

AEROBIC BACTERIA by GC-FAME

0801

METHOD: 0801, Issue 1

EVALUATION: N/A

Issue 1: 15 January 1998

PROPERTIES: viable and culturable, requires oxygen

SYNONYMS: Gram positive (+) bacteria, gram negative (-) bacteria, *Mycobacterium* species (sp)

SAMPLING		MEASUREMENT	
SAMPLER:	ANDERSEN IMPACTOR (15 × 100-mm culture plates containing TSBA media)	TECHNIQUE:	GAS CHROMATOGRAPHY, FID
FLOW RATE:	28.3 L/min [1]	ANALYTE:	fatty acid methyl esters (FAME) of aerobic bacteria or <i>Mycobacterium</i> sp
VOL-MIN:	50 L	DESORPTION:	1 mL hexane/MTBE (1:1)
-MAX:	300 L	INJECTION VOLUME:	2 µL
SHIPMENT:	keep cold, ship overnight.	TEMPERATURE-INJECTION:	250 °C
SAMPLE STABILITY:	transfer bacteria to fresh culture media weekly	-DETECTOR:	300 °C
BLANKS:	not applicable	-COLUMN:	170 °C to 270 °C (5 °C/min)
ACCURACY		CARRIER GAS:	helium, 2.4 mL/min
RANGE STUDIED:	not applicable	COLUMN:	capillary, fused silica, 25 m × 0.20-mm ID, 0.33-µm film, Ultra-2 [2]
BIAS:	not applicable	CALIBRATION:	MIDI fatty acid methyl ester calibration mix (containing various C9–C20 fatty acids)
OVERALL PRECISION (\hat{S}_r):	not applicable	IDENTIFICATION:	comparison with profile library
ACCURACY:	not applicable	ACCEPTABLE GENUS IDENTIFICATION:	similarity index (SI) > 0.30
		ACCEPTABLE SPECIES IDENTIFICATION:	SI > 0.50

APPLICABILITY: This method is applicable to all viable and culturable bacteria containing C9–C20 fatty acids. The method is applicable to bulk solid and liquid samples containing culturable bacteria, as well as air samples.

INTERFERENCES: No specific interferences were identified. However, any fungi, yeasts, or other source of fatty acid materials will affect identification of bacteria. Additionally, any organic contaminants will interfere with the identification process.

OTHER METHODS: Method 0800, Bioaerosol Sampling, is a general procedure for sampling bioaerosols in air.

REAGENTS:

1. Sodium hydroxide pellets (NaOH),* reagent grade.
2. Methanol,* GC/HPLC grade.
3. Hydrochloric acid (HCl),* 6 N.
4. Sodium chloride (NaCl), reagent grade.
5. Hexane,* GC/HPLC grade.
6. Methyl-*t*-butyl ether (MTBE),* GC/HPLC grade.
7. Sodium sulfate, ultrapure grade.
8. TSA nutrient agar.
9. Granulated agar.
10. TSBA agar. Dissolve 30 g trypticase soy broth and 15 g granulated agar to 1 L deionized water.
11. Saponification reagent. Dissolve 45 g NaOH in 150 mL methanol and 150 mL deionized water.
12. Methylation reagent. Mix 325 mL 6 N HCl with 275 mL methanol.
13. Extraction reagent. Mix 200 mL hexane with 200 mL methyl-*t*-butyl ether.
14. Basic wash solution. Dissolve 10.8 g NaOH in 900 mL deionized water.
15. Saturated NaCl solution.
16. MIDI FAME calibration solution (MIDI, Inc., Newark, DE)

*See SPECIAL PRECAUTIONS

EQUIPMENT:

1. Sampler: Andersen impactor, 15 × 100-mm culture plates containing TSBA culture media.
2. Sampling pump, 28.3 L/min, with flexible tubing.
3. Gas chromatograph, flame ionization detector, Ultra-2 capillary column, and microbial identification system (MIS) (page 0801-1).
4. Water baths, 80 °C, 100 °C, and room temperature.
5. Ice bath.
6. Vortex mixer, test tube .
7. Hematology mixer.
8. Test tubes, screw cap, 13-mm × 100 mm.
9. Incubator with humidity adjustment (100%), set at 28 ± 1 °C.
10. Glass beads, 3-mm.
11. Glass pipettes, disposable.
12. Dispensette bottles, 4.
13. Platinum innoculating loop, 4-mm.
14. Autoclave.
15. Autoclave biohazard bags.
16. Bactoincinerator.
17. Refrigerant packs.

SPECIAL PRECAUTIONS: Sodium hydroxide is caustic and may cause burns. Hydrochloric acid causes severe burns. Methanol, hexane, and methyl-*t*-butyl ether are flammable. Methanol is toxic by ingestion. Handle all bacterial cultures in approved biosafety cabinet, level II minimum. Wear appropriate eye protection, rubber gloves, and lab coat/apron.

SAMPLING:

1. Calibrate each pump with a representative sampler in line.
 2. Attach sampler to pump with flexible tubing.
 3. Sample at an accurately known flow rate at 28.3 L/min for a total sample size of 50 to 300 L.
 4. Remove culture plates from sampler, cover, and pack securely for shipment (media side up).
- NOTE: Keep samples cool, but protect from freezing.

