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AGREEMENT

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Procedure guidelines to determinate 3-Hydroxyvalerate Content in PHBV by Nuclear Magnetic Resonance

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CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

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European foreword

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The following organizations and individuals developed and approved this CEN Workshop Agreement:

— BOKU University. Ines Fritz

— CETEC. Fuensanta Monzó Sánchez

— CETEC. Jean David Peltier

— DIGIOTOUCH OU. Maria Aiello

— DIGIOTOUCH OU. Fernanda D'Acosta

— DURUKAN CONFECTIONERY. Bora Yesiltepe

— EUROPEAN BIOPLASTICS E.V. Estela López-Hermoso

— EUROPEAN BIOPLASTICS E.V. Lorette Du Preez

— FOOTWEAR TECHNOLOGY CENTER OF LA RIOJA. María Iñiguez

— FOOTWEAR TECHNOLOGY CENTER OF LA RIOJA. María Martínez

— INSTITUTE FOR DEVELOPMENT AND INNOVATION. Sinisa Borota

— SPANISH ASSOCIATION FOR STANDARDIZATION. Henar Araguzo Rivera

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Introduction

In the last decades, biobased polymers have gained attention from both academia and industry. They propose as a real sustainable alternative to fossil-based plastics (PE, PP, PET, PS, etc).[1] Polyesters are very promising candidates due to their interesting biodegradability profiles.[2,3] The most studied polyesters have been polylactic acid (PLA), polybutylene adipate terephthalate (PBAT), and polybutylene succinate (bioPBS). Other polyesters of interest are the polyhydroxyalkanoates (PHAs) family.[4] They are 100% biodegradable and synthesised by microorganisms from renewable carbon sources (not made by synthetic polymerisation as other biobased polymers such as PLA, bioPBS). Within the PHAs family, the poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer, also known as PHBV, has excellent properties (demonstrated biodegradation in all environments, nontoxic, flexibility-modulated through 3HV content, mechanical resistance) for different applications.

Since the ratio *3-hydroxybutyrate/3-hydroxyvalerate* defines the mechanical and thermal properties of PHBV, an accurate structural determination becomes a crucial step. In this context, techniques such as TGA-MS or GC-MS have been used in the quantification of the comonomer 3-hydroxyvalerate.[5] However, these techniques normally need a previous pyrolysis or hydrolysis step which might induce errors. Moreover, they are considered destructive analytical techniques since the sample cannot be recovered after the experiment has been conducted. As an alternative, nuclear magnetic resonance (NMR) is a very powerful technique for the non-destructive structural analysis of polymers, including the relative quantification of copolymers.[6] However, as far as we are concerned, no standardised protocols have been reported for the correct characterization of PHBV.

1 Scope

This document specifies an accessible methodology for the quantification of the comonomer content in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) using nuclear magnetic resonance as analytical tool. A rapid, simple, and non-destructive strategy is presented for the relative quantification of comonomer 3-hydroxyvalerate in PHBV.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp/

— IEC Electropedia: available at http://www.electropedia.org/

3.1
shimming
process that is carried out to correct any inhomogeneities in the applied magnetic field during a nuclear magnetic resonance (NMR) experiment

Note 1 to entry: The inhomogeneities can come from two main sources: a) Variation in the applied field due to imperfections in the main magnet, or arising from the main magnet b) Deviations in the main magnetic field due to the presence of the sample and probe in the field

3.2
relaxation delay (d1)
time period between scans

3.3
signal-to-noise ratio (S/N ratio)
ratio used to describe the quality of an NMR spectra, it is governed by the magnetic field strength, the number of scans (n), and the number of spins (N) which is related to the sample concentration

4 Important aspects to be considered for 3-hydroxyvaleratevalerate relative quantification

4.1 Spectrometer parameters

4.1.1 Shimming

Accurate shimming is critical for quantitative NMR. If the spectrum shows evidence of poor shimming (i.e. unsymmetrical or broad peaks, poor resolution, see Figure 1), shimming shall be re-done. If shimming side bands make the signal integration difficult, shimming spinning shall be turned off. If the shimming issues cannot be fixed, it shall be checked that the volume is acceptable, the sample is properly mixed, and that the tube is clean.

