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Simultaneous kinetic determination of 3-hydroxybutyrate and 3-hydroxyvalerate in biopolymer degradation processes

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ABSTRACT

A new kinetic method is proposed for the simultaneous determination of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) based on the different rate of the 3-hydroxybutyrate dehydrogenase-catalysed reactions of these compounds with coenzyme NAD^+. A flow injection system with two reactors of immobilised 3-hydroxybutyrate dehydrogenase and dual detection is used. The concentrations of NADH produced after two different reaction times are measured by fluorometry or spectrophotometry and multivariate linear calibration is applied for quantification. Concentrations of 3HB and 3HV between 1×10^{-6} and 1×10^{-4} M can be determined at an average sampling frequency of $20\,h^{-1}$. In contrast to usual methods, the proposed here makes possible the discrimination of 3HB and 3HV without previous separation so that usual extraction with chlorinated solvents and/or chromatographic separation is not required. The method is of interest in a wide variety of fields concerning PHAs, as it can provide information on the degradation rate and mechanism, composition and structure of these polymers. Its applicability has been proved through the determination of 3HB and 3HV in the digests of some chemically degraded commercial PHAs.

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1. Introduction

Polyhydroxyalkanoates (PHAs) [1,2] are a large group of natural polymers that are synthesized by numerous bacteria under unfavourable growth conditions. Tailor-made PHAs, with controlled composition, structure and properties, are industrially produced with genetically modified bacteria. PHAs have thermoplastics properties but unlike synthetic plastics, they are biodegradable and biocompatible. Most current applications of PHAs are based on these features and are effected in the environmental and medical fields [3,4]. PHAs are ecological plastics that are used in packaging and coating as substitutes for non-biodegradable petrochemical polymers. Their main medical applications are the fabrication of biodegradable body implants and the control of drug delivery, this being used also in pharmacy, agriculture and veterinary. Moreover, the degradation of PHAs is a source of optically active monomers of hydroxyalkanoic acids [5] that can be used to synthesize chiral compounds such as aminoacids, vitamins, antibiotics, pheromones, perfumes or biodegradable solvents. PHAs can be degraded by enzymes (biodegradation) but also by chemical or thermal processes. From an applicative point of view, the degradation kinetics is essential since the degradation rate or the resultant products can determine the potential applications. Chemical hydrolysis is also a crucial stage of methods for indirect analysis of PHAs.

Although more than 150 different monomers of hydroxyalkanoic acids can be combined to give PHAs, only very few of them are actually used. The commonest PHA is poly(3-hydroxybutyrate) (PHB), a homopolymer of (R)-3-hydroxybutyric acid (3HB). The high cost and some disadvantages of PHB have lead to the production of copolymers of 3HB and other hydroxyalkanoic acids. The most widespread commercial products are the copolymers of 3HB and (R)-3-hydroxyvaleric acid (3HV) with variable properties depending on the percentage of poly(3-hydroxyvalerate) (PHV). Experimental products include also blends of PHAs (homopolymers, copolymers or both) and blends of PHAs with other biodegradable polymers. As PHB and PHV are by far the commonest components of PHAs, 3HB and/or 3HV are generally among the ultimate degradation products. The quantification of these monomers can provide information not only on the degradation rate and mechanism but also on the composition and structure of PHAs. Indirect quantification of PHB, PHV and their copolymers is commonly performed after alcoholysis with methanol, ethanol or propanol in sulphuric or hydrochloric acids. The polymers are quantitatively transformed into the esters of 3HB and/or 3HV which can be extracted with chloroform or other solvents and determined by gas chromatography with flame ionization (GC–FID) or mass spectrometry (GC–MS) detection [6,7]. In these methods, the recoveries of PHB and PHV are not complete, due to the partition of the esters between the aqueous and organic phases. Alternatively, the

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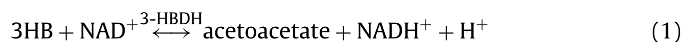
esters can be hydrolysed to their respective hydroxyacids which are then determined directly in aqueous solution by ion-exchange chromatography with conductivity detection [8]. In concentrated sulphuric acid, PHB is quantitatively converted into crotonic acid that can be analysed by HPLC with ultraviolet detection [9]; this is not applicable to PHV [10]. Alkaline hydrolysis of PHB gives a mixture of crotonic acid and 3HB while PHV is converted into 3HV; these products are then usually determined also by chromatographic methods [9,11]. Whatever the degradation process, the 3HB formed can be determined directly in aqueous solution by enzymatic analysis, this being a rapid alternative to chromatographic methods. Enzymatic determinations are based on the selective action of the enzyme (R)-3-hydroxybutyrate dehydrogenase (3-HBDH) which catalyses the oxidation of 3HB with NAD^+ to give acetoacetic acid and NADH. First described by Williamson et al. [12], this reaction is also the basis of a wide variety of methods for the determination of 3HB in biological samples such as blood, serum, urine or milk for clinical or veterinary purposes [12–16]. In its biopolymer applications, the NADH produced is measured directly by photometry at 340 nm [17] or after coupling with other enzymatic reactions using photometric [8] or amperometric detection [18]. The catalytic action of 3-HBDH is highly selective, but not specific for the R-isomer of 3HB. Although the S-isomer does not react, other (R)-3-hydroxyacids, such as (R)-3-hydroxyvaleric or (R)-3-hydroxyhexanoic acids, can also be transformed at slower rates [19]. The existing enzymatic methods are based on measurements under equilibrium conditions (i.e., when the enzymatic reaction is complete) and the quantification of 3HB is only possible if other interfering hydroxyacids are not present. An enzymatic method has been proposed [8] for the determination of the total sum of 3HB and 3HV although these compounds cannot be individually quantified.

