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Preparation of Bacterial Fatty Acid Methyl Esters for Rapid Characterization by Gas-Liquid Chromatography

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Our recent survey (B. L. Brian and E. W. Gardner, *unpublished data*) of fatty acids extracted from a variety of bacterial species (*Escherichia coli, Staphylococcus aureus, Sarcina lutea, Serratia marcescens, Salmonella shottmuelleri, Proteus vulgaris, Bacillus subtilis, Pseudomonas fluorescens*, and several strains of *Vibrio cholerae*) called for a rapid, reproducible technique for extraction, esterification, and analysis of these substances. An extraction procedure was devised; several esterification methods were compared by gas-liquid chromatographic analysis and are here described.

Trypticase Soy Broth (BBL) was chosen as standard culture medium because of low lipid content (C. K. Huston and P. W. Albro, J. Bacteriol. **88**:425, 1964; P. Macleod et al., J. Bacteriol. **83**:806, 1962) and the ability to sustain a variety of organisms. Eighteen-hr broth cultures of bacteria were transferred to Roux flasks containing 3% agar medium. The flasks were incubated for 24 hr at 37 C. The cells were washed off the agar with 0.85% NaCl, centrifuged at 1,750 × *g*, and washed free from the medium with saline. They were then dried in vacuo over $CaCl_2$, powdered with mortar and pestle, and suspended in 30 volumes of $CHCl_3$-CH_3OH (2:1, v/v). The suspension was treated sonically for 15 min at maximal power output (Bronwill Biosonik) and filtered. A second extraction with 30 volumes of $CHCl_3$-CH_3OH (2:1, v/v) was combined with the first, and the total extract was washed by the method of J. Folch, M. Lees, and G. N. Sloane-Stanley (J. Biol. Chem. **226**:497, 1957). Remaining solvent was evaporated in a stream of nitrogen.

Methyl esters of fatty acids from single samples of extractable lipid were prepared by three methods.

(i) Lipids (75 to 100 mg) were refluxed with 10 ml of KOH-CH_3OH-H_2O (3:90:10, w/v/v) for 30 min; the mixture was shaken with 30 ml of petroleum ether (boiling point, 38 to 51 C) and 20 ml of water in a 125-ml separatory funnel. Lower phase was acidified to *p*H 2.0 with 50% H_2SO_4, and fatty acids were extracted with 30 ml of petroleum ether. The solvent was removed with a stream of nitrogen, and fatty acids were esterified with 5 ml of BCl_3 (10%) in methanol (Applied Science Laboratories, College Station, Pa.) by the method of L. D. Metcalfe and A. A. Schmitz (Anal. Chem. **33**:363, 1961).

(ii) Lipids were directly esterified with 5 ml of 1.0% H_2SO_4 in methanol by refluxing for 30 min followed by addition of 3 ml of water. Esters were extracted with 10 ml of $CHCl_3$.

(iii) Lipids were esterified directly by boiling for 5 min with 5 ml of BCl_3-CH_3OH (J. I. Petersen, H. de Schmertzing, and K. Able, Abstr. 142nd Meeting, Am. Chem. Soc., p. 21B, 1962). Three ml of water was added, and esters were extracted with 10 ml of $CHCl_3$.

An Aerograph model 204-1B (Varian Aerograph) gas-liquid chromatograph equipped with stainless steel columns (10 × 1/8 inch) packed

TABLE 1. *Relative percentage of methyl esters of fatty acids with different esterification methods*

Fatty acid[a]	(1) (KOH—CH_3OH and BCl_3—CH_3OH)	(2) (H_2SO_4—CH_3OH)	(3) (Direct BCl_3—CH_3OH)
14:0	5.0	4.0	3.3
14:1	0.5	0.5	0.6
15:0	0.5	0.4	0.6
A[b]	0.7	0.9	0.8
16:0	31.8	34.2	31.2
16:1	40.2	37.8	40.3
17:0	0.9	0.8	1.1
B[b]	0.9	1.0	1.2
18:0	4.1	3.8	3.9
18:1	15.6	16.6	16.9

[a] Numbers preceding colon designate number of carbons and number following indicates degree of unsaturation.

[b] Unidentified components.

with 20% diethylene glycol succinate polyester on 60/80 mesh Chromosorb W was used for fatty acid analyses. The column was maintained isothermally at 180 C. Comparable results were obtained with the three esterification procedures as suggested from data of *V. cholerae* strain NIH 41 (Table 1).

Esterification with methanolic H_2SO_4 or BCl_3-CH_3OH (methods 2 and 3) does not remove unsaponifiable materials which may produce trace peaks when analyzed by gas-liquid chromatography. These peaks, however, do not interfere significantly with the profile pattern of the chromatograms obtained, and thus do not decrease their value for chemical-taxonomic characterization. Direct esterification with BCl_3-methanol (method 3) was preferred, and is recommended for survey work because of the speed with which the esterification is effected.

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