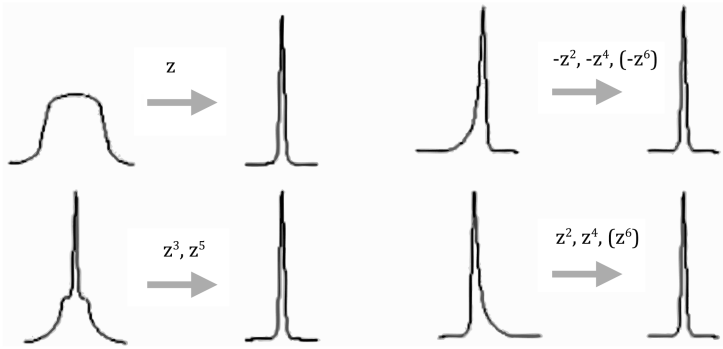


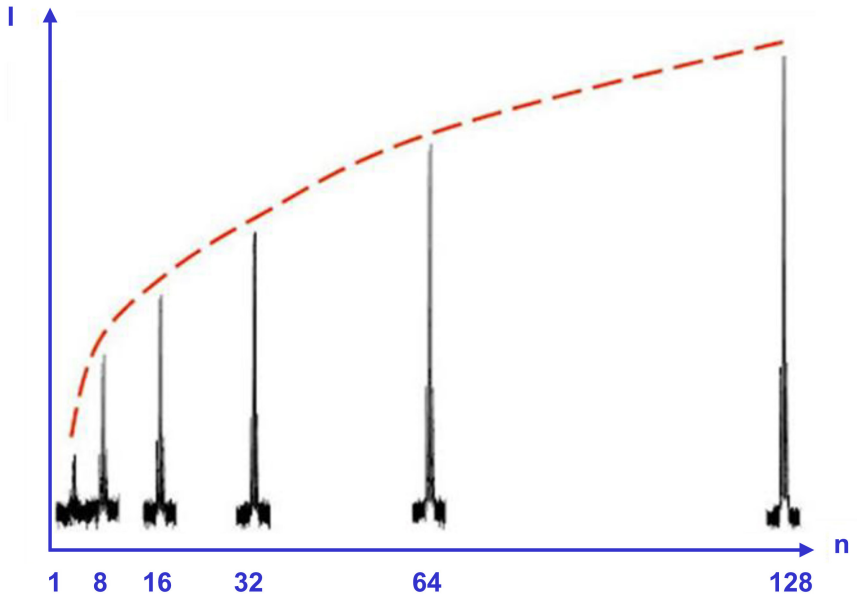
Figure 1 — Illustration of the effect of poor shimming and its resolution[7]

4.1.2 Relaxation delay

A crucial parameter to obtain a correct quantification is the relaxation delay ("d1"). The signals of interest must have relaxed fully between pulses. If the longitudinal relaxation (T1) of the individual signals can be measured accurately (i.e. inversion recovery experiment), then use a relaxation delay of at least five times T1. If accessing T1 data is not possible, a delay time sweep should be done until the signal integration gets stable.

4.1.3 Signal to noise

For accurate integration, a good signal-to-noise ratio is necessary. The number of scans should be set so as to ensure a good S/N (Figure 2).



Key

I is the ntensity

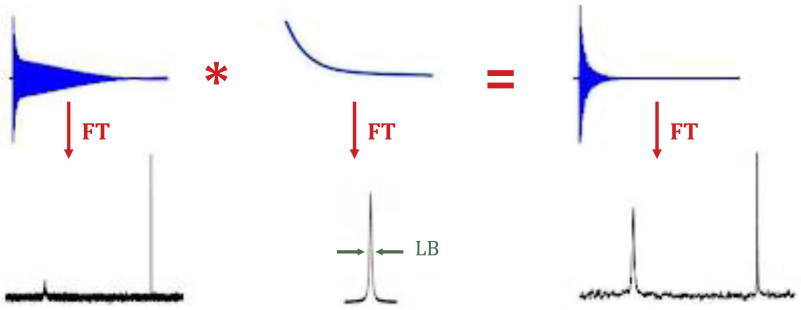
n is the number of scans

Figure 2 — Effect of the number of scans on the signal-to-noise ratio[7]

4.2 Spectrum processing

4.2.1 Line broadening

If the S/N ratio cannot be improved experimentally, line broadening can be used. This will increase the signal-to-noise ratio (but resolution will be affected) by multiplying the FID by a decaying exponential function (Figure 3). Values between 0.2 to 1 Hz are typical for 1H spectra.



