$	1.8 ± 0.3	2.0 ± 0.1
	cis-11,12-$C_{19:0cyclo}$	16.1 ± 2.3[a]	31.9 ± 2.0[a]
Total		100%	100%

Relative percentage compositions calculated without 3-hydroxy fatty acid species for both control and stressed (b). ND: not detected.
[a] Mean values are significantly different between control and stress ($P \leq 0.05$).

5% and 20% for major components and up to 60% for components with abundances below 1%. In summary, it can be concluded that complete transmethylation of lipids can be accomplished at 25 °C with no hydrolysis and minimal losses due to side reactions. Our results are in agreement with those of Christie who reported complete transesterification of lipids at room temperature in a few minutes [10]. Furthermore, hydrolysis losses were negligible when working with the small volumes of reagents and small amounts of lipids (~10 μg) in the micro-scale procedure.

3.5. Fatty acid analysis of S. meliloti cells under two growth conditions using the one-vial method

Results discussed above demonstrated that method A provided the most comprehensive fatty acid profiles while method C and the one-vial version of method C provided the best results for cyclopropane fatty acids. Therefore the one-vial format of method C and conventional method A were used to analyze *S. meliloti* cells cultured under normal and stressed growth conditions, the latter induced by phosphate starvation conditions. Isopropyl tetradecanoate-d_{27} and the recovery standards (Me-$C_{15:0}$, *n*-C_{20}) were added to each sample to determine transmethylation efficiency, extent of hydrolysis and overall recoveries.

Recoveries of *n*-C_{20} and Me-$C_{15:0}$ were good (109–115%) with the one-vial method C. Recovery of methyl tetradecanoate-d_{27} (only 18%) was consistent with results presented above wherein transmethylations of the isopropyl ester were found to be significantly slower that transmethylations of lipids; the lipids were fully transmethylated under these conditions. The one-vial procedure for method C did not show any evidence of hydrolysis (i.e., no trimethylsilyl tetradecanoate-d_{27} was detected).

Relative percentage compositions of fatty acids in *S. meliloti* grown under control and phosphate-stressed conditions are shown in Table 7a using the one-vial method C. The most dramatic result was a spectacular increase in the amount of 3-hydroxybutanoic acid found in the phosphate-stressed cells. 3-Hydroxybutanoic acid (often called 3-hydroxybutyric acid) is formed via hydrolysis of poly-3-hydroxybutyrate (PHB), a biopolymer commonly found in bacteria. A distinct advantage of the one-vial method C is the ability to detect 3-hydroxybutanoate directly in the GC/MS analysis; conventional fatty acid analytical procedures use one or more aqueous extraction steps which result in loss of 3-hydroxybutanoic acid to the aqueous phase. This is consistent with results obtained when method A was applied to the analysis of phosphate-starved cells since 3-hydroxybutanoic acid was not detected (data not shown). In contrast, this polar molecule is fully retained (and detected) using the one-vial protocol which affords the methyl ester of 3-hydroxybutanoic acid; the latter is converted by MSTFA into the readily detected methyl ester of 3-trimethylsilyloxybutanoic acid. Thus, this protocol is the only one we are aware of which allows the detection of PHB on a small scale and which allows the determination of fatty acids and 3-hydroxybutanoate simultaneously.

Previously reported methods for the analysis of PHB are tedious procedures and suffer from losses due to the water extraction steps [39–41]. While the one-vial method has great potential for the analysis of PHB in bacteria, this method has not been optimized nor have appropriate controls been developed yet. However, we are confident that the data reported here are of good quality. Given the increasing interest in the determination of PHB in a number of systems, this method should prove useful to a number of researchers.

Given the large change in 3-hydroxybutanoic acid levels it is difficult to compare the differences in the percentage compositions of the other fatty acids (Table 7a); by removing the 3-hydroxybutanoate data (Table 7b) direct comparisons of the fatty acid levels were possible. The relative percentage composition of *cis*-11,12-methyleneoctadecanoic acid, the most abundant of cyclopropane fatty acids, increased 2-fold under the phosphate starvation conditions. This increase led to a modest decrease in all of the other fatty acids, particularly oleic acid, since it is the precursor of that cyclopropane fatty acid.

It should be remarked, that percentages of cyclopropane fatty acids in phosphate-starved cells were 5–8% lower using method A relative to the one-vial method C due to the acidic conditions used in method A. The distinct advantages of the one-vial protocol over standard method C protocols are 4-fold: (1) the greater ease of using the one-vial protocol, (2) the ability to do multiple samples in parallel, (3) the ability to determine 3-hydroxybutanoic acid directly and reproducibly and (4) free fatty acids generated from hydrolysis as a side reaction can be accounted for in final results.

4. Conclusions

The results presented show that for fatty acid analysis in *S. meliloti* two methods were found to be useful: (1) basic hydrolysis followed by acid-catalyzed methylation was best for the analysis of 3-hydroxy fatty acids while (2) base-catalyzed transmethylation with sodium methoxide was best for determination of cyclopropane-containing fatty acids. The methods reported here included a number of recovery and internal of standards and a silylation step which not only improved the protocols but allowed, for the first time, direct determination of losses caused by sample handling or side reactions as well as the fates of acid- and base-sensitive fatty acid derivatives.

Most importantly, a micro-scale, one-vial method for fatty acid analysis was developed with sample sizes decreased by at least 100-fold compared to conventional procedures. The methodology was designed to analyze small samples sizes, to minimize sample handling, to increase sample throughput, to increase method sensitivity and to improve the overall quality of analysis; the method succeeded on all counts. This method was successfully applied to the analysis of fatty acids in *S. meliloti* and showed very few side reactions, small sample losses and excellent recoveries, identical to conventional, large-scale methods. The use of a range of quality control and recovery standards allowed monitoring for completion of derivatization reactions, side reactions and recoveries. Quantitation of trimethylsilyl esters of fatty acids provided an easy and rapid way to measure the degree of sample loss due to hydrolysis. Hydrolysis side reactions were negligible when transmethylations were conducted at room temperature; reaction temperatures of 50 °C or higher resulted in significant losses of fatty acids, presumably due to aldol-like condensation reactions. One unexpected outcome of these small-scale reactions was the development of a convenient, one-step method for the analysis of 3-hydroxybutanoic acid, resulting in an efficient method for the determination of poly(3-hydroxybutanoic acid) on very small scales. These procedures were applied to the analysis of *S. meliloti* grown in the presence and absence of phosphate. Analysis of phosphate-starved cultures of *S. meliloti* using the one-vial method, revealed a 2-fold increased of cyclopropane fatty acids and a 73% increase in poly(3-hydroxybutanoic acid). The methodologies developed here will be applied in future studies of *S. meliloti* to investigate the effect of phosphate starvation and other stressors on the cyclopropanation of lipids and to determine the genes involved in this process.

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