Methods for Analysis of Poly(3-hydroxyalkanoate) Composition

Ing. Theo C. de Rijk¹, Ing. Pieter van de Meer², Dr. Gerrit Eggink³, Dr. Ruud A. Weusthuis⁴

¹ State Institute for Quality Control of Agricultural Products (RIKILT), PO Box 230, Bornsesteeg 45, NL-6700 AE Wageningen, The Netherlands; Tel: +31-317-475400; Fax: +31-317-417717; E-mail: t.c.derijk@rikilt.wag-ur.nl
² Agrotechnological Research Institute (ATO), PO Box 17, 6700 AA, Bornsesteeg 59, NL-6700 AE Wageningen, The Netherlands; Tel: +31-317-475300; Fax: +31-317-475347; E-mail: p.vandermeer@ato.wag-ur.nl
³ Agrotechnological Research Institute (ATO), PO Box 17, 6700 AA, Bornsesteeg 59, NL-6700 AE Wageningen, The Netherlands; Tel: +31-317-475300; Fax: +31-317-475347; E-mail: g.eggink@ato.wag-ur.nl
⁴ Agrotechnological Research Institute (ATO), PO Box 17, 6700 AA, Bornsesteeg 59, NL-6700 AE Wageningen, The Netherlands; Tel: +31-317-475300; Fax: +31-317-475347; E-mail: r.a.weusthuis@ato.wag-ur.nl
Introduction

The formation of polyesters by microorganisms has received considerable attention, especially for potential application as bioplastics. These polyesters are called poly(hydroxyalkanoic acids) (PHA) and are not one type of polymer, but a whole family of different polyesters. This is caused by the fact that a multitude of monomers can be incorporated in the polymers. PHA comprise of monomers with 3-hydroxy, 4-hydroxy, and 5-hydroxy groups, the length of the carbon backbone varies between 4 and 16 carbon atoms, and a broad range of functional groups can be present, e.g. halogens, phenoxy, acetoxy, phenyl, cyano, and epoxy groups. To date more than 110 different monomers have been identified. The monomer composition of PHA is variable, and can be manipulated by means of the substrate(s) used and the environmental conditions. The material properties of these materials, and therefore also the application potential, depend largely on the monomer composition. This clearly illustrates the importance of the determination of the monomer composition of PHA. This chapter describes the tools and methods that have been used and developed for this analysis.

2 Historical Outline

After the discovery of poly(3-hydroxybutyrate) (Poly(3HB)), Lemoigne (1926) was the first to describe an analytical method for Poly(3HB) analysis. After extraction with hot alcohol and purification with chloroform and diethylether, the polymer was saponi-
fied. The resulting mixture was distilled, resulting in a volatile fraction and a non-volatile residue. The volatile fraction was trapped, crystallized, and weighed accurately, and contained the dehydrated product of 3-hydroxybutyric acid: α-crotonic acid (trans-2-butenioic acid). The nonvolatile fraction was also weighed accurately and contained primarily 3-hydroxybutyric acid. From the weight of both fractions the amount of Poly(3HB) in the starting material Bacillus 'M' could be calculated.

Although Lemoigne's (1926) method was accurate, it was also time consuming and large amounts of biomass were needed. In order to overcome this problem, Williamson and Wilkinson (1958) developed a spectrophotometric method which was considerably faster. After treating Bacillus cells with sodium hypochlorite, a turbid solution was obtained and measured spectrophotometrically. However, the method had to be fine tuned for every new bacterium and oily inclusions caused interference.

A more specific analysis method was developed by Slepecky and Law (1960) and Law and Slepecky (1961). They treated extracted polymer from Bacillus megaterium with concentrated sulfuric acid to convert Poly(3HB) quantitatively to crotonic acid. Due to the α,β-unsaturated bond, crotonic acid has an absorption maximum at 235 nm in concentrated sulfuric acid and this property was used for ultraviolet (UV) measurement. The method was in close agreement with gravimetric analysis and quantities down to 5 μg could be detected. However, due to the laborious extraction and washing procedure, the method was time consuming and, for this reason, Ward and Dawes (1973) developed a disk assay method. A considerable gain of time with comparable results was achieved. A further improvement was achieved by Karr et al. (1983). Crotonic acid originating from Poly(3HB) was analyzed with ion-exchange high-performance liquid chromatography (HPLC) and UV detection. The HPLC method was further improved by Hesselmann (1999).

