Rubber Oxygenase Degradation Assay by UV-Labeling and Gel Permeation Chromatography

*Vico K. B. Adjedje, Yannick L. Wolf, Martin J. Weissenborn, and Wolfgang H. Binder**

A versatile and robust end-group derivatization approach using oximes has been developed for the detection of oxidative degradation of synthetic polyisoprenes and polybutadiene. This method demonstrates broad applicability, effectively monitoring degradation across a wide molecular weight range through ultraviolet (UV)-detection coupled to gel permeation chromatography. Importantly, it enables the effective monitoring of degradation via derivatization-induced UV-maximum shifts, even in the presence of an excess of undegraded polyene, overcoming limitations previously reported with refractive index detectors. Notably, this oxime-based derivatization methodology is used in enzymatic degradation experiments of synthetic polyisoprenes characterized by a cis: trans ratio with the rubber oxygenase Lcp$_{K30}$. It reveals substantial UV absorption in derivatized enzymatic degradation products of polyisoprene with molecular weights exceeding 1000 g mol^{-1} - an unprecedented revelation for this enzyme's activity on such synthetic polyisoprenes. This innovative approach holds promise as a valuable tool for advancing research into the degradation of synthetic polyisoprenes and polybutadiene, particularly under conditions of low organocatalytic or enzymatic degradation activity. With its broad applicability and capacity to reveal previously hidden degradation processes, it represents a noteworthy contribution to sustainable polymer chemistry.

1. Introduction

The production of both natural and synthetic rubbers, derived from polyene-polymers, holds immense economic and environmental significance, with an estimated production of millions of tons per year.[1,2] Polyisoprene is commonly obtained from the Brazilian rubber tree *Hevea brasiliensis*, as *cis*-1,4-polyisoprene, or produced synthetically as a mixture of the 1,4-, 1,2-, and 3,4-isomer, whereas polybutadiene is produced solely synthetically as a mixture of the 1,4- and 1,2-isomers. Both polymers are crucial for car tires and other molded mechanical goods, which are accumulating in the environment.[3,4] Mechanical grinding either at ambient temperature or by cryofracturing is a common method for rubber recycling.[5,6] The C=C double bond in such polyenes can act as a point of attack for the degradation by organometallic-, organo-, and biocatalytic methods, by cross-metathesis using Schrock,[7,8] Grubbs,[9–11] and Grubbs-type catalysts.[12–14] Organocatalytic cleavage of polyisoprene has been achieved using phenyl hydrazine,[15] peroxynitrous acid,[16,17] and periodic acid.[18–20] As a novel method for biodegradation rubber-degrading microorganisms were isolated from various ecosystems and the responsible enzymes for their degradation capability probed,[2,21–27] focusing on the enzymatic degradation of natural rubber, *cis*-1,4-polyisoprene,[28,29] and *trans*-1,4-polyisoprene, gutta-percha.[30] It was demonstrated that for low molecular weight synthetic polyisoprenes the dosage form played a crucial role in the degradation efficacy of the rubber oxygenase Lcp$_{K30}$.[31] However, to further enhance the understanding and improve the sustainable enzymatic degradation of synthetic polyisoprenes and to screen for the enzymatic activity of rubber oxygenases toward synthetic polybutadiene at low enzymatic activity, new analytical tools for high-throughput screening are required.

The analysis of enzymatic and bacterial rubber-degrading activity has been mostly limited to high molecular weight polyisoprene, high molecular weight polybutadiene, and natural rubber so far.[2,27] The occurrence of the aldehyde and keto-functionality in the end-groups of the degradation products can be proven by staining with Schiff reagent[27] or 2,4-dinitrophenylhydrazine,[32] Fourier-transform infrared spectroscopy[33] and nuclear magnetic resonance (NMR) spectroscopy.[27] Ultrahigh performance or high performance liquid chromatography, coupled to mass

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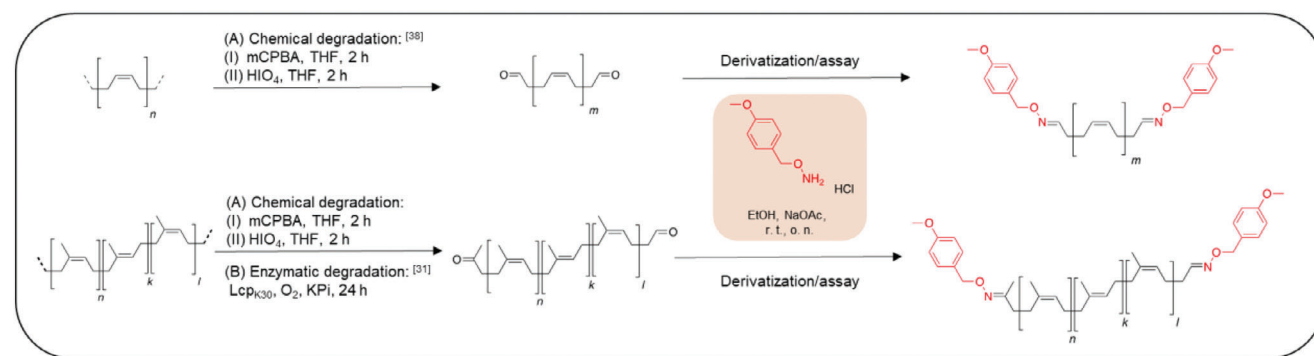E-mail: wolfgang.binder@chemie.uni-halle.de

