Comparison of Rapid Methods for Analysis of Bacterial Fatty Acids

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When rapid gas-liquid chromatography methods for determination of bacterial fatty acids were compared, results showed that saponification was required for total fatty acid analysis. Transesterification with boron-trihalide reagents (BF$_3$-CH$_3$OH, BCl$_3$-CH$_3$OH) caused extensive degradation of cyclopropane acids and was less effective than saponification in releasing cellular hydroxy fatty acids. Digestion of cells with tetramethylammonium hydroxide was unsatisfactory because of extraneous gas-liquid chromatography peaks and because of lower recovery of branched-chain and hydroxy fatty acids. A simple, rapid saponification procedure which can be used for total cellular fatty acid analysis of freshly grown cells is described.

Many laboratories are currently using gas-liquid chromatography (GLC) to study the cellular fatty acid composition of microorganisms (6, 7, 15, 16, 19, 20). These studies have rapidly expanded our knowledge of the chemistry and taxonomy of closely related bacteria. They have also provided the microbiologist with additional criteria for more rapid identification of cultures.

Classical methods for lipid analysis are time consuming and impractical for processing large numbers of samples (2). A number of rapid and simple methods for cellular fatty acid analyses by GLC have been reported, but the results have not been compared (1, 6, 11, 12). Many of these methods require the use of specialized equipment and hazardous chemicals.

We examined the fatty acid composition of lyophilized and fresh bacterial cells by using four different methods of ester preparation. In this report we compare and evaluate these methods; we also describe a GLC procedure for fatty acid analysis which can easily be performed in a clinical laboratory.

MATERIALS AND METHODS

The cultures used were *Pseudomonas diminuta* UC-501 and *P. maltophilia* RYS-67. Extensive studies of these two pseudomonads have shown that they contain most of the common, as well as several unusual, bacterial fatty acids (13; C. M. Kaltenbach, Ph.D. thesis, University of North Carolina, Chapel Hill, 1973). Both cultures were grown and harvested

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as described previously (14). The cells were steam-sterilized, lyophilized, and stored at −4 C. A 10-mg portion of each lyophilized culture was used for the fatty acid analyses. For analysis of fresh cells, cultures were grown for 18 to 24 h at 35 C on plates of Trypticase soy agar containing 0.1% yeast extract (Difco Laboratories, Detroit, Mich.). Two loops of cells were carefully removed from the plates and processed for fatty acids.

All samples were heated in tubes which were fitted with Teflon-lined caps and were free from cracks and chips. Extractions were made by shaking the samples for 20 s with a mixture of chloroform and hexane (1:4, CHCl$_3$-Hex). The organic phase, which contained the methyl ester, was transferred to a beaker and concentrated to approximately 0.3 ml under a gentle stream of dry N$_2$. Anhydrous sodium sulfate crystals were added to the contents of the beaker to absorb any water. The methyl ester samples were transferred to a calibrated tube, and the final volume was adjusted to 0.1 ml. The samples were stored at −4 C.

The fatty acid composition of lyophilized cells was determined by each of the following methods.

Method A. The cells were heated for 1 h at 100 C in 10 ml of 5% NaOH in 50% aqueous methanol (NaOH-CH$_3$OH). Nonsaponifiable material was removed by extracting the saponified material with an equal volume of 1:4 CHCl$_3$-Hex. The aqueous layer was acidified to pH 2 with 6 N HCl, and the fatty acids were extracted twice with 1:4 CHCl$_3$-Hex. The extracts were combined in a beaker and evaporated to dryness under N$_2$. The fatty acids were transferred to a test tube by two successive rinsings with 1 to 2 ml of boron trichloride-methanol reagent (BCl$_3$-CH$_3$OH; Applied Science Laboratories, State College, Pa.). The mixture was heated at 85 C for 5 min, cooled, and transferred to a separatory funnel which contained equal volumes of 1:4 CHCl$_3$-Hex and distilled water.

After extraction, the organic phase which contained the fatty acid methyl esters was concentrated to 0.1 ml as described previously.

