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Enhanced Gas Chromatography-Mass Spectrometry Method for Bacterial Polyhydroxyalkanoates (PHAs) Analysis

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Abstract

A GC-MS 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.

Keywords: Gas Chromatography, Mass Spectrometry, GC-MS, Polyhydroxyalkanoate, PHA, 3-hydroxyalkanoic acid

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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 acids 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, Maryland, 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 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 RF and RT 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, C₄), to mcl-PHA monomers (3-hydroxyoctanoic acid, C₈; 3-hydroxydecanoic acid, C₁₀; 3-hydroxydodecanoic acid, C₁₂) and lcl-PHA monomer (3-hydroxyhexadecanoic acid, C₁₆) 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] H₂SO₄), and incubation at 100 °C for 3 h. Methyl benzoate (5 mg/L) was included as an internal standard. Methanolized sample (1 µL) was injected into an Agilent HP6890 GC Series equipped with the 5975I MS detector and a HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm; Agilent Technologies, Palo Alto, Calif). The temperature of the injection port, interface, quadrupole and ion source was set at 250, 280, 120 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 to 600 with 0.1 mass accuracy).

The analytical response parameters of the method for the various 3-hydroxyalkanoic acid methyl esters were as follow: C₄, 4.69 min; C₈, 10.88 min; C₁₀, 13.67 min; C₁₂, 16.09 min; and C₁₆, 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 alkanolic 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:

$$\text{Response factor, RF} = (A_A \times C_i)/(A_i \times C_A) \quad \text{Eq (1)}$$

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 follow: C₄, 0.302 ± 0.023 ; C₈, 1.176 ± 0.375 ; C₁₀, 1.716 ± 0.322 ; C₁₂, 2.254 ± 0.323 ; and C₁₆, 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 etc (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.

$$RT = 1.299 \times (\text{carbon no.}) + 0.129 \quad \text{Eq (2)}$$

$$RF = 0.235 \times (\text{carbon no.}) - 0.646 \quad \text{Eq (3)}$$

The predicted RTs and RFs for the PHA monomers were calculated using Eq (2) and Eq (3), respectively, and compared against experimentally-observed values. With the exception of C_4 monomer where the observed RT and predicted RT differed by about 13.6%, the difference for the rest of the PHA monomers was smaller and found to be within 4.0% (Table 1). The percentage difference between observed RFs and predicted RFs were between 0.7% (C_{10}) and 4.9% (C_8). Hence, the predicted RT and RF values were generally found to be a reasonable estimate of the observed values.

The predictive value of the linear relationship between carbon number and RT, as described by Eq (2), was evaluated using scl-PHA polymer standards poly(3-hydroxybutyric acid) (PHB) and poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBHV), where PHBHV contained between 11.0 to 13.0 mol% of a C_5 monomeric unit that was not included as an analytical standard in the present study. Both PHB and PHBHV were purchased from Sigma-Aldrich (St Louis, MO, USA). A mcl-PHA polymer, with composition verified by H^1 -

NMR and C^{13} -NMR as C_{10} monomeric repeats, was also included as a standard (Fig. S1). After methanolysis, PHB was chemically converted to C_4 methyl esters, PHBHV was chemically reacted to C_4 and C_5 methyl esters, and mcl-PHA was chemically reacted to C_{10} methyl esters (Fig. 1B). The RT of C_4 methyl esters from PHB was 4.69 min while that of C_4 and C_5 methyl esters from PHBHV were 4.66 min and 6.22 min, respectively. The RT of C_{10} methyl esters from mcl-PHA was 13.58 min (Table 1). The observed RTs of C_4 methyl esters derived from both PHB and PHBHV were more similar to that derived from C_4 PHA monomer standard (4.69 min) as compared to the RT value predicted by Eq (2) (5.33 min). However, the observed RT of C_5 methyl esters from PHBHV and C_{10} methyl esters from mcl-PHA were similar to the predicted RT values (C_5 , 6.62 min; C_{10} , 13.12 min) with a percentage difference of about 6.4% and 3.4%, respectively. Despite a greater discrepancy between the predicted and observed RT value for C_4 methyl esters, the present study also found that the mass fragmentation information provided by GC-MS can help to cross-validate the predicted RT value, minimizing errors in PHA detection. As such, Eq (2) proved to be useful in providing a reliable estimation for the RTs of homologous PHAs, especially when coupled with mass spectra information.