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Scheme 1. Chemical and enzymatic degradations of polyisoprene and polybutadiene with the chosen derivatization strategy of the respective end-groups conducted in this study for the assay.

spectrometry with ultraviolet detectors (U/HPLC-UV-MS) was used for the analysis of the molecular mass of oligoisoprenoidic degradation products[34] or derivatized products via positive-ion ESI-MS.[35] Predominantly gel permeation chromatography (GPC) has been employed to reveal the number-average molar mass of the bioconversion of the polymer into its corresponding degradation products via refractive index (RI) detection.[27]

To the best of our knowledge, a quantitative derivatization via a Schiff-reagent, 2,4-dinitrophenylhydrazine or Girard reagent T has not been shown for polyisoprene and polybutadiene with aldehyde or keto end-groups. There currently is no methodology to investigate the enzymatic degradation of either synthetic polyisoprene or synthetic polybutadiene by GPC at low degradation activity, or via UV detection methods with complete derivatization for high molecular weight polymers without side reactions at mild conditions. Inspired by recent advances in the derivatization strategies for oximes,[36,37] we set out to implement a methodology for the detection of low enzymatic activity of the enzymatic degradation of synthetic polyisoprenes and the detection of enzymatic degradation of synthetic polybutadiene by GPC to track the activity of rubber oxygenase with a new UV-active derivatization methodology (**Scheme 1**).

2. Result and Discussion

The here reported new methodology to track the enzymatic degradation of synthetic polyisoprenes is based on a derivatization strategy, leading to a detectable shift in the UV absorbance of the polymer via GPC. We first established the method with organochemically degraded polyisoprene and polybutadiene and subsequently investigated the applicability for enzymatic degradation of synthetic polyisoprenes. An oxime, based on the derivatization reagent O-(4-methoxybenzyl)-hydroxylamine hydrochloride was used to check for the two easily trackable aldehyde end-groups, generated during oxidative degradation. As oxime conjugates from ketones have higher stability than the respective aldehyde derivatives, the application toward aldehyde derivatives was probed and established herein.[36]

The scope of the investigated synthetic polymers included a low molecular weight polyisoprene (PI 3000), which has previously been shown to lead to the most degradation products with the cosolvent based surfactant free emulsification strategy,[31] a polyisoprene of similar isomeric motifs but higher number av-

erage molar mass (PI 4500), a polybutadiene of low molecular weight but high *cis*-content (PB 3500), a polybutadiene of high *trans*-ratio (PB 12 000), and a high molecular weight 1,4-*cis*-polybutadiene (PB 200k) (**Table 1**). PB 3500 and PB 12 000 were investigated because of their relatively high *cis*-content and a molecular weight which allowed the formation of their surfactant-free emulsions with a cosolvent (Figures S1 and S2, Supporting Information). This makes them ideal candidates for the potential enzymatic degradation investigation of synthetic polybutadiene with the cosolvent emulsified system. For telechelic polybutadienes with well-defined end groups, it is best to start with precursors of the highest molar mass possible.[38] Consequently, a high molecular mass 1,4-*cis*-polybutadiene was included in our investigations. The oxidatively degraded samples, e.g., **PI 3000-Ox**, and the derivatized samples, e.g., **PI 3000-D**, of the respective polymers, are listed in Table 1. The microstructure of the samples did not change significantly when compared to the starting polymers, neither during degradation nor during the subsequent derivatization, as can be observed in the corresponding ^{1}H NMR-spectra (Figures S4–S9, S12–S13, and S16–S19, Supporting Information).

