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Internal Standards for Gas Chromatographic Analysis of Metabolic End Products from Anaerobic Bacteria

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2-Methylpentanoic acid and benzoic acid are suggested for use as routine internal standards for gas chromatographic analysis of microbial end products.

Analysis of short-chain organic acid production in bacteriological media is useful in the taxonomy of certain anaerobic bacteria (1). The routine addition of internal standards may facilitate the identification and quantitation of these acids. Furthermore, the standard acids can function as monitors of the analytical techniques (2).

The volatile marker 2-methylpentanoic acid (α-isocaproic) and the nonvolatile marker benzoic acid were stocked separately as their sodium salts in 0.1% aqueous solutions. The 1.0-ml samples of acidified broth culture drawn for volatile and nonvolatile analyses were each supplemented with 0.1 ml of the appropriate standard. Volatile acids were directly extracted from one tube with 1.0 ml of diethyl ether. The other tube was supplemented with 1.0 ml of boron trifluoride methanol (Applied Science), incubated for 30 min at 80°C, and extracted with 0.5 ml of chloroform. Volumes, 2.0 μl, of each organic phase were injected.

A Shimadzu GC4BMPF gas chromatograph with flame ionization detectors was operated at an input sensitivity of 10^2 MΩ and an output range of 320 mV. Chromatograms were drawn by a Shimadzu R201 strip chart recorder (5.0 mm/min). The silanized Pyrex columns (4 m long by 3-mm ID) contained 6% Carbowax 20M-terephthalic acid coated onto 80/100-mesh Gas-Chrom Q (Applied Science). Operating conditions included: injection and detector blocks, 200°C; oven, 170°C (isothermal); nitrogen, hydrogen, and air flows, respectively, at 37, 30, and 350 ml/min.

Figure 1 depicts chromatograms of known acid mixtures and illustrates the elution relationships between the marker compounds and other acids of interest. The appropriate marker is clearly resolved from the nine volatile acids and the six methylated acids.

The retention times of the volatile acids relative to 2-methylpentanoic acid are: acetic acid, 0.28; propionic acid, 0.39; isobutyric acid, 0.45; butyric acid, 0.57; isovaleric acid, 0.67; valeric acid, 0.89; 2-methylpentanoic acid, 1.0; 3-methylpentanoic acid, 1.11; 4-methylpentanoic acid, 1.15; and hexanoic acid, 1.35. The retention times of the derivatized (nonvolatile) acids relative to benzoic acid are: pyruvic acid, 0.18; lactic acid, 0.25; oxalic acid, 0.37; malonic acid, 0.56; fumaric acid, 0.70; succinic acid, 0.79 and benzoic acid, 1.0.

Chromatograms from organic extracts of a 24-h broth culture of *Bacteroides fragilis* subsp. *vulgatus* are shown in Fig. 2. The presence of each marker gives a clear internal reference point for the volatile acids (acetic, propionic, isobutyric, butyric, and isovaleric) and the methylated acids (lactic, oxalic, malonic, fumaric, and succinic). Two deflections of the methylated trace that do not conform to elution times of known compounds are recorded relative to methyl benzoic acid: I, 0.63; II, 1.58.

We have recovered neither 2-methylpentanoic acid nor benzoic acid from clinical anaerobic isolates. These substances are not significant in routine classification and identification of anaerobic bacteria (1).

Internal standardization may be useful in culture analysis. The observation of unknown eluants may be made more systematic and meaningful by recording them as relative retention times. Comparisons between the sensitivity of flame ionization and thermal conductivity traces may be roughly clarified by relating the areas of marker peaks. In quantitative studies the internal standards may be useful foundations for ratio recovery curves or molar response correction factors. This is especially significant when highly volatile solvents such as diethyl ether are used. However, due to disparate partitioning of the volatile acids into diethyl ether and to variations in methylation efficiencies among the nonvolatile acids, it would be necessary to carefully define a fixed quantitative relationship between chromatographic recovery of each short-chain organic