The discovery of PHA by Wallen (1974) in activated sludge proved the presence of a more complex polyester. After chloroform extraction, PHA was separated from Poly(3HB) with hot ethanol (95%) and analyzed by nuclear magnetic resonance (NMR). A methylene group adjacent to a methyl group was found, indicating the presence of longer hydroxy fatty acids. After saponification and gas chromatography-mass spectroscopy (GC-MS) analysis, the presence of 3-hydroxybutyric acid, 3-hydroxyvaleric acid, and 3-hydroxyhexanoic acid was confirmed. The identification of 3-hydroxyheptanoic acid was tentative.

However, despite progress being made in the development of faster and more specific analytical methods, the available methods were relatively inaccurate or rather time consuming. The development of fermentation processes based on process kinetics also made it necessary to develop faster and more reproducible analytical methods. The GC method described by Braunegg et al. (1978) was a major advance in this respect. First, a dilute acid (3% H₂SO₄) was used to hydrolyze the polymer. In contrast to the alkaline hydrolysis used by Wallen and Rohwedder (1974), which inevitably led to a mixture of a 3-hydroxy fatty acid methyl ester and a 2-alkenoic fatty acid methyl ester (Capon et al., 1983), the mild acidic hydrolysis resulted in only one methyl ester per component. Second, extraction, hydrolysis, and derivatization took place in one vessel, so an elaborate and error-introducing sample pretreatment was avoided. Third, the resulting mixture of methyl esters was analyzed with the powerful separation technique of GC, which made it possible to use an internal standard to improve quantitation. The meth-
od was compared to the methods developed by Lemoigne (1926), Williamson and Wilkinson (1958), and Slepecky and Law (1960), and showed excellent performance.

The method consisting of acidic extraction, hydrolysis, and methylation developed by Braunegg et al. (1978) was used and adapted by many authors, e.g., Lägeveen et al. (1988), Brandl et al. (1988), Gross et al. (1989), Timm and Steinbüchel (1990), Abe et al. (1990), Huijbers et al. (1994a,b), Jan et al. (1995), and Kato et al. (1996).

As the acidic extraction, hydrolysis, and acetylation proved to be very successful in the analysis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (Poly(3HB-co-3HV)) and medium-chain-length (MCL)-PHA, several authors introduced derivative methods. De Smet et al. (1983), Findlay and White (1983), Riis and Mai (1988), and Bear et al. (1997) used HCl to hydrolyze PHA.

A further important step forward was the introduction of NMR. Although it had already been used by Wallen and Rohwedder (1974) and Capon et al. (1983), it was Doi et al. (1986, 1988, 1990, 1995), and Doi and Abe (1990) who used the technique to its full potential. Huijbers et al. (1992, 1994a), Doi et al. (1993), Eggink et al. (1995), and Casini et al. (1997) used NMR with great success in the analysis of polyunsaturated or epoxidized PHA. As NMR is a noninvasive technique, it was also very useful in the analysis of other types of PHA.

Several other analytical methods lack the specificity to discriminate between different monomers in PHA. Among them are the spectrophotometric method developed by Slepecky and Law (1960) and Law and Slepecky (1961), the infra-red method described by Jüttner et al. (1975), the pyrolysis method of Morikawa and Marchessault (1981), and the sensitive enzymatic method of Parry et al. (1980), which was commercialized by Boehringer Mannheim. However, the described methods could be useful as fast screening techniques.

3 Analytical Methods

The methods used in the analysis of PHA are very similar to the methods which are being used in the analysis of fats and oils. GC is being used in the quantitative analysis of complicated mixtures of fatty acids, HPLC is being used in the analysis of triglycerides, and NMR is being used in qualitative analysis and spatial conformations of triglycerides.

GC analysis of PHA offers quantitative information about the total amount and percent composition of PHA. When combined with MS detection it also adds information about the mass and identity of the monomer involved. Combination of GC with other specialized detectors like the atomic emission detector (AED) gives relevant information about whether the monomers contain atoms like chlorine, bromine, or iodine. However, GC can only be used with (semi)volatile compounds, so PHA has to be hydrolyzed and acylated to produce the volatile monomers which can be subjected to GC analysis.