PB 200k was degraded by epoxidation with 3-chloroperoxybenzoic acid (mCPBA) and subsequent oxidative chain cleavage of the epoxide C—C bond by periodic acid as reported in literature.[38] The resulting telechelic polybutadiene (**PB 200k-Ox**) was then subjected to derivatization with O-(4-methoxybenzyl)-hydroxylamine hydrochloride and sodium acetate in ethanol overnight for derivatization.[39] ^{1}H-NMR showed that the aldehyde proton (**Figure 1**, Index I) at 9.77 ppm disappeared in the derivatized sample, and the adjacent methylene protons α to the aldehyde (Figure 1, Index 2/3) at 2.38 and 2.47 ppm changed as well. In the derivatized telechelic polybutadiene (**PB 200k-D**) the methyl group of the derivatization reagent was detectable at 3.80 ppm (Figure 1, Index 6), together with the aromatic protons at 7.30 and 6.88 pm (Figure 1, Index 7/8) and the methylene group at 5.0 ppm. The protons at 7.41 and 6.66 ppm could be assigned to the oxime proton of the respective *E/Z* isomer (Figure 1, Index 10). [1]H—[1]H correlation spectroscopy (COSY) NMR revealed that the proton of the *E/Z* isomers of the oxime at 7.41 and 6.66 ppm correlated to the protons at either 2.40 or 2.22 ppm (**Figure 2**). The assignment of the carbons with heteronuclear single quantum spectroscopy (HSQC) and heteronuclear multiple bond spectroscopy (HMBC)

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Table 1. Polymers investigated in the oxidative degradation and their subsequent derivatization strategy.

Polyenes	Microstructure [%][a)d]				M_n [g mol^{-1}][b)]	Đ[b)]	ID	M_n [g mol^{-1}][b)]	Đ[b)]
	3,4-units	1,4-units cis	1,4-units trans	1,2- units					
PI 3000	16	56	27	1	3100	1.1	PI 3000-Ox	1700	1.6
							PI 3000-D	1800	1.5
							PI 3000 WT-D	2600	1.1
PI 4500	16	56	27	1	4500	1.1	PI 4500-Ox	2300	1.5
							PI 4500-D	2400	1.5
							PI 4500 WT-D	3300	1.1
PB 3500	–	75	24	1	3500	2.0	PB 3500-Ox	2400	2.3
							PB 3500-D	2200	2.5
PB 12000	33	55	12	33	11500	1.2	PB 12000-Ox	5400	2.0
							PB 12000-D	5100	1.7
PB 200k	98	–	2	98	190000	1.3	PB 200k-Ox	6100	2.9
							PB 200k-D	5900	2.4

[a)] Determined by ^{1}H NMR; [b)] Determined by GPC; M_n = number average molar mass. Đ = polydispersity index; PI = polyisoprene; PB = polybutadiene; PI 3000 = polyisoprene M_n of 3000.