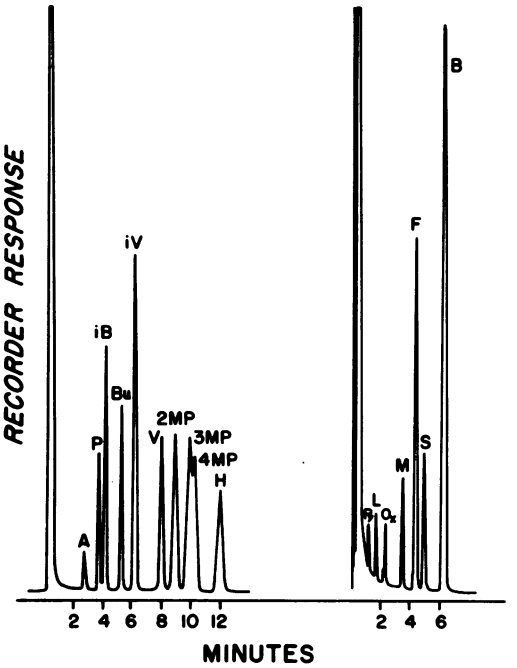

FIG. 1. *Volatile (pattern on left) and nonvolatile (pattern on right) chromatograms of known acid mixtures. A, Acetic; P, propionic; iB, isobutyric; Bu, butyric; iV, isovaleric; V, valeric; 2MP, 2-methylpentanoic; 3MP, 3-methylpentanoic; 4MP, 4-methylpentanoic; H, hexanoic; Py, pyruvic; L, lactic; Ox, oxalic; M, malonic; F, fumaric; S, succinie; B, benzoic.*

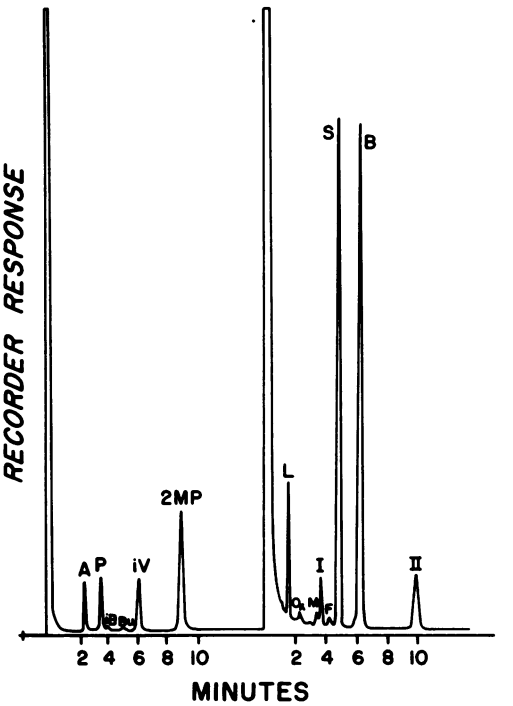

FIG. 2. *Volatile (pattern on left) and nonvolatile (pattern on right) chromatograms of extracts from B. fragilis broth culture. A, Acetic; P, propionic; iB, isobutyric; Bu, butyric; iV, isovaleric; 2MP, 2-methylpentanoic; L, lactic; M, malonic; F, fumaric; S, succinic; B, benzoic. Recorded relative to benzoic: I, 0.63; and II, 1.58.*

acid and that of the appropriate internal standard. Semiquantitative comparisons between cultures may be simplified by first comparing marker peak areas. The presence and area of the markers give positive assurance that the techniques of sample preparation and analysis were correct. This feature is notably helpful when no metabolic acids are recoverable from the broth culture.

LITERATURE CITED

1. **Holdeman, L. V., and W. E. C. Moore.** 1972. Anaerobe laboratory manual. Virginia Polytechnic Institute and State University Anaerobe Laboratory, Blacksburg.
2. **Littlewood, A. B.** 1970. Gas chromatography principles, techniques, and applications. Academic Press Inc., New York.