SAMPLE PREPARATION:

5. Isolate individual bacteria by pure culture technique.
NOTE: See APPENDIX for *Mycobacterium* conditions.
6. Select a single pure colony from unknown field samples and inoculate the method-specific TSBA agar using the quadrant streaking technique.
NOTE: Identification with the clinical library of the FAME system requires incubation on blood/chocolate agar plates at 35 °C.
 - a. Incubate at 28 °C for 24 to 48 hours.

- b. Harvest approximately 40 mg (either by weighing into test tube or harvesting an amount about the size of a half moon on the platinum loop) of the pure cultured bacteria from the 3rd quadrant (or quadrant with confluent growth).
 - c. Place into a 13-mm × 100-mm test tube and cap.
NOTE: A harvest of 40 mg should ideally correspond to approximately a total area count of 300,000 as measured on the GC chromatogram.
7. To each test tube, add 1 mL of saponification reagent and tightly cap.
 - a. Vortex 30 seconds, then place in a 100 °C water bath for 5 min.
 - b. Remove from water bath, vortex for 30 seconds, and replace in the water bath for 25 min to complete the saponification process.
 8. Cool test tubes in a water bath (room temperature).
 - a. Add 2 mL of methylation reagent and cap tightly.
 - b. Vortex for 30 seconds and place in an 80 °C water bath for exactly 10 min.
 9. Cool the test tubes in an ice bath for several minutes.
 - a. Add 1.25 mL of extraction reagent and cap tightly.
 - b. Place the test tubes in the hematology mixer and mix end over end for 10 min.
 - c. Remove the bottom layer by pipetting and add 3 mL of basic wash mixture. Mix end over end for 5 min.
 10. Remove the top layer (except for *Mycobacterium* analyses) by pipetting, transfer to an autosampler vial, and attach a crimp cap.
NOTE: If no definitive separation occurs, add several drops of saturated sodium chloride solution and agitate to facilitate separation.

CALIBRATION AND QUALITY CONTROL:

11. Calibrate daily with a fresh solution of the MIDI FAME calibration standard. The system automatically recalibrates after every ten injections.
12. Use *Xanthomonas maltophilia* as a positive QC culture (SI > 0.90). Other bacterial cultures such as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Micrococcus roseus*, and *Mycobacterium smegmatis* (SI > 0.80) serve as suitable blind QC cultures.

MEASUREMENT:

13. Set gas chromatograph according to manufacturer's recommendations and to conditions given on page 0801-1. Inject a 2- μ L sample aliquot with an autosampler.
14. The FAME profile generated for each unknown bacteria analyzed is electronically compared to a computer generated library containing the fatty acid profiles of over 5,000 bacteria. Bacterial identifications are generated for each sample and ranked in order based upon similarity indices.
NOTE: Identification is based on comparison with a profile library; therefore, sample identification is not definitive. The similarity index (SI) indicates how closely the sample compares to known bacteria in the library collection.

EVALUATION OF METHOD:

Approximately 500 analyses of bacterial cultures comprising 40 different genus and 80 plus species were completed in the evaluation of this method [3,4]. Overall accuracy of the GC-FAME-MIS in this evaluation was > 98%. Correct identification of *Mycobacterium* cultures was highly dependent upon the addition of glycerol to the Middlebrook 7H10 culture media.

REFERENCES:

- [1] Jensen PA, Scarpino P [1992]. Evaluation of eight bioaerosol samplers with bacteria. *Am Ind Hyg Assoc J* 53(10):660–667.
- [2] Sasser M [1990]. Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note # 101.
- [3] Pendergrass SM, Jensen PA [1997]. Application of the gas chromatography-fatty acid methyl ester (GC-FAME) system for the identification of environmental and clinical isolates of the family *Micrococcaceae*. *Appl Occup Environ Hyg* 12(8):543–546.
- [4] Schafer M, Pendergrass SM [in preparation]. Identification and differentiation of selected members of the genus *Mycobacterium* by gas chromatography-fatty acid methyl ester (GC-FAME) analysis. *J Applied and Environmental Microbiology*.

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APPENDIX. MYCOBACTERIUM CONDITIONS AND CULTURE MEDIA.

For the analysis of *Mycobacteria*, follow the method as written with exception of the following steps.

- Step 6. Culture media: Middlebrook 7H10 culture media containing Middlebrook OADC Enrichment (with 0.5% glycerol).
Incubation: 35 °C in the presence of 5 to 10% CO₂ for 2 to 14 days; slow growing cultures like *Mycobacterium tuberculosis* may require up to six weeks.
- Step 7. Vortex mixing: Add 3 to 5 glass beads to the mixture prior to vortexing.
- Step 9. Extraction: For *Mycobacterium* analyses, remove the top layer and add approximately 10 mg of sodium sulfate to remove any water from the FAME solution.
- Step 10. Transfer: The FAME solution is pipetted to a new autosampler vial. Take care not to carry over any sodium sulfate, and attach crimp cap.
- Step 12. Quality control: Use *Mycobacterium smegmatis* as a positive QC culture (SI > 0.80) for *Mycobacterium* analyses.