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TECHNICAL NOTE

Enhanced gas chromatography-mass spectrometry method for bacterial polyhydroxyalkanoates analysis

Giin-Yu Amy Tan,[1,2] Chia-Lung Chen,[1] Liya Ge,[1] Ling Li,[1] Lin Wang,[1,2] Lei Zhao,[1,2] Yu Mo,[1,2] Swee Ngin Tan,[3] and Jing-Yuan Wang[1,2,*]

Residues and Resource Reclamation Centre, Nanyang Environment and Water Research Institute, Nanyang Technological University, 1 Cleantech Loop, Singapore 637141, Singapore,[1] Division of Environmental and Water Resources Engineering, School of Civil and Environmental Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore,[2] and Natural Sciences and Science Education Academic Group, Nanyang Technological University, 1 Nanyang Walk, Singapore 637616, Singapore[3]

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A gas chromatography-mass spectrometry method for quantification of polyhydroxyalkanoates (PHAs), containing 4-carbon to 16-carbon monomers, even in the absence of standards, was developed. Strong linear correlations existed between PHA carbon number and retention time/response factor ($R^2 \geq 0.987$). Based on the correlations, high recovery values, between 100.5% and 114.3%, were obtained for PHA polymers.

[**Key words:** Gas chromatography; Mass spectrometry; Polyhydroxyalkanoate; 3-Hydroxyalkanoic acid; Homologous]

Polyhydroxyalkanoates (PHAs) are biopolyesters naturally synthesized by bacteria. They have attracted much commercial interest due to its biodegradability, biocompatibility, and its synthesis from renewable resources. At the molecular level, PHA is made up of (*R*)-3-hydroxyalkanoic acid repeat units of varying carbon lengths. Depending on the functional *R* group, PHAs may vary between 3 and 5 carbon atoms (short-chain length PHA, scl-PHA), 6 and 14 carbon atoms (medium-chain length PHA, mcl-PHA), and 15 or more carbon atoms (long-chain length PHA, lcl-PHA). The properties and chemical diversity of PHAs have given rise to various applications ranging from biodegradable packaging materials to medical products (1). Despite the potential benefits that PHAs may bring, the commercialization of PHAs is hindered by its high production cost (2). This has led to a considerable amount of interest to explore different means to reduce the production cost. This includes characterizing new and more efficient PHA-accumulating microorganisms or exploring cheaply available waste substrates for PHA production (3). Thus, a simple and reliable analytical method for identification and quantification of PHAs would greatly facilitate the future development of PHA-related research.

To date, many analytical methods for PHAs have been reported, e.g., nile red staining coupled with flow cytometry (4) or fluorescence spectrometry (5) and high-performance liquid chromatography (HPLC) (6) can provide quantitative information about PHAs. However, their capacity to provide qualitative information about PHA monomeric constituents is limited. Conversely, methods such as nuclear magnetic resonance (NMR) (7), and gas chromatography (GC) (8) can yield both qualitative and quantitative information about PHAs. GC-based methods are usually preferred over NMR as a first-line analytical method due to the relative ease of sample preparation and analysis, and lower cost (9).

GC coupled with flamed ionization detector (GC-FID) is one of the most commonly-used methods to identify and quantify PHAs (8), however, the robustness of GC-FID is greatly dependent on the inclusion of appropriate PHA analytical standards. On the other hand, GC coupled with mass spectrometer (GC-MS) enables putative PHAs to be identified through the comparison of mass spectra pattern against the NIST Standard Reference Library (National Institute of Standards and Technology, Gaithersburg, MD, USA) which makes it more robust in the detection of new putative PHAs (9). Nevertheless, GC-MS can only provide a tentative identification of PHAs. Further validation using suitable PHA analytical standards is pivotal in ensuring the accuracy of the detection result.

Currently, the lack of readily- or commercially-available PHA analytical standards to represent the chemical diversity of PHAs has made their analysis particularly challenging (8,10). Existing literary descriptions of GC-MS method are mostly confined to commercially-available PHA standards (11,12). Some of the ways to circumvent this problem include the chemical synthesis of PHA analytical standards or deriving analytical standards through the biosynthesis of PHAs by well-characterized PHA accumulators (13). These approaches may be more tedious, time-consuming and expensive. This warrants a need to develop a GC-MS method that enables PHAs analysis even in the absence of analytical standards.