Method B. The cells were heated for 15 min at 100 C in 5 ml of NaOH-CH$_3$OH. The saponified material was cooled and acidified to pH 2. A 4-ml portion of either boron trifluoride methanol (BF$_3$-CH$_3$OH, Applied Science Laboratories, State College, Pa.) or BCl$_3$-CH$_3$OH reagents was added, and the mixture was heated for 5 min at 100 C. The contents of the tube were added to 10 ml of a saturated sodium chloride solution, and the methyl esters were extracted twice with an equal volume of 1:4 CHCl$_3$-Hex. The combined extracts were evaporated to 0.1 ml.

Method C. The cells were mixed with 2 ml of either of the boron trihalide methanol reagents (BCl$_3$-CH$_3$OH or BF$_3$-CH$_3$OH) and heated at 100 C for 60 min. The esterified mixtures were added to 10 ml of distilled water and extracted twice with 1:4 CHCl$_3$-Hex. The extracts were evaporated as described previously.

Method D. The cells were mixed with 0.5 ml of a saturated solution of tetramethylammonium hydroxide (Eastman Chemical Co., Rochester, N.Y.) in anhydrous methanol. The mixture was heated at 100 C for 5 min. The contents of the tube were cooled and centrifuged. Three to five microliters of the supernatant was used for GLC analysis.

GLC. A Perkin-Elmer model 900 gas chromatograph (Perkin-Elmer, Norwalk, Conn.) equipped with flame ionization detectors and a disk integrator recorder was used for analysis of samples prepared by methods A, B, and C. Samples were analyzed on a coiled-glass column (3.66 m × 0.635 cm outer diameter) that was packed with 3% OV-1 and coated on 80- to 100-mesh acid-washed, dimethylchlorosilane-treated Chromosorb W (Applied Science Laboratories, State College, Pa.). After the sample was injected, the column was temperature-programmed from 180 to 260 C at 5 C/min. The injector port temperature was 250 C and the detector temperature was 275 C. The carrier gas was prepurified N$_2$ at a flow rate of 45 ml/min. The sample size was usually 3 μliters.

Samples prepared by method D were analyzed with a Barber-Colman model 5000 gas chromatograph equipped with a flame ionization detector. The instrument contained a U-tube glass column (2.4 m × 0.635 cm outer diameter) which was packed with 3% OV-1. The column was temperature-programmed from 150 to 260 C at 5 C/min. The injector port temperature was 325 C and the detector temperature was 260 C. The carrier gas was prepurified N$_2$ at a flow rate of 50 ml/min.

Analysis time for the fatty acid methyl ester samples was 20 min. This time interval was sufficient to detect fatty acid methyl esters with carbon chains from 10 to 21 carbons in length. The methyl ester peaks were identified by comparison of their retention times on the OV-1 column with the retention times of highly purified methyl ester standards (Applied Science Laboratories, State College, Pa.). In addition,

the identity of the methyl esters of the uncommon fatty acids was confirmed by mass spectrometry (18). Peak areas were determined by disk integrator; the percentage of each acid was calculated from the ratio of the area of its peak to the total area of all peaks.

RESULTS

Method A has been used in this laboratory for GLC analysis of the cellular fatty acid composition of many different bacteria. Although the results from this method are consistent and reproducible, it is time consuming and requires relatively large volumes of solvents and reagents.

Method B is an adaptation of the method described by Metcalf et al. for rapid analysis of fatty acid esters (12). The saponification reagent and extraction solvents used in method A were substituted for those recommended by Metcalf et al. In their original procedure, BF$_3$-CH$_3$OH was added directly to the cooled saponified material. However, we found that esterification of the fatty acid was more complete when the saponified material was acidified to pH 2 before the methylating reagent was added.

Method C is similar to those described first by Abel et al. (1) and later by Drucker et al. (6). The interesterification of the cells was carried out with commercially available reagents in tubes instead of flasks or sealed ampules. Care was taken when the esterified cells were added to water and extracted with CHCl$_3$-Hex because these mixtures readily emulsified. However, when saturated NaCl was substituted for the water, emulsions rarely occurred.