Although the separation power of HPLC is considerably less compared to GC, there are some advantages. There is no need for lyophilizing the sample material, so there is no loss of time needed for drying. Furthermore, as the HPLC methods analyze the dehydrated free fatty acids, no further derivatization is needed, thus reducing the total analysis time even more. The HPLC method is useful in the analysis of Poly-(3HB-co-3HV) types of PHA.

NMR is a very useful technique in analyzing PHA containing double bonds. With the aid of two-dimensional (2D) homonuclear or
heteronuclear techniques the exact location of double bonds in the monomer and the cis/trans configuration can be determined. It is also very useful in the analysis of all kinds of specialized PHA such as halogenated or acetylated PHA. It is essential in the analysis of epoxidized PHA, as epoxy groups will split into diols in the acidic hydrolysis of the PHA (Sykes, 1986).

It is beyond the scope of this chapter to cover in detail the theory and background of the described analytical methods. Relevant background information is available from a wide range of renowned sources. General information on instrumental analytical techniques is available from Skoog (1985), Settle (1997), and Silverstein and Webster (1998). Specific information on HPLC is available from Lindsay (1992), and Snyder and Kirkland (1996). Detailed information on GC is given by Grob (1995). In-depth information on NMR is available from Friebolin (1991), Sanders and Hunter (1993), and Günther (1995), and thorough information on the principles and use of MS is available from McLafferty and Turecek (1993), and Chapman (1993).

4 Analysis of SCL-PHAs

Several methods for the determination of the monomer composition of SCL-PHAs have been developed based on analytical methods as GC/(MS), NMR, and HPLC.

4.1 GC and MS

Braunegg et al. (1978) developed a method in which wet bacterial biomass of Algaligenes eutrophus and Mycoplana rubra was analyzed. After centrifugation the watery layer was discarded, and the residual cell material was hydrolyzed and methylated in a mixture of 2 ml acidified methanol (3% v/v H$_2$SO$_4$) and 2 ml chloroform at 100 °C for 3.5 h. Phase separation was achieved by adding 1 ml water to the mixture. Methylated 3-hydroxybutyric acid was extracted into the chloroform phase and injected directly into the gas chromatograph equipped with a packed column filled with either 2% Reoplex 400 or Carbowax M 20 TPA on Chromosorb. Addition of the internal standard benzoic acid improved the accuracy and reproducibility (maximum standard deviation ± 0.5%) and amounts as low as 0.5 µg could be detected. However, reaction time and acid concentration had to be optimized carefully. An acid concentration above 3% resulted in the formation of degradation products, whereas acid concentrations below 3% resulted in only partial hydrolysis. The acidic hydrolysis was compared to a treatment with 1 N KOH in methanol, but the yield of the methyl ester of 3HB was considerably less in comparison to the acidic hydrolysis (no data given).

The method was used by several researchers in their analysis of Poly(3HB-co-3HV): Slater et al. (1992, 1998) in Escherichia coli, Anderson et al. (1995) in Rhodococcus ruber, and Madden et al. (1999, 2000) in Ralstonia eutropha. The method proved to be upgradeable to MCL-PHA analysis and was the starting point of many researchers in this area, e.g. Lageveen et al. (1988), Haywood et al. (1989), Jan et al. (1995, 1996), Kato et al. (1996), and Valentin et al. (1999).

Comeau et al. (1988) expanded the method developed by Braunegg et al. (1978) to poly(3-hydroxyvalerate) (Poly(3HV)). Several improvements were also introduced. Activated domestic waste water sludge was lyophilized and a washing step was introduced after the methylation reaction to remove column-degrading components. The use of a capillary DB-Wax column was
described and the identity of the analytes was confirmed by MS. The reproducibility \((n = 6)\), calculated as coefficient of variation, was 2–3\% and amounts as low as 50 ng could be detected. However, the recovery was dependent of the weight of the sample. The method developed by Comeau et al. (1988) was used by Satoh et al. (1992) and Louie et al. (2000) in the analysis of the PHA content of activated sludge.