GC-MS quantification for hydrocarbon compounds such as PHAs is typically performed through calculating a response factor (RF) for each PHA analytical standard while the identification of PHAs is

* Corresponding author at: Residues and Resource Reclamation Centre, Nanyang Environment and Water Research Institute, Nanyang Technological University, 1 Cleantech Loop, Singapore 637141, Singapore. Tel.: +65 6790 4100; fax: +65 6792 7319.

E-mail addresses: amytangy@ntu.edu.sg (G.-Y.A. Tan), jywang@ntu.edu.sg (J.-Y. Wang).

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done by comparing the retention times (RTs) of the putative PHAs against the RTs of analytical standards (9,11). Previous studies have reported correlations between the carbon number (i.e., molecular weight) of homologous hydrocarbon series and RF (14,15), as well as between carbon number and retention time (16). These correlations can help to estimate the RFs and RTs of other hydrocarbon homologues for which analytical standards are unavailable. The present study postulated that such correlations may also exist between the carbon number and RF/RT for homologous PHAs. Based on this postulation, the objective of the present study was to develop a GC-MS method that enables reliable qualitative and quantitative analysis of PHAs in the absence of reference standards.

To meet the objective, homologous saturated PHA monomers 3-hydroxyalkanoic acids were chosen to test this study's postulation due to the commercial-availability of these analytical standards. 3-Hydroxyalkanoic acids of varying carbon number ranging from scl-PHA monomer (3-hydroxybutyric acid, C_4), to mcl-PHA monomers (3-hydroxyoctanoic acid, C_8; 3-hydroxydecanoic acid, C_{10}; 3-hydroxydodecanoic acid, C_{12}) and lcl-PHA monomer (3-hydroxyhexadecanoic acid, C_{16}) were procured from Sigma–Aldrich (St. Louis, MO, USA). Five milligram (1250 mg/L) of each PHA monomer standard was chemically converted to their respective 3-hydroxyalkanoic acid methyl esters via methanolysis according to the procedure adapted from Oehmen et al. (12) using equal volumes of chloroform and acidified methanol (15% [v/v] H_2SO_4), and incubation at 100°C for 3 h. Methyl benzoate (5 mg/L) was included as an internal standard. Methanolyzed sample (1 μL) was injected into an Agilent HP6890 GC Series equipped with the 5975I MS detector and an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies, Palo Alto, CA, USA). The temperature of the injection port, interface, quadrupole and ion source was set at 250°C, 280°C, 120°C and 250°C, respectively. Oven temperature was programmed at an initial temperature of 40°C and subsequently raised at rate of 10°C/min to 280°C and held for 5 min. Helium carrier gas was set at a flow rate of 1.2 mL/min. Solvent delay was set at 2.5 min. MS detector using electron impact (EI) ionization at 70 eV was operated in full scans (mass range of m/z 40–600 with 0.1 mass accuracy).

The analytical response parameters of the method for the various 3-hydroxyalkanoic acid methyl esters were as follow: C_4, 4.69 min; C_8, 10.88 min; C_{10}, 13.67 min; C_{12}, 16.09 min; and C_{16}, 20.28 min (Fig. 1A and Table 1). Internal standard methyl benzoate was detected at 8.53 min. The mass spectra of the 3-hydroxyalkanoic acid methyl esters were characteristic of fragmentation patterns previously reported (17) with four main fragment ions present at m/z 103, formed by an α cleavage to the hydroxyl functional group; at m/z 74, arising from McLafferty rearrangement; at m/z 71, possibly from the expulsion of methanol from m/z 103; and at m/z 43, attributed to either the saturated alkanoic portion or methyl ester moiety of the molecule (data not shown). The observed RF for each PHA monomer analytical standard was calculated using the expression:

$$\mathrm{RF} = (A_A \times C_i)/(A_i \times C_A) \quad (1)$$

where A_A is the sum of peak areas of the four main fragment ions of the PHA analytical standard, A_i is the peak area of the characteristic m/z 105 ion of the methyl benzoate internal standard; C_A and C_i are the concentrations of the PHA analytical standard and methyl benzoate internal standard, respectively. The observed RFs, based on at least three independent sample determinations ($n \geq 3$), for the various 3-hydroxyalkanoic acid methyl esters were as follows: C_4, 0.302 ± 0.023; C_8, 1.176 ± 0.375; C_{10}, 1.716 ± 0.322; C_{12}, 2.254 ± 0.323; and C_{16}, 3.078 ± 0.805 (Table 1).