Method D is a modification of that suggested by MacGee (11). The cells were digested with a methanolic solution of tetramethylammonium hydroxide instead of the aqueous solution originally described. Although preparation of the samples was simple and rapid, the results of the GLC analyses were unsatisfactory. There was a very large solvent peak on the chromatograms which interfered with the analysis of the shorter-chain fatty acid methyl esters. Also, the reagent blank contained peaks which eluted at the same retention times as did some of the fatty acid methyl ester standards. In addition, the relative sizes of the methyl ester peaks of branched-chain and hydroxy acids were markedly smaller than those obtained with other methods. For these reasons, method D was considered unreliable for fatty acid analysis, and further evaluations of this procedure were not made.

Percentages of the major fatty acids in lyophilized cells of *P. diminuta* and *P. maltophilia* are

shown in Tables 1 and 2, respectively. These values are an average of at least three analyses. The data show that, with the exception of hydroxy and cyclopropane acids, the average values for other fatty acids were comparable, regardless of the method used. The values for the hydroxy acids in *P. maltophilia* were consistently higher in samples processes by methods A and B. For example, the percentage of 3-OH lauric acid in *P. diminuta* processed by methods A and B was 8 and 10%, respectively, compared to only 4 and 5% by method C (Table 1). Similarly, the values for the hydroxy acids in *P. maltophilia* were lower in samples prepared by method C than by methods A and B (Table 2).

The relative percentage of the C_{19} cyclopropane acid in *P. diminuta* (Table 1) was markedly influenced by the method of sample preparation. Samples processed by methods A and B contained 16 and 18%, respectively, whereas samples prepared by method C when BCl_3-CH_3OH was used contained 11%. Samples processed by method C when BF_3-CH_3OH was used, however, contained only 2% of the C_{19} cyclopro-

pane acid. The large decrease in the percentage of this acid when BF_3-CH_3OH was used was accompanied by the appearance of several unidentified peaks in the chromatogram. The total percentages of these peaks together with the C_{19} cyclopropane acid peak was essentially the same value as that observed for the C_{19} cyclopropane acid in samples prepared by methods A and B. The largest of the unknown peaks was also present (7%) in samples prepared by method C when BCl_3-CH_3OH was used, but it was not present in samples prepared by methods A and B. The other unidentified peaks observed in samples prepared by method C with BF_3-CH_3OH were not detected in method C samples processed with BCl_3-CH_3OH.

The destructive effects of BF_3-CH_3OH on cyclopropane acids were evaluated further in tests with a highly purified methyl ester standard of lactobacillic acid (C_{19} cyclopropane acid) and with cells of *Escherichia coli*. Portions of the C_{19} cyclopropane acid standard were mixed with BF_3-CH_3OH and placed at 25 C or 100 C. After 15 min at either temperature, the samples were extracted and analyzed by GLC.

TABLE 1. *Percentages of major cellular fatty acids of lyophilized P. diminuta*

Method	Fatty acids														
	3-OH 12[a]	Un	14	15	Un	16:1	16	17	18:1	18	Un	19 cyc	Un	Un	Un
A	8[b]	5	4	1	—	2	30	TR	33	TR	—	16	—	1	—
B	10	4	2	1	—	2	27	2	30	3	—	18	—	1	—
C-(BCl₃)	4	3	3	2	1	2	30	2	34	1	—	11	—	7	—
C-(BF₃)	5	2	2	1	3	2	28	1	36	2	2	2	2	8	4

[a] 3-OH, Hydroxy acid; Un, unidentified acid; 16:1 and 18:1, monounsaturated fatty acids; cyc, cyclopropane acid. Numbers refer to number of carbon atoms.

[b] —, Not detected; TR, less than 1%. Numbers refer to percentages of total acids. Values are an average of three different determinations.

