Supplementary Information (SI) for RSC Sustainability.

Supporting Information

Analysis of the Product-Spectrum during the Biocatalytic Hydrolysis of PEF (Poly(ethylene furanoate)) with Various Esterases

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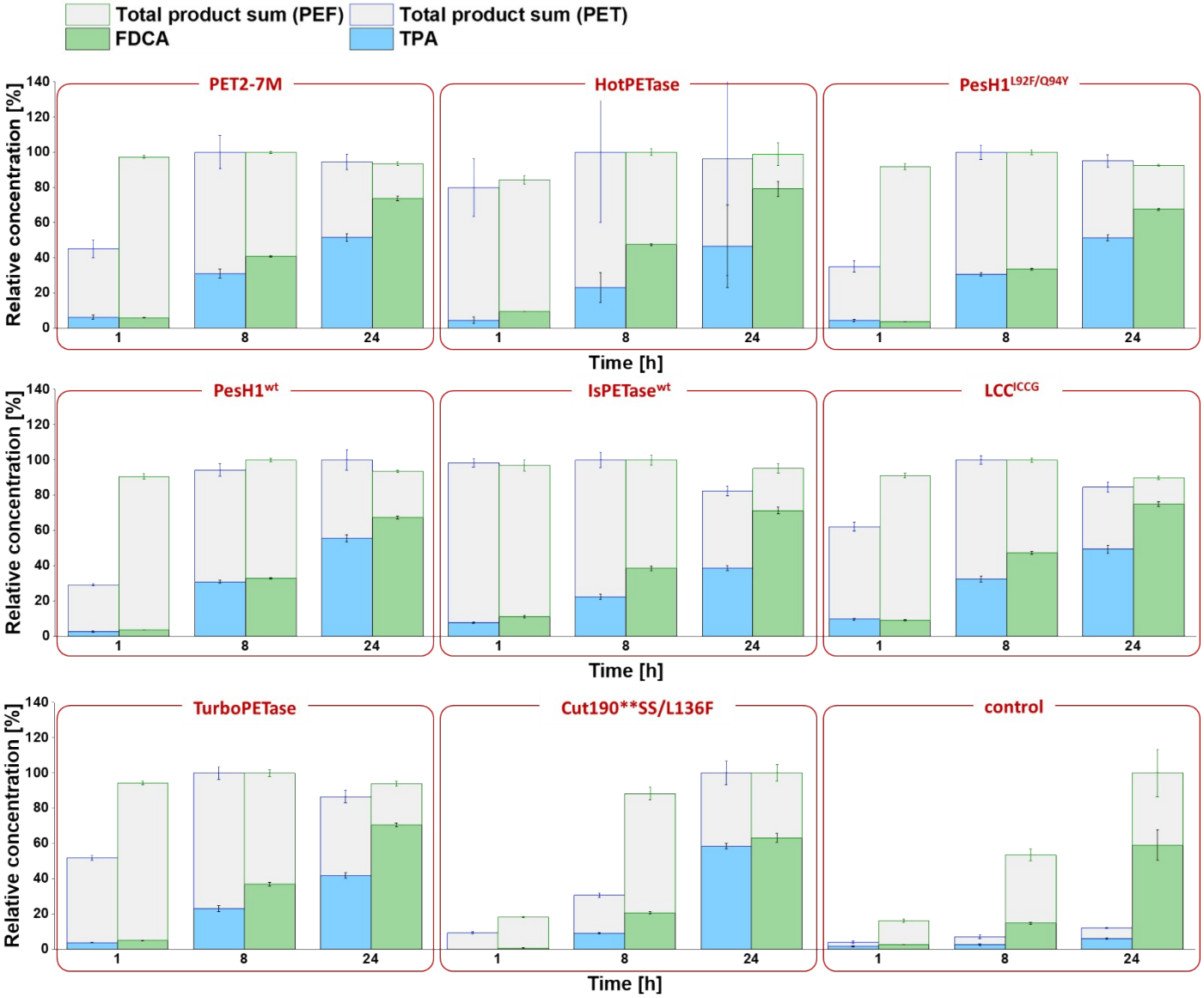

Figure S1. Comparison of PEF vs. 3PET hydrolysis under equal conditions. Comparison of hydrolysis of Nano-PEF (this study; shown in green, right bars) and of 3PET (previous study; [1] raw data were used for creating this figure; shown in blue, left bars). The highest concentration of the total product concentrations within one data set (individual for Nano-PEF and 3PET) was set to 100%, and the other concentration were related to that. The total amount is depicted in grey with the respective frame and the basic building blocks (i.e., TPA and FDCA in the case of 3PET and Nano-PEF, respectively) are shown in the respective color. Reactions were performed in triplicates, and the error bars represent the standard deviation. The background due to self-hydrolysis of 3PET and Nano-PEF was always subtracted.