The relationships between the carbon number of PHA analytical standards and their respective RTs as well as their respective RFs were analyzed using OriginPro 8.5.1 (OriginLab Corporation, Northampton, MA, USA). PHA carbon number was observed to correlate positively with both RT and RF (Fig. 2). A linear relationship was found between the carbon number of PHA monomer standards and RT (Eq. 2) with an adjusted coefficient of determination (R^2) of 0.987 (Fig. 2A). Similarly, a linear relationship was observed between PHA carbon number and RF (Eq. 3) with an adjusted R^2 of 0.997 (Fig. 2B). These results indicated strong linear relationships, suggesting that the equations may predict the RT and RF for saturated PHA monomeric homologues with reasonable accuracy. Such correlations have been previously demonstrated for homologous series of n-alkanes (15), ketones, secondary alcohols, nitrogen heterocycles, and so on (14). To our best knowledge, the present study presents the first reference demonstrating linear correlations between PHA carbon number and RT/RF under GC-MS analysis.

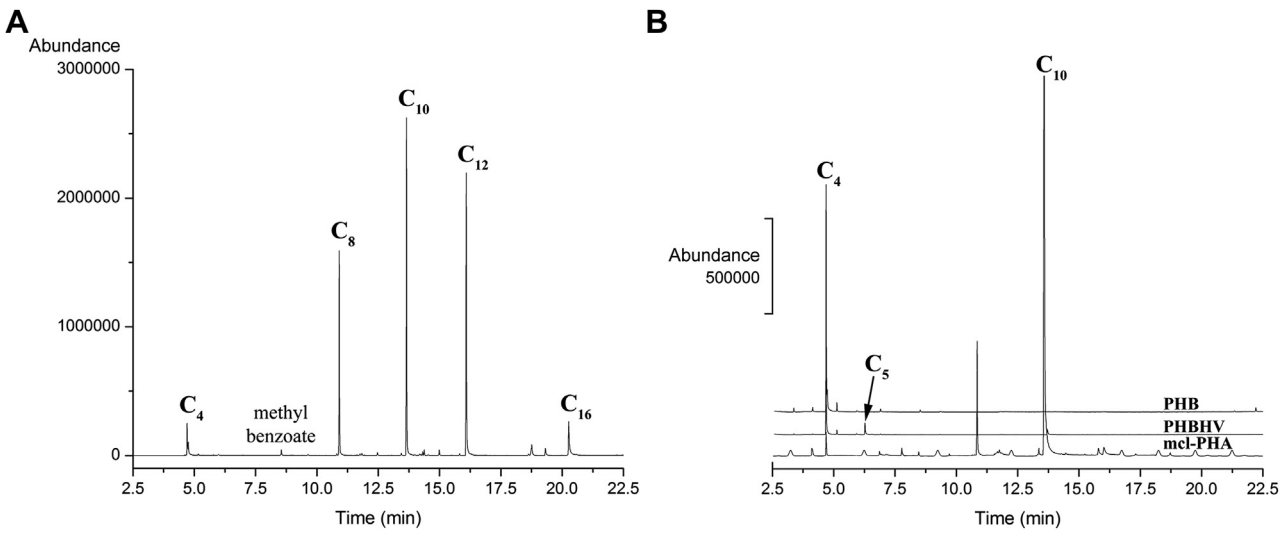

FIG. 1. Total ion chromatograms of GC-MS. (A) 3-Hydroxyalkanoic acid methyl esters derived from PHA monomer analytical standards and methyl benzoate internal standard. (B) 3-Hydroxyalkanoic acid methyl esters derived from PHA polymers PHB, PHBHV and mcl-PHA. C_4, C_5, C_8, C_{10}, C_{12}, C_{16} represent methyl esters of 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxyhexadecanoic acid, respectively.