TABLE 2. *Percentages of major cellular fatty acids of lyophilized P. maltophilia*

Method	Fatty acids													
	10[a]	i 11	i 2-OH 11	i 3-OH 11	2-OH 12	3-OH 12	14[b]	i 15	16:1	16	Un	Un	18:1	18
A	1[c]	5	5	7	1	8	15	36	9	7	1	2	2	1
B	1	6	5	6	TR	8	11	39	8	10	1	3	1	1
C-(BCl₃)	1	4	3	4	1	4	9	38	11	14	2	6	2	1
C-(BF₃)	2	3	3	4	1	4	9	31	13	16	4	6	3	1

[a] See footnotes to Table 1. i, Iso acid; 2-OH, hydroxy acid.

[b] The percentage of the C_{14} acid also includes the percentage of C_{13} iso 3-OH acid fatty acid. These two acids did not separate under the GLC conditions used.

[c] See footnotes to Table 1.

The chromatograms in Fig. 1 clearly show that extensive degradation of the C_{19} cyclopropane acid methyl ester standard occurred at 100 C but not at 25 C. Only one large peak (confirmed by mass spectrometry as the methyl ester of C_{19} cyclopropane acid [4]) was present in the chromatogram of the 25 C sample, whereas several major unidentified peaks were present in the chromatogram of the 100 C sample. The retention time of these major peaks was identical to that of the unidentified peaks observed in chromatograms from *P. diminuta*. Cells of *E. coli* processed by methods A and B contained relatively large amounts of C_{17} cyclopropane acid, whereas extensive degradation of this acid occurred with cells processed by method C and BF_3-CH_3OH.

On the basis of these results, experiments were made to compare various time intervals for saponification (methods A and B) and transesterification (method C). Similar results were obtained when the saponification time of method A was reduced from 60 min to 15 min. No differences were observed when the saponification time was extended to 30 min or to 60 min in method B. No attempts were made to evaluate saponification times of less than 15 min with either method A or B. Transesterification (method C) at 15 and 60 min gave comparable results; the extensive degradation of cyclopropane acids observed at 60 min with BF_3-CH_3OH also occurred at 15 min.

Experiments with the 15-min period of saponification or transesterification were made with freshly grown cells of *P. diminuta* and *P. maltophilia* to compare their cellular fatty acid composition with that of lyophilized cells.
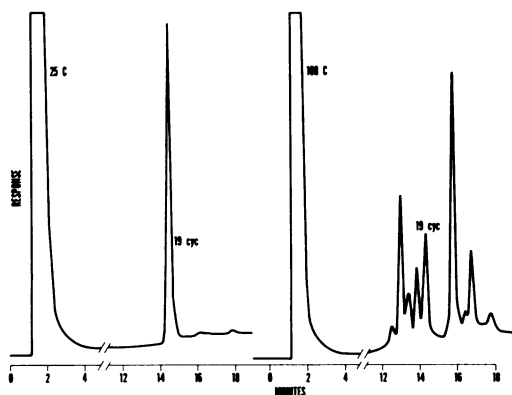

FIG. 1. *Gas-liquid chromatograms of lactobacillic acid* (C_{19} *cyclopropane*) *methyl ester mixed with 14% BF_3-CH_3OH reagent. The sample on the left was held for 15 min at 25 C before analysis, and the sample on the right was held for 15 min at 100 C.*

Growth from each culture was carefully removed from the plate of Trypticase soy agar containing 0.1% yeast extract and dispensed into tubes containing 2 ml of each of the reagents used in methods A, B, and C. The samples were heated at 100 C for 15 min and each was then processed as described previously. The data (Tables 3 and 4) show that the fatty acid composition of freshly grown cells is essentially the same as that found for lyophilized cells (Tables 1 and 2). Values for the hydroxy fatty acids were again higher in samples processed by methods A and B than in those processed by method C. The degradation of C_{19} cyclopropane acid in freshly grown cells of *P. diminuta* processed by method C was also observed (Table 3). As noted with lyophilized cells, this destruction was more pronounced with BF_3-CH_3OH than with BCl_3-CH_3OH. Repeated experiments with freshly grown cells of each culture processed with the 15-min heating period gave results essentially identical to those in Tables 3 and 4.