However, the recovery of 3-hydroxybutyric acid from Poly(3HB) was only partial, as mentioned by Riis and Mai (1988) and Huijberts et al. (1994b). The partial recovery was thought to be caused by an unfavorable partitioning coefficient of the 3-hydroxybutyric acid methyl ester after phase separation during the sample clean up. To prevent this problem, Riis and Mai (1988) developed a new method based on the derivatization of 3-hydroxybutyric acid to a propyl ester. As the propyl ester is more lipophilic compared to the methyl ester, the partitioning coefficient should improve. Dry bacterial mass was hydrolyzed and methylated with a mixture of 2 mL 1,2-dichloromethane and 2 mL acidified propanol (20\% \(v/v\) HCl) at 100 °C for 2 h. Phase separation was achieved by adding 4 mL water to the mixture. The propylated 3-hydroxybutyric acid was extracted into the 1,2-dichloroethane phase and injected directly into the gas chromatograph equipped with a packed column filled with 10\% Reoplex 400 on Porolith. The reproducibility \((n = 10)\), calculated as coefficient of variation, was 2–4\%, the recovery for Poly(3HB) was 99.5 ± 2.1\%, and amounts as low as 20 ng could be detected. In contrast to the sulfuric acid hydrolysis performed by Braunegg et al. (1978), no degradation of hydroxybutyric acid was observed. Jan et al. (1995) confirmed the hypothesis of Riis and Mai (1988). The partitioning coefficient of 3-hydroxybutyric acid methyl ester was calculated at 1.59 and resulted in a 40\% loss of recovery. The method developed by Riis and Mai (1988) was used by Hahn et al. (1999) in the analysis of PHA in transgenic Arabidopsis thaliana plants.

In the investigation of marine bacteria, Odhiam et al. (1986) developed a method based on the Bligh and Dyer extraction method (Bligh and Dyer, 1959). After extraction of lyophilized bacteria with 3 mL chloroform/methanol/water \((1/2/0.8, \, v/v/v)\), phase separation was achieved by addition of 1 mL water. The chloroform phase was dried and the obtained residue was hydrolyzed at 80 °C for 30 min in 1 mL methanol/water containing 15\% NaOH. After washing with hexane, the watery layer was lyophilized and the free fatty acids were dissolved in dichloromethane. The acids were derivatized with 2,3,4,5,6-pentafluorobenzyl bromide and the hydroxy group of 3HB was acylated with heptafluorobutyric anhydride to improve chromatographic separation. The obtained fatty acid derivatives were separated on a SE-54 capillary column and detected by mass spectrometry in \(EI^+, \, Cl^-(CH_4)\) or \(Cl^-(NH_3)\) mode. The method proved to be extremely sensitive with a detection limit of 0.05 pg. The coefficient of variation for a typical marine bacterium sample containing 0.12\ \mu g 3-hydroxybutyric acid was 27\% \((n = 5)\).

An interesting development was mentioned by Seebach and Fritz (1999). A method based on titinate-catalyzed transesterification of Poly(3HB-co-3HV) into trifluoroacetoxy ethyl esters and separation on a chiral GC column made it possible to separate the positional isomers R/S-hydroxybutyric acid and R/S-hydroxyvaleric acid. Unfortunately no analytical data were available.

4.2 NMR

The determination of the monomer composition of Poly(3HB) and Poly(3HB-co-3HV)
is relatively simple, and can easily be acquired by means of GC, GC-MS, and HPLC. However, with these methods it is not possible to detect the presence of two or more distinctive polymers, because the polymers are hydrolyzed before analysis.

It is, however, possible to determine this by means of NMR: the complexity of the carbonyl signals in the $^{13}$C-NMR spectrum can be used to determine whether PHA consists of homopolymers or a copolymer.

This method was used by Doi et al. (1986) to prove that *A. eutrophus* growing on acetate and propionate produced the copolymer Poly(3HB-co-3HV). In another study, Doi et al. (1988) reported an unusual copolyester of 3-hydroxybutyric acid and 4-hydroxybutyric acid. Kunioka et al. (1988) and Doi et al. (1990a) also reported a copolyester of 3-hydroxybutyric acid and 4-hydroxybutyric acid isolated from *A. eutrophus*.