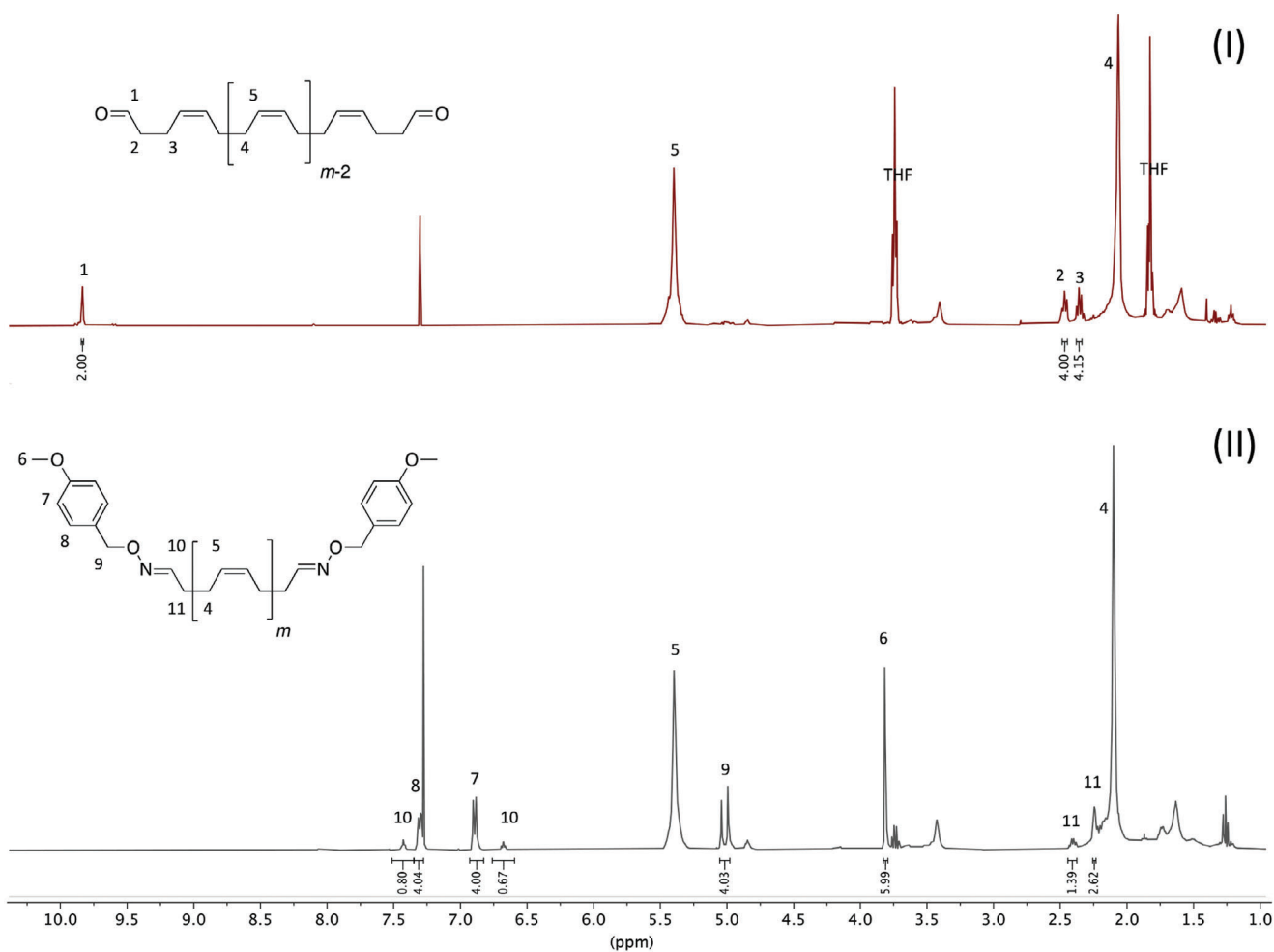**Figure 1.** ^{1}H NMR spectrum of the derivatized and chemically degraded **PB 200k-Ox** (I) and the derivatized telechelic polybutadiene **PB 200k**-D (II).
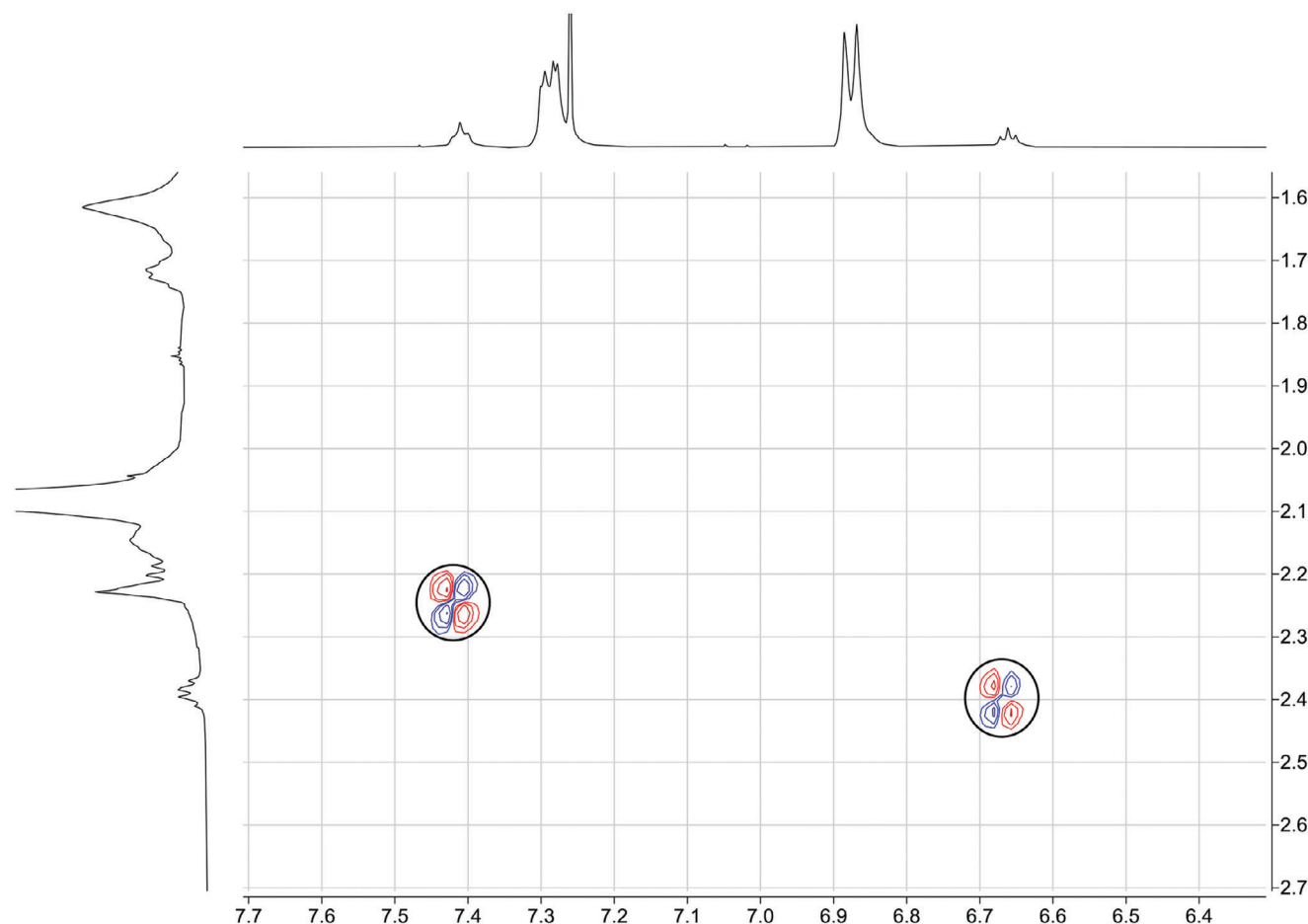
Figure 2. ^{1}H-^{1}H COSY 2D NMR of the respective derivatized end groups of **PB 200k-D**.