The predictive value of the linear relationship between carbon number and RF, as described by Eq (3), was evaluated in terms of method recovery using known amounts of PHA monomers. Method recovery was calculated as a percentage of deviation of the measured values from the actual values using the following expression:

$$\text{Method recovery (\%)} = [(W_m - W_A)/(W_A)] \times 100\% \quad \text{Eq (4)}$$

where W_m and W_A are the measured weight and actual weight of the PHAs, respectively. Based on predicted RF values, the estimated recovery for all the tested PHA monomers (i.e., C_4 , C_8 , C_{10} , C_{12} , and C_{16}) were between $95.3 \pm 30.4\%$ and $122.6 \pm 28.8\%$, which were similar

to the observed recoveries obtained using observed RF values (between $95.6 \pm 14.3\%$ and $124.0 \pm 26.1\%$) (Table 1).

The RF values predicted by Eq (3) were subsequently applied to quantify PHA polymers (i.e., PHB, PHBHV, and C₁₀-containing mcl-PHA). Estimated recoveries for PHB and mcl-PHA were $113.8 \pm 14.1\%$ and $100.5 \pm 10.4\%$, respectively. To quantify the estimated recovery for PHBHV, the predicted RF values for both C₄ and C₅ methyl esters had to be considered. Based on Eq (3), the RF value for C₄ and C₅ methyl esters was predicted to be 0.294 and 0.529, respectively (Table 1). Based on the predicted RF values, the estimated recovery was $114.3 \pm 4.2\%$. The estimated recovery values for all tested PHA polymers were within the range of 80 to 120%, which is the acceptable range according to the quality control criteria prescribed for most US EPA methods. In addition, the monomeric composition of PHBHV was found to be 90.6 ± 1.5 mol% of C₄ monomer and 9.4 ± 1.5 mol% of C₅ monomer, which was a reasonable estimation of the actual values (87.0 to 89.0 mol% of C₄ monomer and 11.0 to 13.0 mol% of C₅ monomer). Hence, Eq (3) can be used to estimate the RFs of PHA homologues, enabling quantification without the need to include analytical standards.

Collectively, the results suggested that the proposed method can provide a reliable identification and quantification of saturated PHA monomer homologues between the carbon number of C₄ and C₁₆. The proposed method may be particularly advantageous in facilitating the analysis of some saturated PHA monomers with odd-number carbon length for which analytical standards are less readily available or expensive to procure (10). This also eliminates the need to include a full spectrum of PHA standards, lowering the analysis cost and greatly simplifying method development.