This method has also been used for non-SCL-PHAs. Doi et al. (1995) established with this method that the PHA produced by *A. caviae* from sodium salts of alkanolic acids of different chain lengths was a copolymer, consisting of 3-hydroxybutyrate and 3-hydroxyhexanoate. Valentin et al. (1991) discovered that *Burkholderia* sp. grown on sucrose produced two distinctive homopolymers rather than a copolyester of 3-hydroxybutyric acid and 3-hydroxy-4-pentenoic acid. This was confirmed by $^{13}$C-NMR spectroscopy. NMR methods have also been used to determine the position of the hydroxyl group in the alkyl chain. In a study by Valentin et al. (1991) on 4-hydroxyvaleric acid as a carbon source for growth and accumulation of PHA, 2D-$^{13}$C-$^1$H-correlation spectroscopy (COSY) was applied to show that 4-hydroxyvaleric acid was a constituent of the polyester. This was confirmed with NMR measurements on a standard solution.

In addition to the determination of the monomer composition of SCL-PHAs, NMR has also been used in the determination of the chain dynamics (Doi et al., 1986), the crystallinity (Doi et al., 1988), the pathways involved in SCL-PHA synthesis (Williams et al., 1994; Anderson et al., 1995), the molecular weight (Spyros et al., 1997), the PHA content of biomass (Jan et al., 1996), and the end groups of SCL-PHAs (Madden et al., 1999).

### 4.3 HPLC

Karr et al. (1983) avoided a large part of the time-consuming sample purification which is inevitable in the method of Slepecky and Law (1960) and Law and Slepecky (1961) by introducing HPLC separation followed by UV measurement. After quantitative conversion of Poly(3HB) to crotonic acid, the obtained free acid was chromatographed on a ion-exchange HPLC column. No further pretreatment was necessary and samples containing 0.01–14 μg of Poly(3HB) could be analyzed. The method was used to analyze the Poly(3HB) content of *Rhizobium japonicum*.

The HPLC method of Karr et al. (1983) was applied by Del Don et al. (1994) to analyze the content of PHA in the biomass of phototrophic sulfur bacteria. An interesting phenomenon is the ability of the HPLC method to discriminate between cis- and trans-crotonic acid, formed by the hydrolysis of *R* - and *S*-Poly(3HB). Furthermore 3-hydroxybutyric acid was separated from 3-hydroxyvaleric acid.

Gerngross and Martin (1995) used the HPLC method of Karr et al. (1983) to analyze Poly(3HB) which was synthesized *in vitro* by combining purified PHA synthase from *A. eutrophus* with synthetically prepared R-3-hydroxybutyryl coenzyme A. Jossek et al.
(1998) used the same method to investigate Poly(3HB) formed in vitro by combining R-3-hydroxybutyryl coenzyme with purified recombinant PHA synthase from Chromatium vinosum.

Hesselmann et al. (1999) combined a propanol/sulfuric acid digestion with ion-exchange HPLC and conductivity detection to determine the PHA content of activated sludge. The method could be used with wet material, thus avoiding time-consuming lyophilization. The relative yield was 100 ± 2% in wet material and 98 ± 7% in dry material.

In summary, the GC method described by Braunegg et al. (1978) underestimates the total amount of Poly(3HB) by about 40%. The GC method developed by Riis and Mai (1988) is a major improvement as it analyses the much more lipophilic propyl ester of 3-hydroxybutyric acid, and should therefore be the method of choice. The HPLC methods of Karr et al. (1983) and Hesselmann et al. (1999) offer an interesting alternative.

5 Analysis of other PHA

With the discovery of PHA with other monomers than 3-hydroxybutyric acid and 3-hydroxyvaleric acid by Wallen and Rohwedder (1974) and De Smet et al. (1983), the need for a more specific analytical method was eminent. The method had to be specific and accurate. Unfortunately the available analytical methods were largely focussed on the carboxyl group of the 3-hydroxy group of PHA, a common feature of all 3-hydroxy fatty acids (Slepecky and Law, 1960; Law and Slepecky, 1961; Jüttner et al., 1975; Morikawa and Marchessault, 1981). The more specific enzymatic method of Parry et al. (1980) detected the combined amount of R-3-hydroxybutyric acid and R-3-hydroxyvaleric acid, and the HPLC methods of Karr et al. (1983) and Hesselmann et al. (1999) were only able to separate 3-hydroxybutyric acid, 3-hydroxyvaleric acid, and 3-hydroxyhexanoic acid.