measurements also showed this correlation (see Figures S8–11, Supporting Information). This is a clear indication that the aldehyde functionality of the telechelic polybutadiene is fully converted to the oxime-derivative by this derivatization strategy.

The derivatized sample, **PB 200k-D** was measured via GPC with a UV detector indicating absorption maximum shifts from 244 nm for an unreacted polybutadiene (**Figure 3**, black) to the absorption maximum of the free derivatization agent (Figure 3, blue) at 274 nm but with a shoulder of the polybutadiene backbone at 244 nm for **PB 200k-D** (Figure 3, red). This shift allows for the distinction between the unreacted polymer and the derivatized sample in the same GPC-measurement by a change in the tracking wavelength from 244 to 274 nm for derivatized polybutadienes. Derivatization therefore enables the tracking of degradation activity at the derivatized UV absorption maximum of 274 nm for polybutadienes. One of the key advantages of O-(4-methoxybenzyl)-hydroxylamine hydrochloride as the chromophore is a quantitative conversion with the carbonylendgroups, with its elution peaks fully separated in GPC at distinctively higher elution volumes (Figure S21, Supporting Information).

To check for the strength of the analytical method "under real conditions," mixtures of telechelic polymer and undegraded polymer were generated. This approach probed the derivatiza-

tion strategy with a larger background of still unreacted polymer, just as expected for the enzymatic degradation at low activities. Chemical degradation was conducted according to a
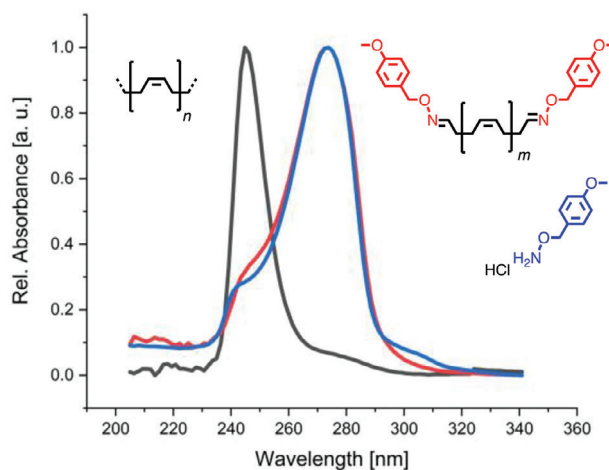

Figure 3. UV absorption spectra of polybutadiene (black), **PB 200k-D** (red), and the free derivatization agent O-(4-methoxybenzyl)-hydroxylamine hydrochloride (blue).