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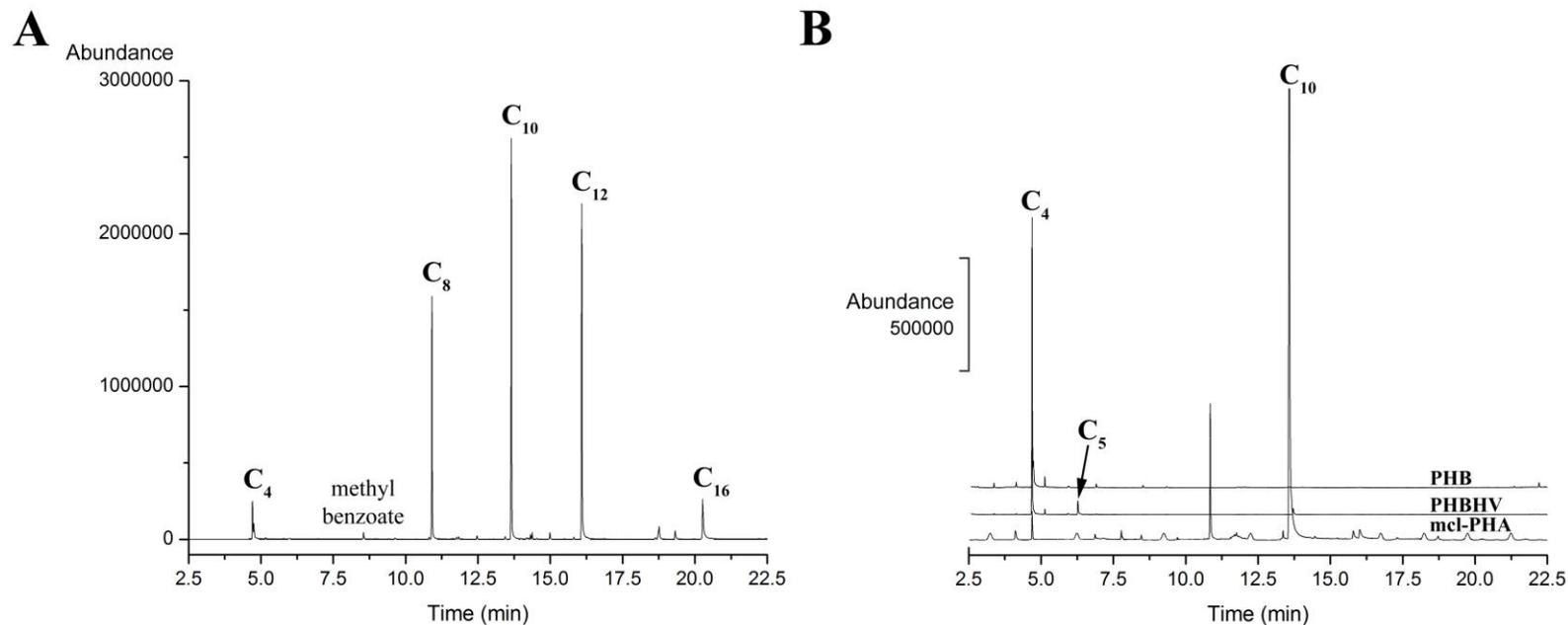


FIG. 1. Total ion chromatograms of GC-MS. (A) 3-Hydroxyalkanoic acid methyl esters derived from PHA monomer analytical standards and methyl benzoate internal standard. (B) 3-Hydroxyalkanoic acid methyl esters derived from PHA polymers PHB, PHBHV and mcl-PHA. (C₄, C₅, C₈, C₁₀, C₁₂, C₁₆ represent methyl esters of 3-hydroxybutyric acid, 3-hydroxyvaleric acid, 3-hydroxyoctanoic acid, 3-hydroxydecanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxyhexadecanoic acid, respectively.)