Key

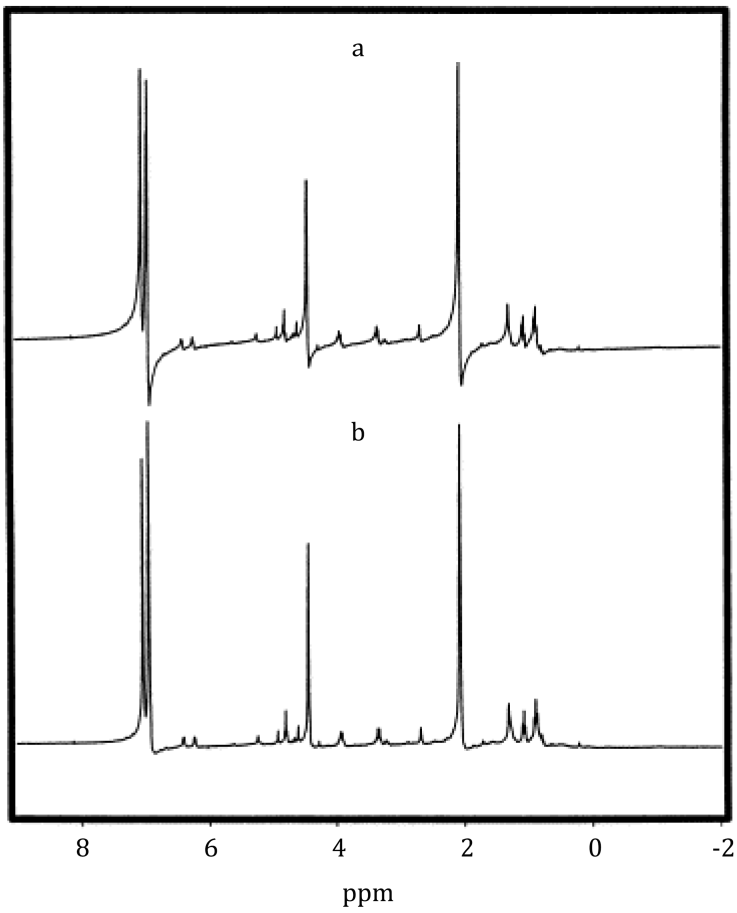
FT is the Fourier transform

LB is the line broadening

Figure 3 – Effect of line broadening on the S/N ratio

4.2.2 Accurate phasing

Even though the spectra are automatically phased, the phasing should be checked to make sure it is acceptable (see Figure 4). If not, phase correction shall be done (automatically or manually).



Key

a	is the Fourier transform NMR spectrum
b	is the result of Fourier transform NMR spectrum after automatic phase correction
ppm	are the parts per million

Figure 4 — A Fourier transform NMR spectrum (a) and the result (b) after automatic phase correction[8]

4.2.3 Baseline correction

A flat baseline is important for accurate integration. Similarly to the spectrum phase, the baseline correction is automatically applied. However, this can be corrected manually if necessary.