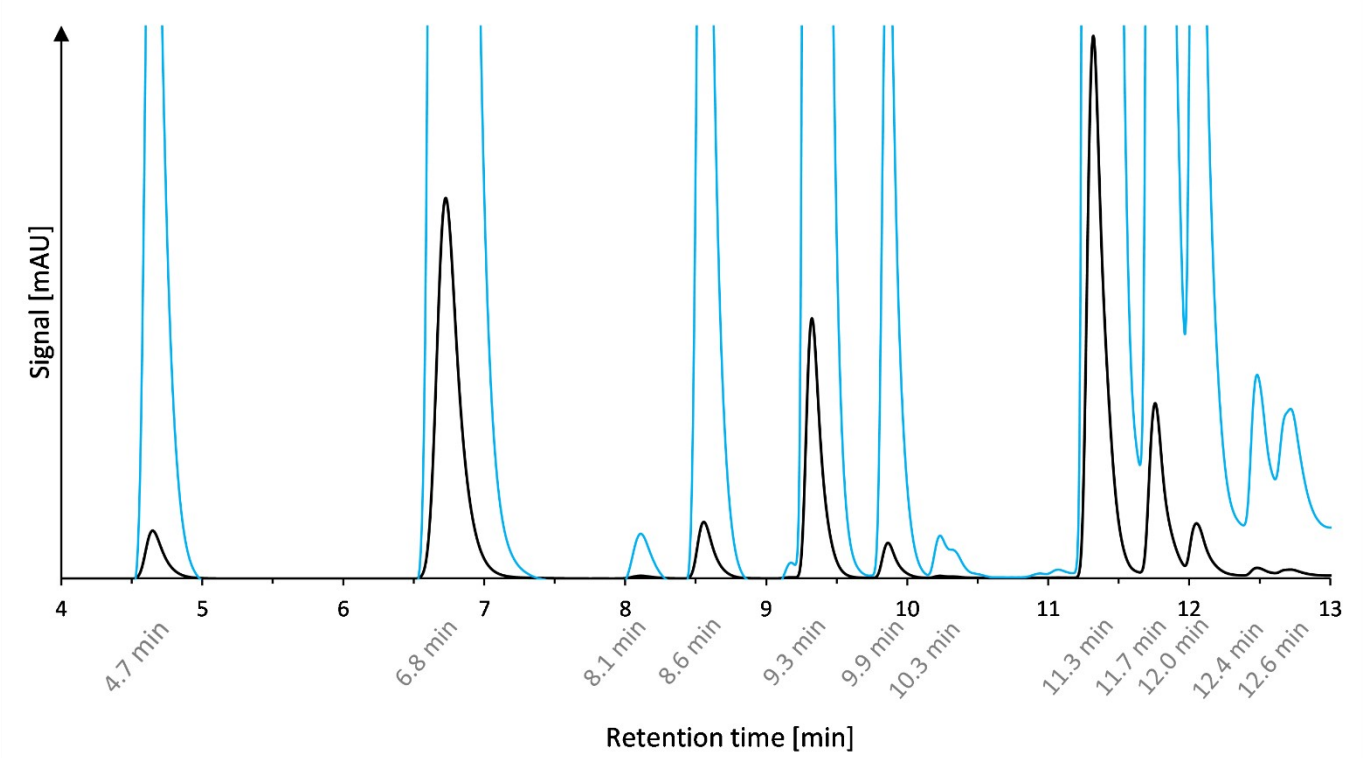

Figure S2. Exemplary HPLC chromatogram of a partially hydrolyzed Nano-PEF sample. During hydrolysis, numerous detectable intermediates are formed and quantified using FDCA calibration data, enabling the determination of the overall hydrolysis rate of the enzymes under study. The blue curve depicts data at 20x magnification, enhancing the visibility of the small peaks.