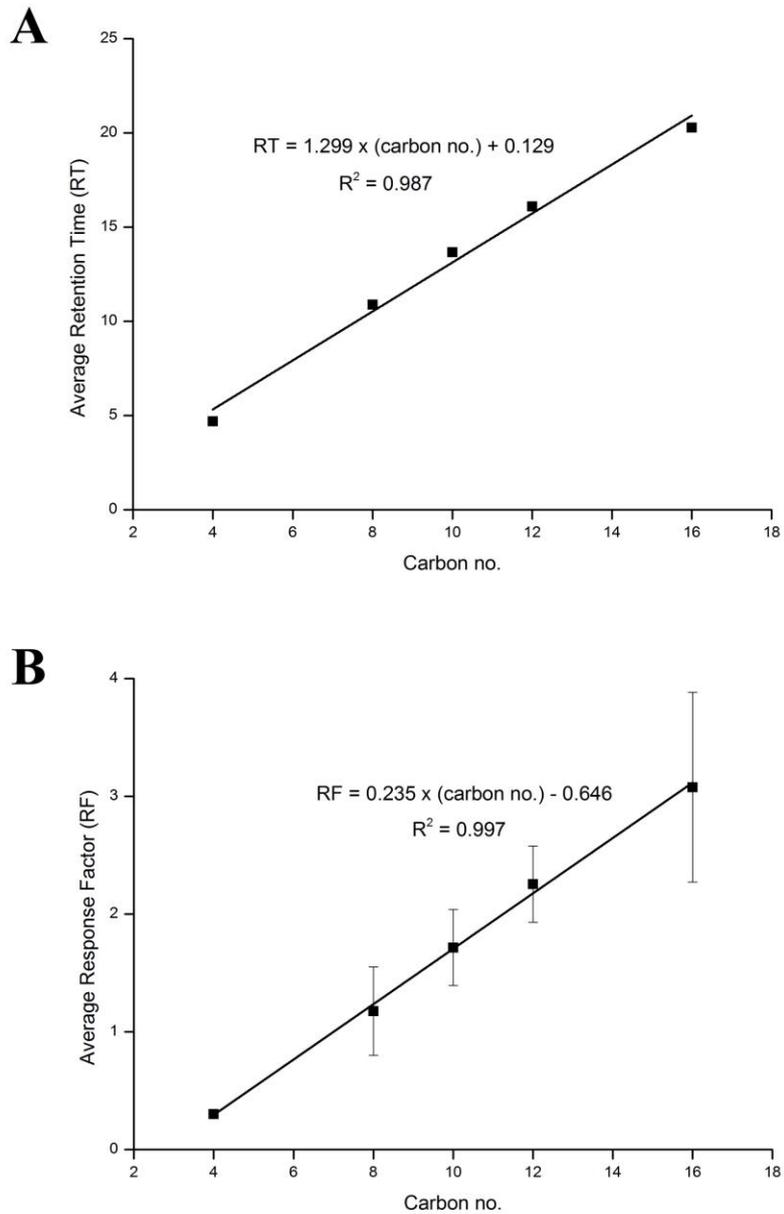


FIG. 2. Graphs depicting the linear relationship between PHA carbon number and (A) average retention time (RT), and (B) average response factor (RF). The mean values represent values from at least three independent experiments ($n \geq 3$) and the error bars represent standard deviations.