4.2.4 Definition of appropriate integration regions

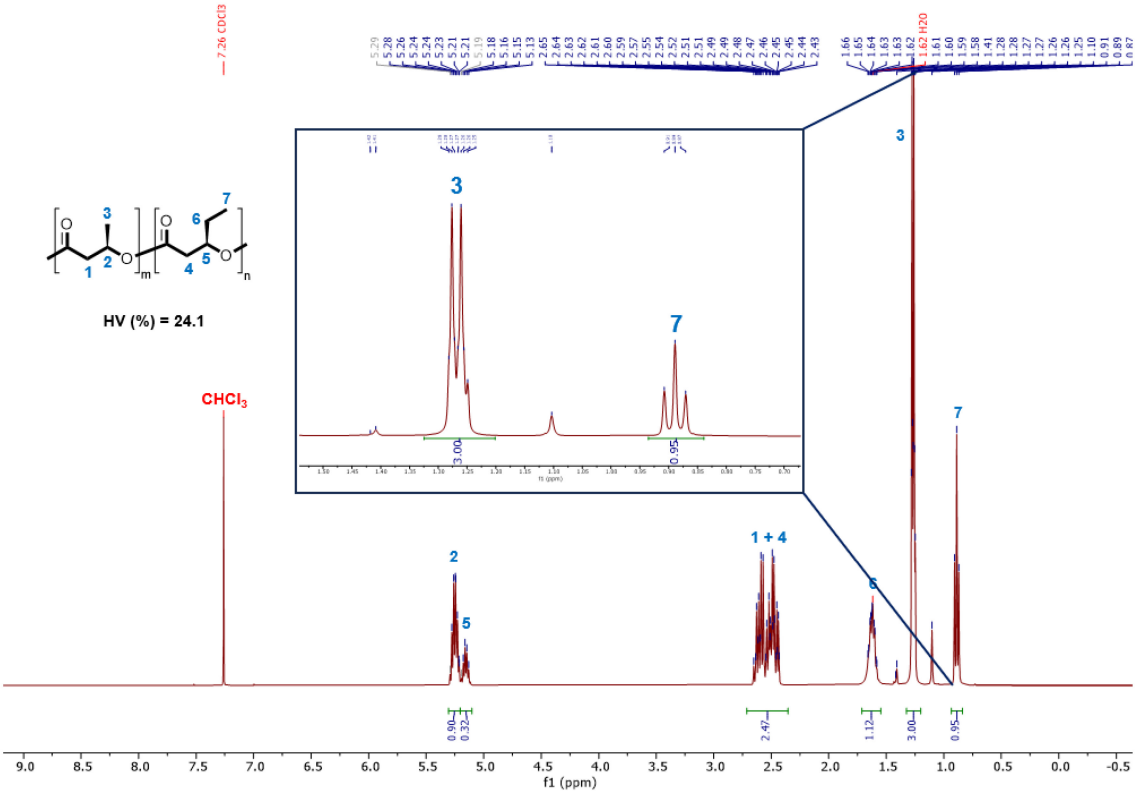
Theoretically, the integration region should cover at least 20 times the line width in each direction to ensure an accurate value. However, this is not always possible due to the presence of other signals. If that is the case, the integral limits can be reduced but the same limits should be applied to all the peaks. Moreover, if the integration process includes the 13C satellites or spinning sidebands, this must be applied to all the peaks.

5 Sample preparation

PHBV in the form of film or powder (10-15 mg) is placed in a glass vial and dissolved in deuterated chloroform (500 µL). It is recommended to use anhydrous solvent to avoid overlap of the water peak with signals attributed to the polymer. The polymer concentration must be kept within the established limits to obtain suitable spectra for quantification.

6 Results and discussion

^{1}H Nuclear magnetic resonance of one type of PHBV enriched in comonomer 3-hydroxyvalerate was carried out following the methodology described below (see supporting information > NMR methods). As an example, Figure 5 shows the spectrum of PHBV in deuterated chloroform at 25 °C and a delay time (d1) of two seconds. All the peaks have been assigned to the respective protons in the polymer structure. As can be seen, there are two well-resolved peaks ascribed to the methyl protons of the two monomers: a doublet centred at 1.27 ppm (3-hydroxybutyrate monomer) and a triplet at 0.89 ppm (3-hydroxyvalerate monomer). These are the peaks to be integrated for the relative quantification of 3-hydroxyvalerate in the polymer.



Key

HV is the percentage of the comonomer 3-hydroxyvalerate in the polymer (%)

f1 is the chemical shift (ppm)

Figure 5 — ^{1}H NMR spectrum of PHBV (400 MHz, $CDCl_3$, 25 °C, d1 = 2 s)

The percentage of 3-hydroxyvalerate can be straightforwardly obtained using the equation below:

$$\%\ \mathrm{HV} = \frac{I_7}{I_3 + I_7} \times 100 \quad (1)$$

where:

HV is the percentage of the comonomer 3-hydroxyvalerate in the polymer (%);

I_3 is the integral of the methyl protons attributed to the monomers 3-hydroxybutyrate;

I_7 is the integral of the methyl protons attributed to the monomers 3- hydroxyvalerate.

As aforementioned, a crucial parameter to keep in mind to get accurate integration values is the delay time (d1). In this context, five spectra were run increasing this parameter from 1 second up to 20 seconds. The percentage in 3-hydroxyvalerate was then calculated and the values are gathered in Table 1. No meaningful changes were observed with an increase in the delay time, suggesting fast relaxation times for the methyl protons of both monomers or a similar longitudinal relaxation profile (remember the quantification is relative to each monomer).