5.1 GC

Wallen and Rohwedder (1974) saponified PHA to a mixture of free 3-hydroxy fatty acids and the corresponding α,β-unsaturated derivatives. The obtained free acids were esterified with Tri-Sil/BSA to a trimethylsilyl ether, whereas the 3-hydroxy group was derivatized into a trimethylsilyl ester. The mixture of 3-trimethylsilyl ethers/esters was finally analyzed by GC and GC-MS. Although useful for qualitative analysis, the method was slow and the partial dehydration of 3-hydroxy fatty acids resulted in large experimental errors.

Lageveen et al. (1988) expanded the method described by Braunegg et al. (1978) to MCL-PHA by using 15% H2SO4 in methanol as the transmethylation reagent. After 140 min at 100 °C, the methyl esters were extracted into the chloroform phase and analyzed on a nonpolar GC column. The method was linear between 0.15 and 15 mg of PHA, and the identity of the analytes was confirmed by GC-MS (EI and CI (NH3)). The method described by Lageveen et al. was used by Abe et al. (1990, 1994), Fritzche et al. (1990b), Fritzche and Lenz (1990), Huijberts et al. (1992, 1994a), Kellerhals et al. (2000), and Klinke et al. (1999), among many others. Brandl et al. (1988), Gross et al. (1989), Timm and Steinbüchel (1990), Kim and Fuller (1992), Kim et al. (2000), Valentin et al. (1992, 1994, 1999), and Fichtenbusch et al. (1996, 1998, 1999, 2000) used the method with minor modifications.

Huijberts et al. (1994b) investigated the influence of a prolonged hydrolysis time on the yield and stability of the methyl esters.
originating from MCL-PHA. It was found that the hydrolysis time, described by Lageveen et al. (1988), had to be increased from 140 min to 4 h. Even after 24 h of hydrolysis no degradation of the methyl esters could be observed.

Findlay and White (1983) developed a GC method in which lyophilized estuarine sediment was hydrolyzed and ethylated with 2 N HCl for 4 h at 100 °C. The resulting ethyl esters were separated on a nonpolar SE-30 capillary column and identified with MS detection. The recovery was estimated at 99 ± 3.5%, a reproducibility of 5–7% was obtained, and the detection limit was estimated at 100 fmol of ethylbutyrate.

5.2 MS

The mass spectra of the methyl esters of saturated 3-hydroxy alkanoic acids are being dominated by $m/z = 103$, caused by an $\alpha$ cleavage between C3 and C4. Further important ions are $m/z = 74$, caused by the McLafferty rearrangement (McLafferty and Turecek, 1993) of the methyl ester, and $m/z = 71$, probably caused by expulsion of methanol from $m/z = 103$ (Wallen and Rohwedder, 1974; Huijberts et al., 1994a; He et al., 1998). The ion with $m/z = 43$ is not indicative of a special functionality in the molecule as it can originate from the saturated alkanoic part of the molecule as well as from the methyl ester moiety (McLafferty and Turecek, 1993). See Figure 1. Unfortunately the methyl esters of saturated 3-hydroxyalkanoic acids are not capable of absorbing the large amount of energy (70 eV) which is being used in electron impact (EI)-MS and hence the signal intensity of the molecular ion is very low. To obtain this vital information two methods can be applied: chemical ionization (CI)-MS or EI-MS of the trimethylsilyl derivatives of the available methyl esters. CI-MS with NH$_3$ as ionization gas was applied by Lageveen et al. (1988) in the analysis of 3-hydroxyoctanoic acid. Huijberts et al. (1994a) describes the use of CI-MS with isobutane and He et al. (1998) describes the use of CI-MS in the analysis of PHA obtained from Pseudomonas stutzeri 1317.

The use of trimethylsilyl derivatives of the methyl esters of 3-hydroxy alkanoic acids has been described by Lee and Choi (1995) and Lee et al. (1995, 1996). After acidic transmethylation of PHA, the resulting methyl

![Fig. 1](image-url)
esters were silylated with bis(trimethylsilyl)acetamide and analyzed with GC-MS (EI\(^+\)). The obtained mass spectra were characterized by ions with \(m/z = 73\) and \(m/z = 89\), both originating from the trimethylsilyl group. Also present was an ion with \(m/z = 175\), caused by the cleavage between C3 and C4. The molecular weight of the monomers could easily be calculated from the intense signals of the \([\text{M-15}]^+\) ion, and the \([\text{M-73}]^+\) ion which were present in all mass spectra. Casini et al. (1997) analyzed polyunsaturated monomers obtained from linseed oil-grown PHA after derivatization with N,O-bis(trimethylsilyl)trifluoroacetamide.