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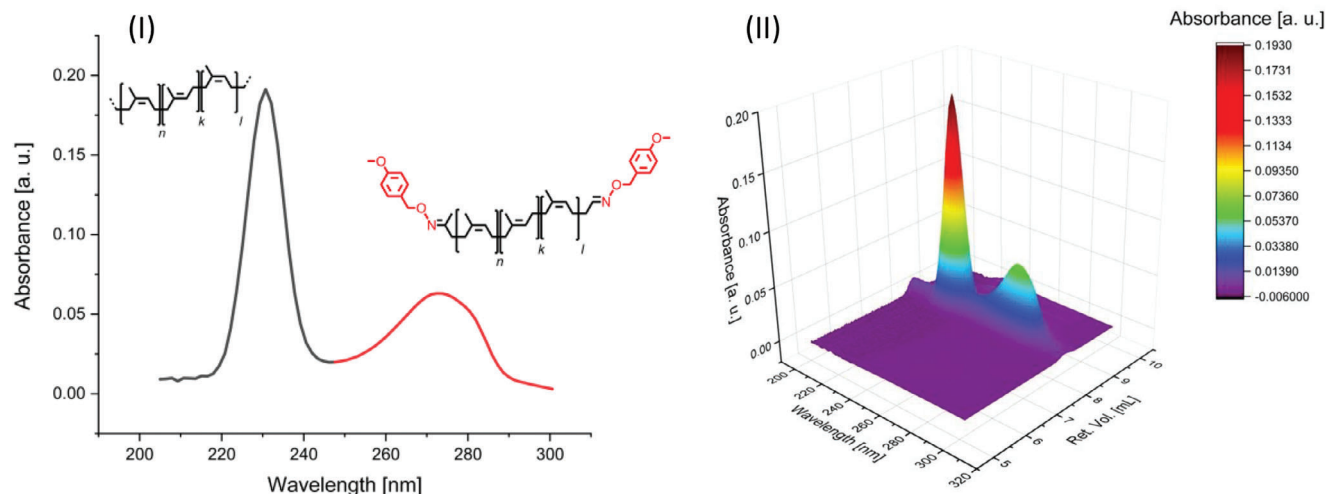www.mrc-journal.de

Figure 4. UV absorption spectra of the underivatized (black) and derivatized (red) **PI 3000-D (I)**, corresponding 3D visualization of the **PI 3000-D** intensity with an additional axis displaying the retention volume (II).

modified procedure of Pillard and coworkers,[38] who established and optimized the chemical degradation of high molecular mass 1,4-*cis*-polyisoprene and 1,4-*cis*-polybutadiene degradation. The polymers were first epoxidized with mCPBA and then oxidatively cleaved with periodic acid, selecting a concentration of the mCPBA to obtain epoxidation rates of ≈2%.

Theoretical and experimental epoxidation values, ranging from 0.7% to 1.5% (see Table S1, Supporting Information), differ due to deviations in the microstructure of the internal double bonds in the investigated polymers (see Table 1) from the all cis-polybutadienes or all cis-polyisoprenes reported in the literature.[38] Periodic acid concentration was set to 1.2 eq. of the respective mCPBA concentration to avoid an uncontrolled degradation of the double bonds in polyisoprene.[18] The molecular weights of **PI 3000-Ox**, **PI 4500-Ox**, **PB 3500-Ox**, and **PB 12000-Ox** decreased significantly, according to gel permeation chromatography (**Table 1**; and Table S3, Supporting Information). ^{1}H-NMR showed that the aldehyde proton at 9.77 ppm as well as the microstructure around the aldehyde appeared in all of the chemically degraded samples (Figures S4–S7, Supporting Information). Determination of the $M_{n \text{ (NMR)}}$ via end group correlation to the repetitive units in the polymer backbone led to higher values than the $M_{n \text{ (GPC)}}$ due to the intended residue of undegraded polymer (Table S2, Supporting Information).

The oxime-derivatized **PI 3000-Ox** (**PI 3000-D**) exhibited two absorption maxima in the UV absorption spectrum (**Figure 4**). The maximum at 227 nm can be attributed to unreacted polyisoprene when compared to a GPC run with solely unreacted **PI 3000** (Figure S18, Supporting Information). The other maximum at 272 nm was assigned to the derivatization of **PI 3000-D**. The ^{1}H-NMR spectrum of **PI 3000-D** showed the expected shift of the aldehyde proton (^{1}H: 9.83 ppm) to the corresponding oxime derivate as a mixture of E- and Z-isomers (^{1}H: 7.39 and 6.65 ppm) (Figures S12 and S13, Supporting Information). The microstructure around the derivatized oxime functionality was also detected by ^{1}H—^{1}H COSY NMR for **PI 3000-D** revealing that the proton of the E/Z isomers of the oxime correlated to protons of the adjacent methylene (Figure S14, Supporting Information). The methyl

group of the derivatized keto-functionality was partially overlapped by the methyl-groups of the polyisoprene backbone. For further confirmation of the conversion of the keto-functionality UHPLC-ESI-HRMS measurements were conducted. UHPLC-ESI-HRMS measurements of **PI 3000-D**, **PI 4500-D**, **PB 200k-D**, **PB 3500-D,** and **PB 12000-D** showed the expected derivatized low-molecular weight degradation products (Tables S5–S8 and Figures S32, S37, S39–S43, Supporting Information). The UHPLC-ESI-HRMS measurements did neither show partially derivatized nor underivatized degradation fragments in the analysis of the extracted ion chromatograms, which is further proof for the full conversion of both telechelic end-groups by the here established derivatization strategy (Figures S32, S33, and S41, Supporting Information). The derivatization of **PB 3500-Ox** and **PB12000-Ox** also showed full conversion of the aldehyde functionality and a shift to the corresponding oxime in ^{1}H NMR with the standard procedure for derivatization of polybutadienes (Figures S16–S19, Supporting Information). The molecular weights and the PDIs were not significantly affected by the derivatization procedure for all of the derivatization attempts (**Table 1**).