Table 1 — Influence of the delay time (d1) in the relative quantification of 3-hydroxyvalerate

NMR assay	Delay time (d1), s	3-HV, %
1	1	24.2
2	2	24.1
3	5	24.1
4	10	24.1
5	20	24.1

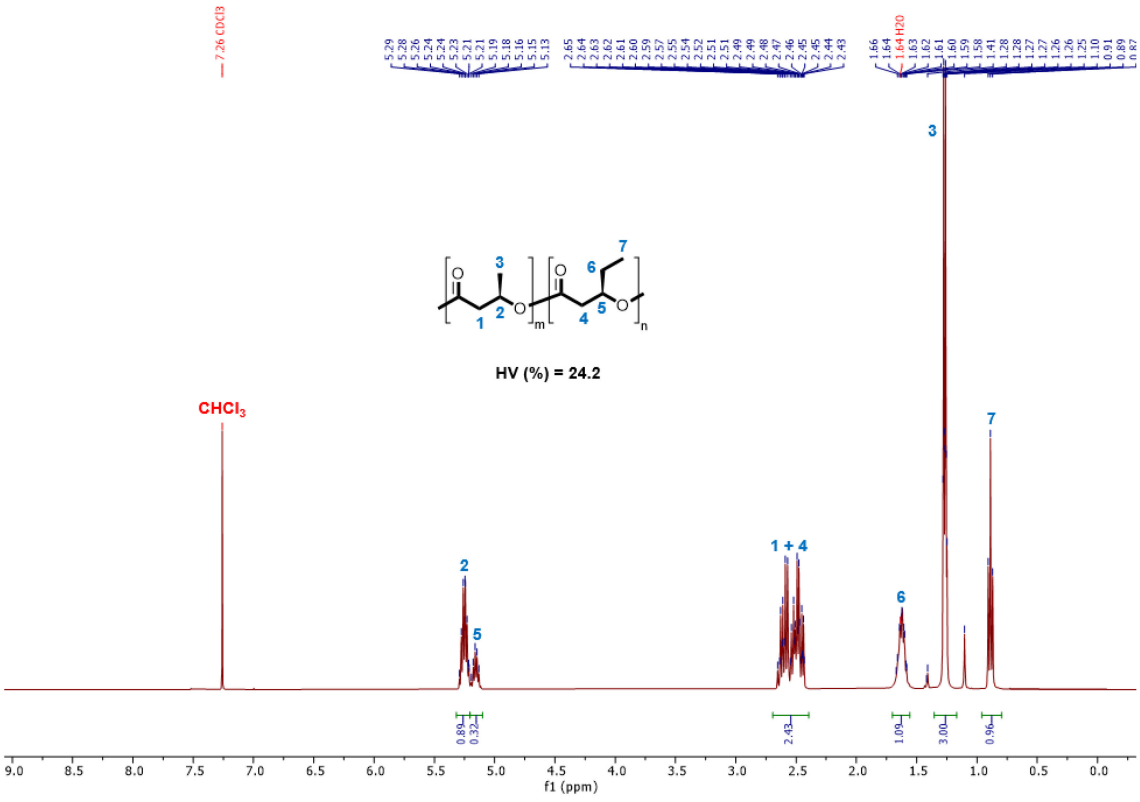
Annex A
(informative)

Supporting information

A.1 NMR method

All NMR spectra were obtained in $CDCl_3$ at 25 °C using a Bruker 400 MHz Avance Neo Nanobay spectrometer. Chemical shifts are reported in parts per million relative to the internal solvent peak ($CDCl_3$: δ 7.26). Initial delay time (d1) was fixed to 1 s and it was increased up to 2 s. A minimum of 128 scans per measurement are recommended.