Eight unsaturated 3-hydroxyalkanoic acids could be identified including 3-hydroxy-\(\text{cis,cis,cis-5,8,11}\)-tetradecatrienoic acid and 3-hydroxy-\(\text{cis,cis,cis-7,10,13}\)-hexadecatrienoic acid. The exact position and cis/trans configuration of the double bonds was determined with NMR because it cannot be determined with mass spectrometry. Double bonds within an alkenoate ester migrate rapidly during the ionization process, hence obscuring the mass spectrum (McLafferty and Turecek, 1993).

One of the first applications of NMR spectroscopy published on the research of PHA was reported by Wallen and Rohwedder (1974). The \(^1\)H-NMR spectrum was used only to distinguish between 3-hydroxybutyric acid and other 3-hydroxy fatty acids in a PHA heteropolymer present in activated sludge of a sewage plant.

The presence of 3-hydroxybutyric acid was shown by a doublet in the \(^1\)H-NMR spectrum. 3-hydroxy fatty acids with a longer carbon chain by a triplet and a quartet. The presence of unsaturated monomers was determined by Fritzsche et al. (1990a) using \(^{13}\)C-NMR spectroscopy. Both cis and trans octenoic acid in MCL-PHA produced by \(P.\) oleovorans grown on 3-hydroxy-\(\text{cis-octenoic}\) acid were detected.

Based on the NMR data and the substrate used, it was suggested that the unsaturated monomers were cis and trans 3-hydroxy-6-octenoic acid. In Table 1 the \(^{13}\)C chemical shifts of the carbon atoms of 3-hydroxyalkanoic acids are shown.

### Tab. 1 Chemical shifts in p.p.m. relative to tetramethylsilane of isolated PHA samples

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From Gross et al. (1989) and de Waard et al. (1993).
$^1$H-NMR and 2D-DQF COSY was used to unambiguously establish the structure of unsaturated monomers in MCL-PHA produced by *P. putida* grown on glucose (Huijberts et al., 1992). Eggink et al. (1992) used the same technique in combination with $^{13}$C-enriched substrates, and showed the incorporation of unsaturated fatty acids as 3-hydroxy-5-cis-dodecenoate, 3-hydroxy-5-cis-tetradecenoate, and 3-hydroxy-7-cis-tetradecenoate.

Heteronuclear multiple bond coherence (HMBC) was introduced in PHA structure elucidation by de Waard et al. (1993). With the combination of $^1$H and $^{13}$C chemical shift values and the corresponding coherences it is possible to establish the length of the monomer and the exact position of the unsaturations. An example of a HMBC spectrum with declaration of the polymer structure is given in Figure 2. Nevertheless, determination of the chain length by NMR is not always accurate because complete assignment for longer alkyl chains is not possible due to overlap of carbon and proton signals (de Waard et al., 1993). Mono and di-unsaturations were found. Casini et al. (1997) also found triple unsaturated monomers.

Eggink et al. (1995) used 2D-HMBC spectroscopy to identify the position of the

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*Fig. 2* $^1$H-, $^{13}$C- and HMBC NMR spectra of PHA from *P. putida* grown on oleic acid. Crosspeaks are indicated by two numbers, the first one referring to the proton and the second to the carbon atom involved in the coherence (de Waard, 1993).
hydroxy group and the positions of the double bonds in the hydroxy-fatty acid of MCL-PHA produced by P. aeruginosa grown on euphorbia oil and castor oil.

The techniques described for the analysis of the monomer composition of PHA are summarized in Table 2 for saturated 3-hydroxyalkanoic acids and Table 3 for unsaturated 3-hydroxyalkanoic acids.

The reports on the wide array of other PHA monomers will not be discussed in detail, but are summarized in several tables: Table 4 for branched 3-hydroxyalkanoic acids, Table 5 for halogenated 3-hydroxyalkanoic acids, Table 6 for phenolic 3-hydroxyalkanoic acids, Table 7 for miscellaneous 3-hydroxyalkanoic acids, and Table 8 for other hydroxyalkanoic acids.