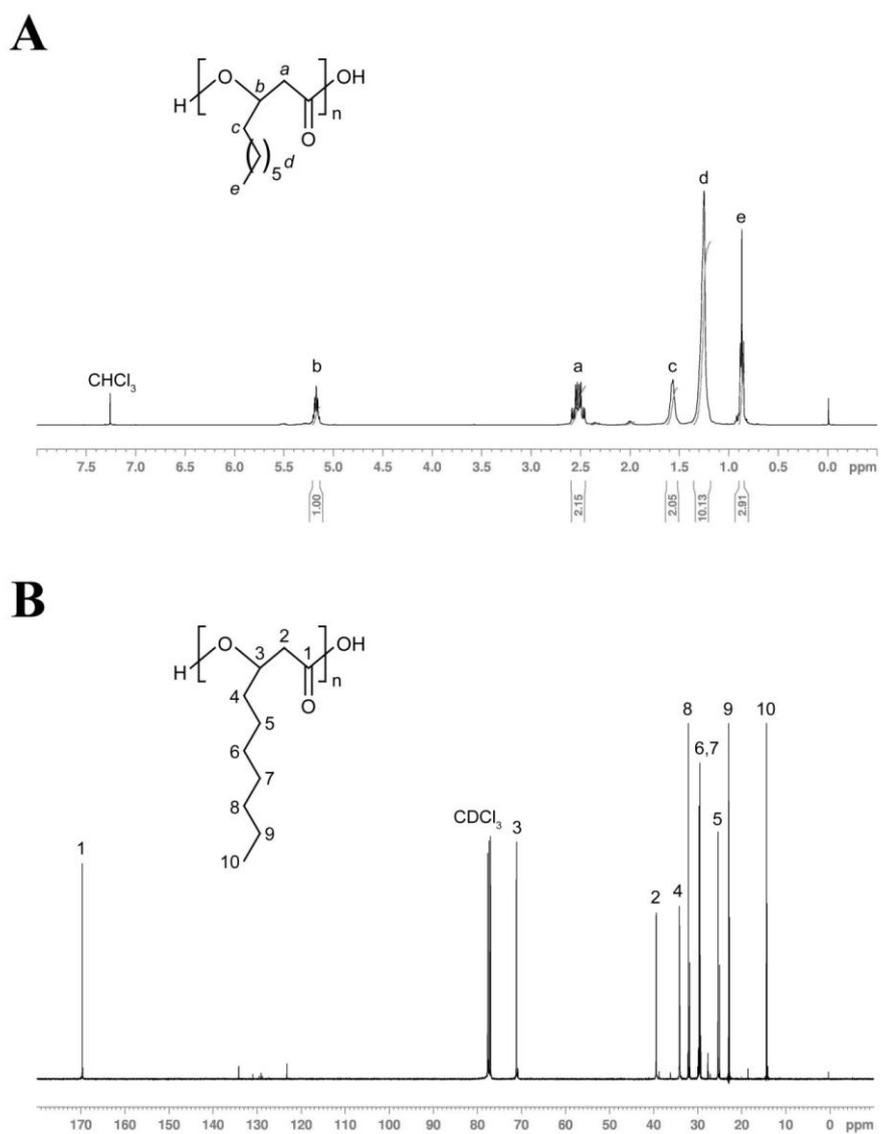


FIG. S1. (A) ^1H -NMR and (B) ^{13}C -NMR spectra of mcl-PHA polymer. The signals corresponding to hydrogen atoms in ^1H -NMR spectrum are denoted by alphabet letters while the signals corresponding to carbon atoms in ^{13}C -NMR spectrum are denoted by numericals.

TABLE 1. Analytical parameters of the developed method for PHA determination.

PHA	Observed Retention Time \pm s.d. (min) ¹	Predicted Retention Time (min) ²	Observed Response Factor \pm s.d. ¹	Predicted Response Factor ³	Actual Concentration (mg/L)	Observed Recovery \pm s.d. (%) ^{1,4}	Estimated Recovery \pm s.d. (%) ^{1,5}
Monomer							
C ₄	4.69 \pm 0.01	5.33	0.302 \pm 0.023	0.294	1250	102.3 \pm 7.2	105.1 \pm 7.4
C ₈	10.88 \pm 0.03	10.52	1.176 \pm 0.375	1.234	1250	100.0 \pm 31.9	95.3 \pm 30.4
C ₁₀	13.67 \pm 0.03	13.12	1.716 \pm 0.322	1.704	1250	100.0 \pm 18.8	100.7 \pm 18.9
C ₁₂	16.09 \pm 0.03	15.72	2.254 \pm 0.323	2.174	1250	95.6 \pm 14.3	99.1 \pm 14.9
C ₁₆	20.28 \pm 0.01	20.91	3.078 \pm 0.805	3.114	1250	124.0 \pm 26.1	122.6 \pm 28.8
Polymer							
C ₄ (PHB)	4.69 \pm 0.00	5.33	-	0.294	1275	-	113.8 \pm 14.1
C ₄ (PHBHV)	4.66 \pm 0.01	5.33	-	0.294	6925 - 7775	-	114.3 \pm 4.2
C ₅ (PHBHV)	6.22 \pm 0.00	6.62	-	0.529			
C ₁₀ (mcl-PHA)	13.58 \pm 0.01	13.12	-	1.704	1250 - 1925	-	100.5 \pm 10.4

¹ Tabulated based on results from at least three independent sample determinations ($n \geq 3$)² Tabulated using Eq (2)³ Tabulated using Eq (3)⁴ Tabulated using Observed Response Factor values⁵ Tabulated using Predicted Response Factor values