A.2 NMR spectra

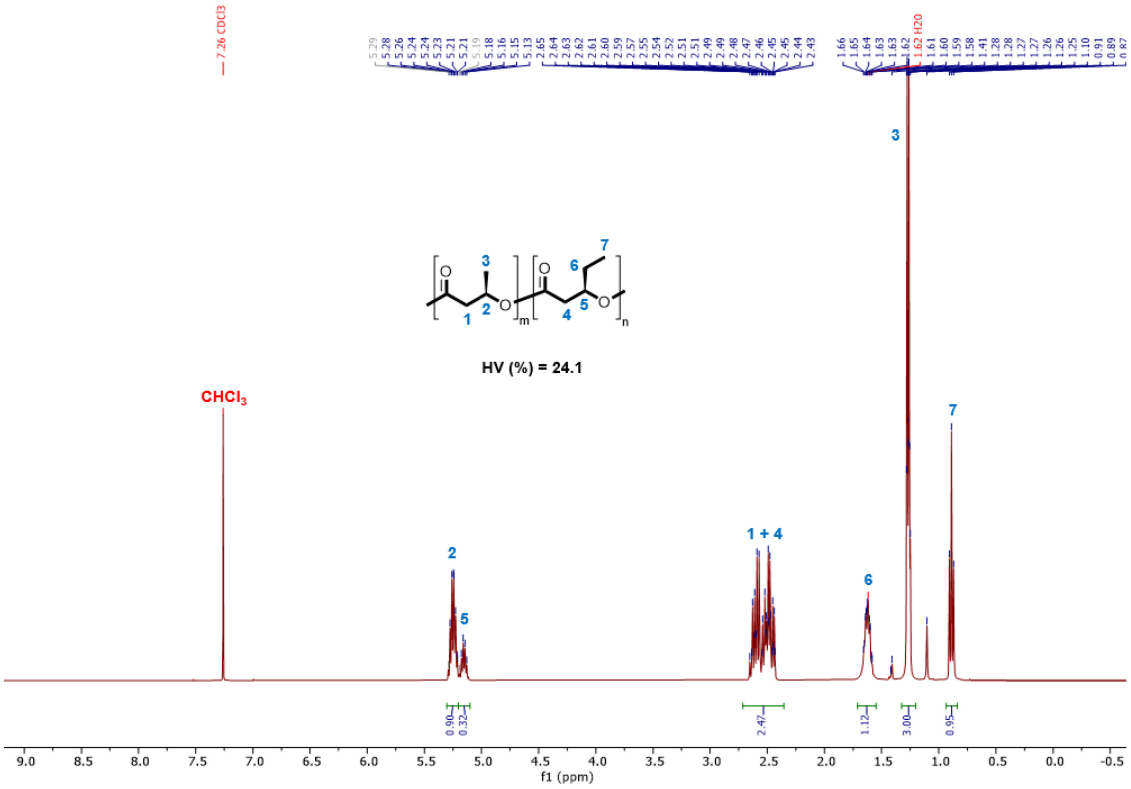


Key

HV is the percentage of the comonomer 3-hydroxyvalerate in the polymer (%)

f1 is the chemical shift (ppm)

Figure A.1 — ^{1}H NMR spectrum of PHBV (400 MHz, $CDCl_3$, 25 °C, d1 = 1 s)