In this article, an enzymatic method is described which makes possible the individual determination of 3HB and 3HV in mixtures of these compounds. It is based on the different rate of the about mentioned 3-HBDH-catalysed reactions:



$$3HB + NAD^+ \overset{3\text{-HBDH}}{\longleftrightarrow} \text{acetoacetate} + NADH^+ + H^+ \quad (1)$$

$$3HV + NAD^+ \overset{3\text{-HBDH}}{\longleftrightarrow} 3\text{-ketovalerate} + NADH + H^+ \quad (2)$$

The total NADH produced is measured under two different kinetic conditions and the concentrations of 3HB and 3HV are calculated by mathematical computation using multivariate linear calibration. Highly reproducible measurements are achieved using flow injection analysis with immobilised 3-HBDH. The proposed procedure is applied to the determination of 3HB and 3HV in chemically degraded commercial PHAs.

2. Experimental

2.1. Apparatus and materials

A RF-10AXL fluorescence detector and a SPD 6AV UV–vis spectrophotometric detector were used. Both detectors were simultaneously connected to a SCL-10AVP System Controller and a computer with LC Solution software (all from Shimadzu, Madrid) for data acquisition. Reagents were propelled with a Minipuls 2-HP4 peristaltic pump (Gilson, Madrid) with calibrated vinyl pump tubes ($0.60\,cm^3\,m^{-1}$). Samples and standards were injected with a V-1451 injection valve (Upchurch Scientific, Barcelona, Spain) electronically actuated and provided with loops of different volume; the sample loop was fed with another peristaltic pump. Flangeless fittings, unions and tees (Upchurch Scientific) and 0.5 mm i.d. PTFE tubing were used in the flow system. The working temperature was controlled with a Lauda E-103 circulation thermostat (Hucoa-Erlöss, Madrid). Statistica 6 software (StatSoft, Tulsa, USA) was used for statistical computation.

2.2. Reagents

The following chemicals were supplied by Sigma–Aldrich (Madrid): controlled-pore glass PG 240-120, pore size 240, mesh size 80–120; 3-aminopropyltriethoxysilane; glutaraldehyde, 50% (w/v), aqueous solution; (R)-(−)-3-hydroxybutyric acid, sodium salt; (R)-3-hydroxybutyrate dehydrogenase (E.C. 1.1.1.30); (−)-methyl (R)-3-hydroxyvalerate, >98%; crotonic acid (trans-2-butenoic acid), 98%; Trizma® hydrochloride (Tris(hydroxymethyl)aminomethane hydrochloride), reagent grade, >99%. Oxidized β-nicotinamide-adenine dinucleotide, NAD^+, free acid, grade I, 100%, was from Roche Diagnostics (Barcelona). All other chemicals were of analytical-reagent grade. Working solutions of 3-hydroxybutyrate were prepared from a stock solution (5.0×10^{-3} M) that was renewed daily. Stock solutions of 3-hydroxyvalerate (1.8×10^{-3} M) were freshly prepared by the hydrolysis of the methyl-ester in 0.05 M sodium hydroxide (30 min at room temperature).

2.3. Enzyme reactors

The enzyme 3-HBDH (100 U) was immobilised on controlled-pore glass (CPG, 0.1 g) by indirect covalent binding, as described earlier [20]. The immobilised enzyme was stored wet (in deoxygenated 0.1 M phosphate buffer, pH 6.0) and refrigerated (4 °C) in sealed bottles. Under such conditions, 3-HBDH was very stable: after more than 2 years, the stored immobilised enzyme showed about 55% of its initial activity (activities were compared under the proposed working conditions with newly made enzyme reactors). Enzyme reactors were made by packing the CPG-immobilised enzyme into PTFE tubes (1 mm i.d., 5–6 cm long) with 10 μm frits (Upchurch Scientific) at the ends. The reactors showed no measurable loss of activity after at least 150 injections under the proposed working conditions.

2.4. Samples and sample preparation

Three commercial PHAs were used (Goodfellow, Huntingdon, England). One of them (sample BP-1) was described as polyhydroxybutyrate/polyhydroxyvalerate 12%-biopolymer (PHB88/PHV12), granule, 10% plasticised pellets (citric ester). The second product (sample BP-2) was described as polyhydroxybutyrate-biopolymer (PHB) sheet, technical grade. The label of the third product (sample BP-3) described a polyhydroxybutyrate-biopolymer (PHB), non-woven fabric, research grade. Since the information given was ambiguous or incomplete, the samples were analysed by NMR to estimate their composition. The commercial products were dissolved in deuterated chloroform and their ^{1}H NMR spectra were registered in a Varian 200 VX spectrometer at 200 MHz. In sample BP-1, the molar ratio PHB:PHV was found to be 7.6:1 and the plasticiser was identified as tributyl O-acetylcitrate. The NMR analysis indicated that both BP-2 and BP-3 were in fact copolymers of 3HB and 3HV with an approximate molar ratio PHB:PHV of 4.2:1 and 4.1:1, respectively.

An adequate amount (20–40 mg) of commercial product was dissolved in 1 mL of chloroform in the 100 mL round bottom flask of a reflux setup. After chloroform was evaporated by heating at 40 °C, 4 mL of propanol and 1 mL of concentrated hydrochloric acid were added and reflux was applied for 3 h at 90 °C to perform propanolysis. After diluting and cooling with 80 mL of water, 4 mL of 5 M sodium hydroxide was added to hydrolyse the propyl esters. The mixture was left for 30 min at room temperature and then it was neutralized (pH 7) with hydrochloric acid and made up to 100 mL with water. The resultant sample solutions were analysed for 3HB and 3HV according to the proposed flow injection procedure.

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