DISCUSSION

Boron trihalides in methanol are widely used reagents for esterification of carboxylic acids and for transesterification of various lipids. Several reports, however, have indicated that these reagents are not satisfactory for some applications and, in fact, cause extensive destruction of unsaturated and cyclopropane fatty acids (8-10). Artifacts, which appear to be methoxy-substituted fatty acid esters, have been observed when highly purified standards of oleic acid or triolein were treated with BF_3-CH_3OH reagent (10). This reagent has also been shown to cause extensive degradation of cyclopropane acids in *E. coli* and in lipids which contain dihydrosterculic acid (5). The artifacts from the degradation of cyclopropane acids were identified by mass spectrometry as methoxy-methyl esters (13).

The results from this study clearly show the limitations of boron trihalide reagents for direct transesterification of bacterial cells (method C). In addition to destruction of the C_{19} cyclopropane acid, the recovery of cellular hydroxy fatty acids with the transesterification procedure was less than that with saponification. Transesterification with either BF_3-CH_3OH or BCl_3-CH_3OH resulted in degradation of the C_{19} cyclopropane acid although the destruction was somewhat less with BCl_3-CH_3OH (Table 1). The reaction products from the destruction of the acid (Fig. 1) were not identified; however, they may represent a series of isomeric me-

TABLE 3. *Percentages of major fatty acids of freshly grown cells of P. diminuta*

Method	Fatty acids														
	3-OH 12[a]	Un	14	15	16:1	16:0	17	18:1	18	Un	19 cyc	Un	Un	Un	Un
A	8[b]	6	4	1	3	35	1	27	3	—	12	—	—	—	—
B	7	5	3	1	2	32	1	35	1	—	13	—	—	—	—
C-(BCl₃)	4	4	2	2	2	31	2	36	3	—	10	—	4	—	—
C-(BF₃)	3	3	3	2	2	29	2	32	4	4	3	2	6	2	3

[a] See footnote to Table 1.
[b] See footnote to Table 1.

TABLE 4. *Percentages of major fatty acids of freshly grown cells of P. maltophilia*

Method	Fatty acids														
	10[a]	i 11	i 2-OH 11	i 3-OH 11	2-OH 12	3-OH 12	Un	14	i 15	16:1	16	Un	Un	18:1	18:0
A	2[b]	6	5	6	1	4	1	14	33	13	7	2	2	2	2
B	2	8	6	8	1	5	2	10	34	7	11	2	3	1	TR
C-(BCl₃)	1	4	4	3	1	1	2	8	36	13	15	4	5	2	1
C-(BF₃)	TR	2	2	4	1	1	2	10	28	16	18	5	6	4	1

[a] See footnotes to Tables 1 and 2.
[b] See footnotes to Table 1 and 2.

thyoxy fatty acid esters similar to those produced when the acid was heated with 50% BF₃-CH₃OH (13).

Results obtained with methods A and B were consistent and reproducible. No destruction of the cyclopropane acid was observed and the recovery of hydroxy acids was substantially higher with these procedures than with transesterification. These results confirm that acid or base hydrolysis is required to release hydroxy acids which generally are tightly bound through ester or amide linkages to other cellular components (3, 17). Since a number of bacteria are known to contain hydroxy or cyclopropane acids or both (2, 3, 14), it is clear that an hydrolysis procedure such as method A or B is essential for accurate and meaningful analysis of cellular fatty acids.

Although the results from methods A and B were comparable, we recommend method B because the entire procedure (saponification, methylation, and extraction of methyl esters) is carried out in a single test tube. We also recommended BCl₃-CH₃OH reagent for methylation since some destruction of cyclopropane acids was observed when samples prepared by methods A and B were methylated with BF₃-CH₃OH. The time of methylation should not exceed 5 min and the temperature should be maintained between 85 and 100 C. The speed, the simplicity, and the fact that freshly grown cells can be analyzed directly are features which indicate that method B can be applied on a routine basis. The analysis of bacterial fatty acids will provide the microbiologist with additional information which will be useful for rapid identification of a variety of microorganisms (6, 7, 15, 16, 19, 20).

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