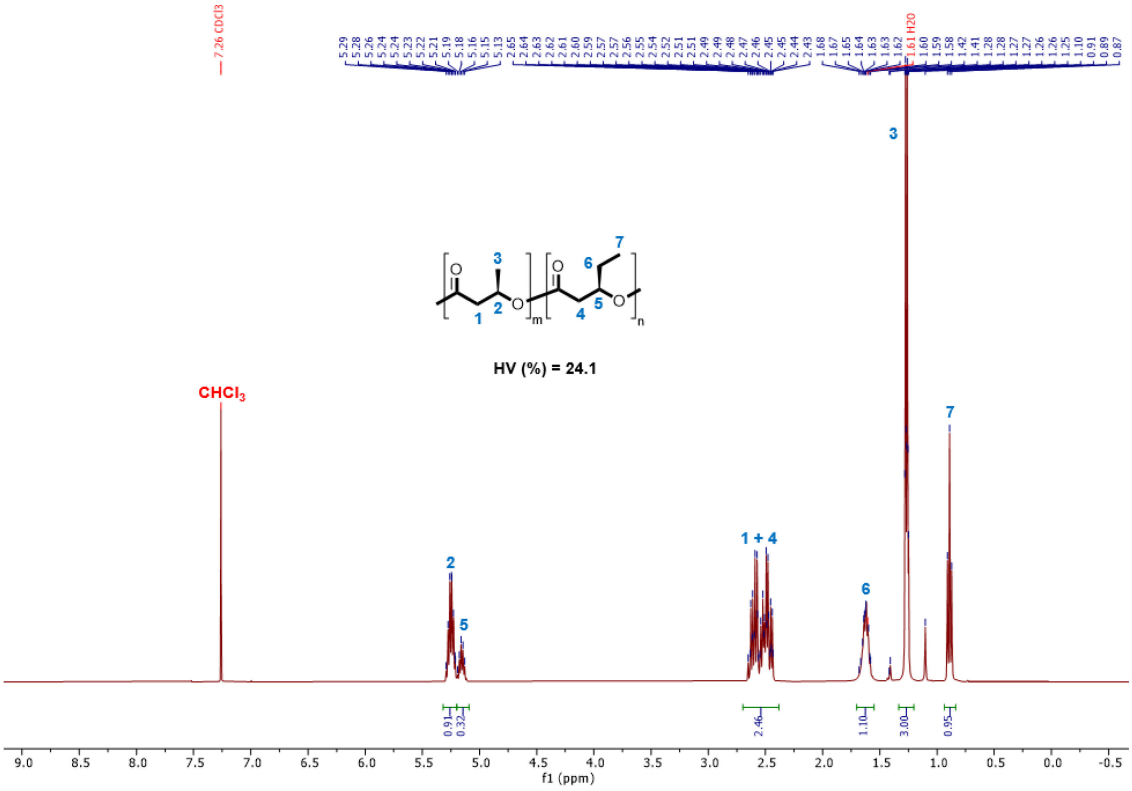


Key

HV is the percentage of the comonomer 3-hydroxyvalerate in the polymer (%)

f1 is the chemical shift (ppm)

Figure A.2 — ^{1}H NMR spectrum of PHBV (400 MHz, $CDCl_3$, 25 °C, d1 = 2 s)

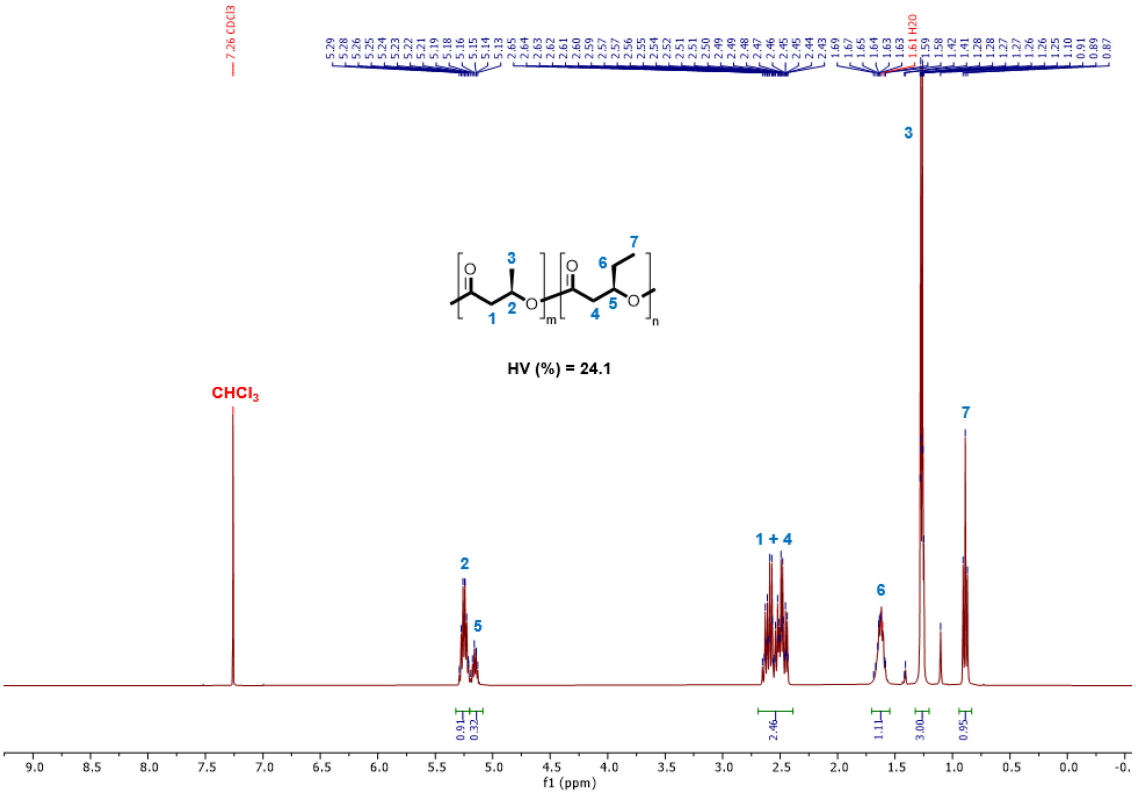


Key

HV is the percentage of the comonomer 3-hydroxyvalerate in the polymer (%)

f1 is the chemical shift (ppm)