In summary, the analytical method described by Lageveen et al. (1988), and adapted and updated by Brandl et al. (1988), Timm and Steinbüchel (1990), and Huijberts et al. (1994b) has proven to be a reliable analytical method in the analysis of saturated chain MCL-PHA. The molecular weight of unsaturated monomers can be determined after silylation of the methyl esters, followed by GC-MS analysis (Lee and Choi, 1995; Lee et al., 1995, 1996). However, if specialized types of PHA have to be measured, NMR has to be used to determine the structure of the monomers unambiguously.

### Tab. 2 Saturated 3-hydroxyalkanoic acids

<table>
<thead>
<tr>
<th>3-OH monomers</th>
<th>Analytical technique</th>
<th>Reference</th>
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<tr>
<td>Propionic acid</td>
<td>$^1$H-NMR, $^{13}$C-NMR</td>
<td>1</td>
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<tr>
<td>Butyric acid</td>
<td>GC(MS), $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$C-COSY NMR, $^1$H-$^1$H-COSY NMR, $^1$H-$^1$C-HSQC-TOCSY NMR</td>
<td>2, 3, 4, 5, 6, 7, 8, 9</td>
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<tr>
<td></td>
<td>HPLC</td>
<td>10, 11</td>
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<tr>
<td>Valeric acid</td>
<td>GC, $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$C-COSY NMR, $^1$H-$^1$H-COSY NMR</td>
<td>2, 4, 6, 8</td>
</tr>
<tr>
<td></td>
<td>HPLC</td>
<td></td>
</tr>
<tr>
<td>Hexanoic acid</td>
<td>GC(MS), $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$C-COSY NMR, $^1$H-$^1$H-COSY NMR, $^1$H-$^1$C-HSQC-TOCSY NMR</td>
<td>2, 4, 13, 6, 14, 15, 7, 8, 9</td>
</tr>
<tr>
<td>Heptanoic acid</td>
<td>GC, $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$C-COSY NMR, $^1$H-$^1$H-COSY NMR</td>
<td>2, 4, 13, 6, 8</td>
</tr>
<tr>
<td>Octanoic acid</td>
<td>GC(MS), $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$C-COSY NMR, $^1$H-$^1$H-COSY NMR, $^1$H-$^1$C-HSQC-TOCSY NMR</td>
<td>16, 4, 13, 6, 14, 15, 7, 8</td>
</tr>
<tr>
<td>Nonanoic acid</td>
<td>GC, $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$C-COSY NMR, $^1$H-$^1$H-COSY NMR</td>
<td>17, 13, 6, 8</td>
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<tr>
<td>Decanoic acid</td>
<td>GC(MS), $^1$H-NMR, $^{13}$C-NMR, $^1$H-$^1$C-COSY NMR, $^1$H-$^1$H-COSY NMR</td>
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<td>Undecanoic acid</td>
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<tr>
<td>Dodecanoic acid</td>
<td>GC(MS), $^1$H-NMR, $^{13}$C-NMR</td>
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<tr>
<td>Tetradecanoic acid</td>
<td>GC(MS), $^{13}$C-NMR, $^1$H-$^1$H-COSY NMR</td>
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<tr>
<td>Hexadecanoic acid</td>
<td>GC(MS)</td>
<td>21, 22</td>
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</tbody>
</table>

1: Doi et al. (1990b); 2: Wallen and Rohwedder (1974); 3: Braunegg et al. (1978); 4: Findlay and White (1983); 5: Riis and Mai (1988); 6: Gross et al. (1989); 7: Abe et al. (1994); 8: Choi and Yoon (1994); 9: Jung et al. (2000); 10: Karr et al. (1983); 11: Hesselman et al. (1999); 12: Del Don et al. (1994); 13: Lageveen et al. (1988); 14: Huijberts et al. (1992); 15: Huijberts et al. (1994a); 16: De Smet et al. (1983); 17: Brandl et al. (1988); 18: Kim et al. (1991); 19: Cronwick et al. (1996); 20: He et al. (1998); 21: Lee et al. (1996); 22: Kellerhals et al. (2000).