We aimed to demonstrate the benefits of the new derivatization method by applying it to the enzymatic degradation of synthetic polyisoprenes. Bioinspired, metastable, surfactant-free cosolvent stabilized emulsions have been shown to lead to increased enzymatic degradation of synthetic polyisoprene with *cis*: *trans* ratios of 56:27 with the rubber oxygenase Lcp_{K30} compared to nonemulsified synthetic polyisoprene.[31] For the formation of these metastable emulsions, the ratio of hydrophobic solvent, polymer, and water was identified to be of crucial importance to form stable emulsions. As a consequence of its high dispersion stability, *n*-hexadecane was chosen as the hydrophobic solvent.[40] The stabilizing effect of the cosolvent stabilized polyisoprene stems either from the coalescence depression through a rise of viscosity of the droplet or the steric repulsion among the polymer chains adsorbed on the droplet surface.[41]

The workflow for the emulsification, the enzymatic degradation, and the derivatization is depicted schematically in **Figure 5**. First, *n*-hexadecane, **PI 3000** or **PI 4500**, and water were

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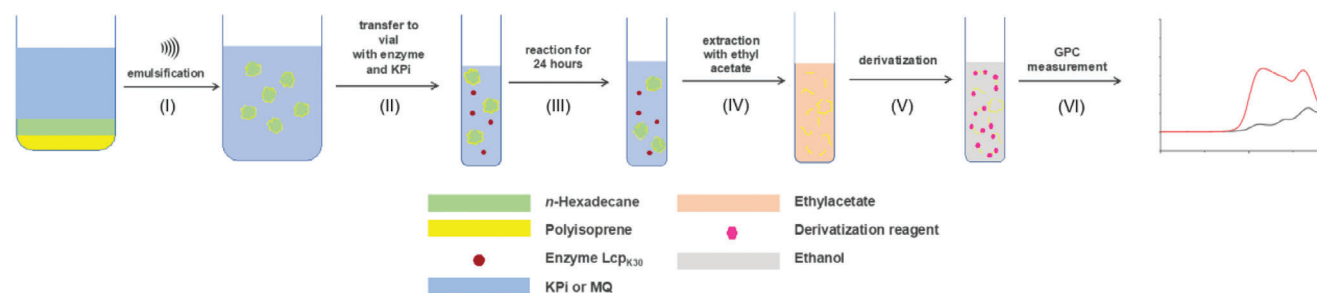Rapid Communications**

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Figure 5. Workflow of the cosolvent stabilized synthetic polyisoprene enzymatic degradation and derivatization procedure.

emulsified (I) (Figure S3, Supporting Information). The emulsion was then transferred to a vial containing the enzyme Lcp_{K30} and potassium phosphate buffer (II). After a reaction duration of 24 h (III), the reaction mixture was extracted twice with ethyl acetate (IV). The solvent of the extracts was removed, and the residue was redissolved in the derivatization solution and shaken overnight (V). The solvent of the derivatized samples was exchanged to tetrahydrofuran for GPC measurements (VI). Subsequently, the samples were subjected to GPC to detect the derivatized products using a UV-detecting system at 272 nm (see **Figure 6** (I)). Negative controls, samples without the addition of enzyme, were prepared in triplicates using the same procedure as reference samples.

As the absorption of the derivatized oximes is strongest at a wavelength of 272 nm, this one was chosen as the tracking wavelength to determine the absorption of the enzymatically degraded and derivatized polyisoprenes (**PI 3000 WT-D**, **PI 4500 WT-D**). The area under the curve of the 272 nm absorption was integrated for the retention volumes from 6 to 9 mL, which was correlated to molecular masses above 1000 according to external standard calibration and distinctively excluded the solvent peak (Figures S21, S27, and S28, Supporting Information). This provides a new insight into the degradation capabilities of Lcp_{K30} even with a background of unreacted polymer above the detection limit of UHPLC-ESI-HRMS.[31] Using the new absorption maximum with the derivatization agent we can track and com-

pare the degradation of endo-type rubber oxygenases (Figure 6 II), not readily observable in GPC using sole RI detection. The integrated area of absorption at 272 nm of the cosolvent emulsified system **PI 3000 WT-D** was significantly higher than the integrated area of absorption at 272 nm of the cosolvent emulsified system **PI 4500 WT-D**. Based on these experimental results we could demonstrate the viability and applicability of the newly developed assay to probe enzymatic activity of rubber oxygenases with synthetic polyisoprene. Thus both, the detection of oxidatively degraded polymers and the enzymatic activity with new rubber oxygenases or protein-engineered enzymes, could be achieved within one GPC-measurement using both, RI and UV-detection.