Figure A.3 — ^{1}H NMR spectrum of PHBV (400 MHz, $CDCl_3$, 25 °C, d1 = 5 s)

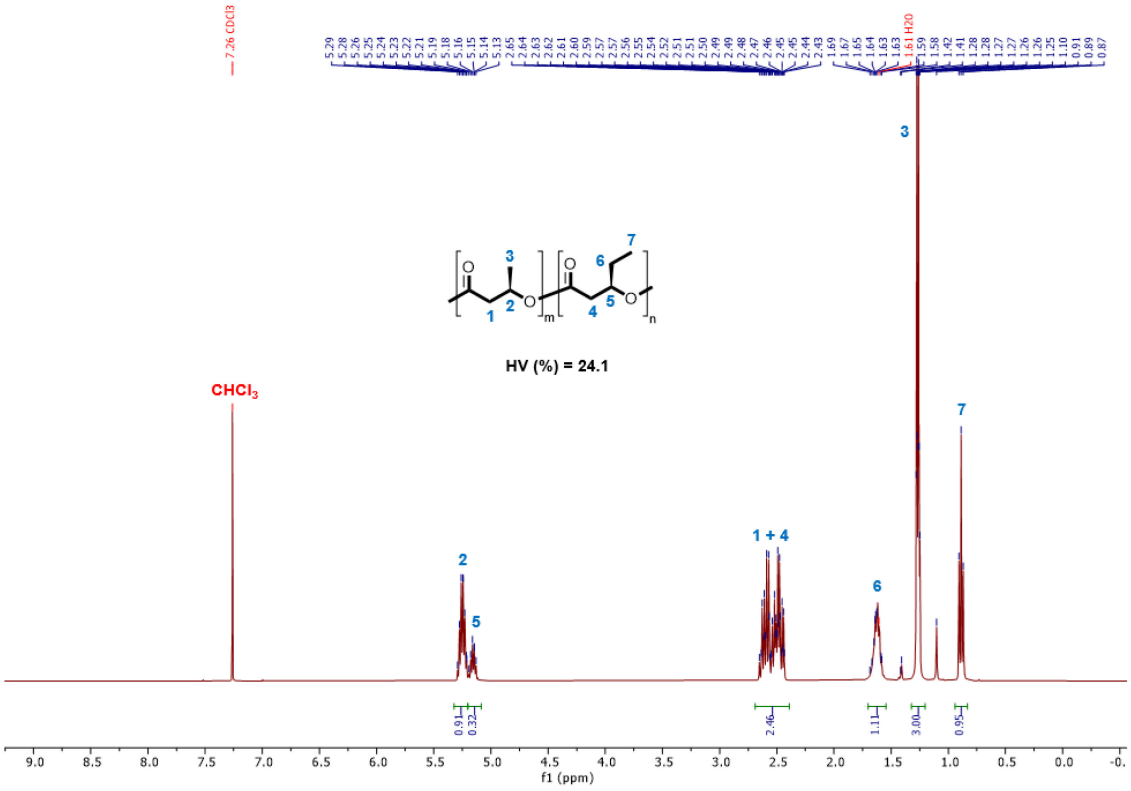


Key

HV is the percentage of the comonomer 3-hydroxyvalerate in the polymer (%)

f1 is the chemical shift (ppm)

Figure A.4 — ^{1}H NMR spectrum of PHBV (400 MHz, $CDCl_3$, 25 °C, d1 = 10 s)



Key

HV is the percentage of the comonomer 3-hydroxyvalerate in the polymer (%)

f1 is the chemical shift (ppm)

Figure A.5 — ^{1}H NMR spectrum of PHBV (400 MHz, $CDCl_3$, 25 °C, d1 = 20 s)

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