3. Conclusion

A new method has been developed to investigate the oxidative degradation of synthetic polyisoprenes and polybutadienes. This method employs an end-group derivatization approach using oximes, coupled to GPC/UV detection. The efficacy of this derivatization was confirmed through various techniques, including ^{1}H NMR, ^{13}C NMR, 2D NMR, and UHPLC-ESI-HRMS. This approach was applied to chemically degraded synthetic polyisoprenes and polybutadienes, with unreacted polymer as a surplus background. It demonstrated broad applicability by effectively monitoring degradation, as evidenced by end-group
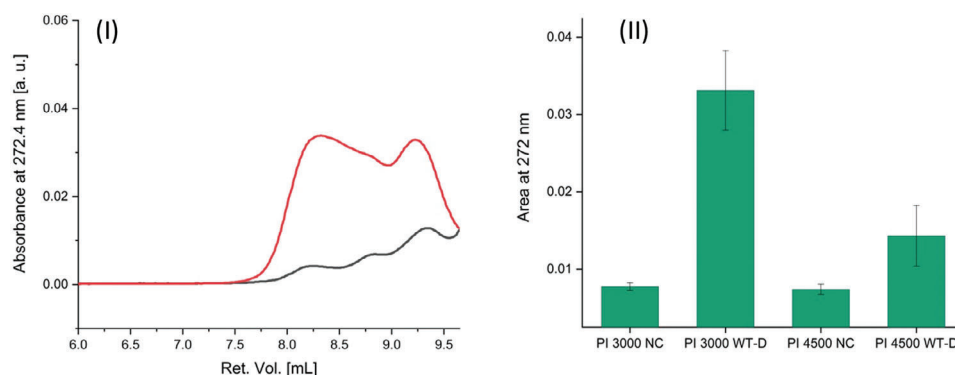

Figure 6. GP-C measurements at a wavelength of 272.4 nm for **PI 3000 WT-D** (red) and the respective negative control (black) (I) and the resulting qualitative area integration of the enzymatic degradation of the triplicates of **PI 3000 WT-D**, **PI 4500 WT-D**, and their respective negative controls (NC) (II) (see related data Figures S22–S28, Supporting Information).

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derivatization-induced UV-maximum shifts across a wide molecular weight range by UV-detection coupled to GPC.

The method was employed to investigate enzymatic degradation of co-solvent emulsified synthetic polyisoprenes. The results revealed that the cosolvent emulsified system **PI 3000 WT-D** produced significantly more degradation products with molecular weight above 1000 g mol^{-1} than for **PI 4500 WT-D**. This insight became possible due to the presented method's ability to surpass the limit of detection in UHPLC-ESI-HRMS analysis to low molecular weight polyisoprenoidic degradation fragments.

Additionally, the newly developed degradation assay could detect shifts in the molecular weight of end-group derivatized absorption maxima of enzymatically degraded samples, even when no significant shifts above 1000 g mol^{-1} were observable in conventional GPC detection by RI techniques. Consequently, this methodology represents a valuable new tool for screening and understanding the enzymatic degradation of synthetic polyisoprenes, particularly at low enzymatic activity levels. It may also find utility in other enzymatic or organocatalytic oxidative degradation investigations of polyenes with an excess of unreacted polymer and serve as basis for further investigations of the enzymatic degradation of synthetic polyisoprenes and potentially synthetic polybutadienes. These two polymers are used ubiquitously in day-to-day life and require novel more sustainable and controlled degradation methodologies. The derivatization approach could enhance the screening capabilities for rubber oxygenases or protein-engineered enzyme variants, as it is applicable across a wide molecular weight range, offers high sensitivity even in the presence of polymer background, and provides information about the end-groups of degradation products. Potentially this method is transferable for the screening in higher throughput screenings. Consequently, this methodology can be seen as a valuable tool in the endeavor of developing novel methods of degrading rubbers in the future and the quest for a more circular polymer chemistry world.

With its broad applicability and capacity to reveal previously hidden degradation processes, this method represents a noteworthy contribution to sustainable polymer chemistry.

4. Experimental Section

A detailed Experimental Section can be found in the Supporting Information.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the Supporting Information of this article.

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