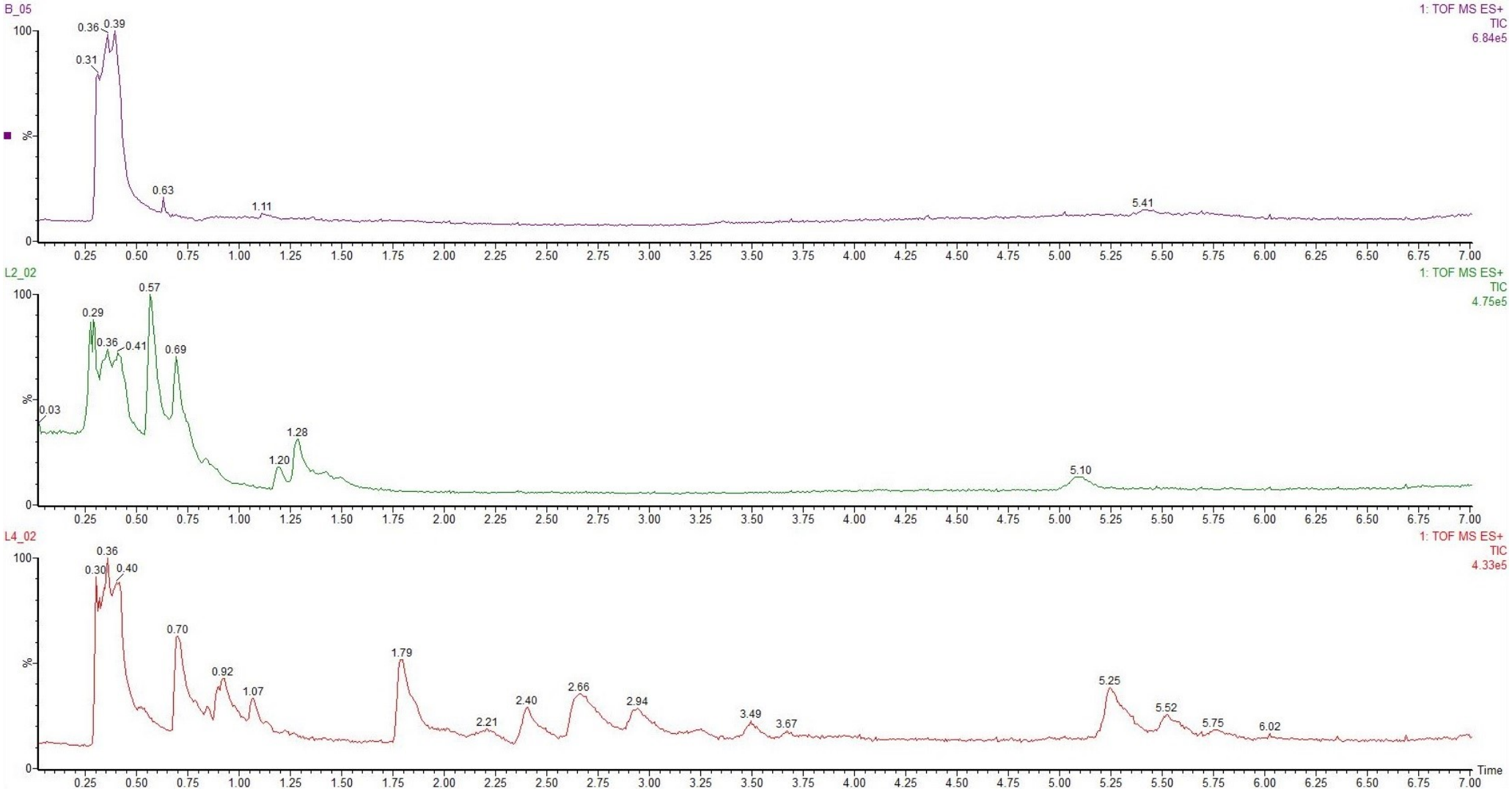

Figure S3. LC/MS chromatograms of different biocatalytic samples. PEF hydrolysis was performed under standard conditions with LCC^{ICCG} (L4, lower panel, red) or Turbo-PETase (L2, middle panel, green) both for 1 h reaction time. All peaks are assigned to their individual retention time. The blank (B, upper panel, pirple) represents the buffer only (i.e., without enzyme and PEF) and shows the background. All hydrolysis intermediates eluted before 7 min.

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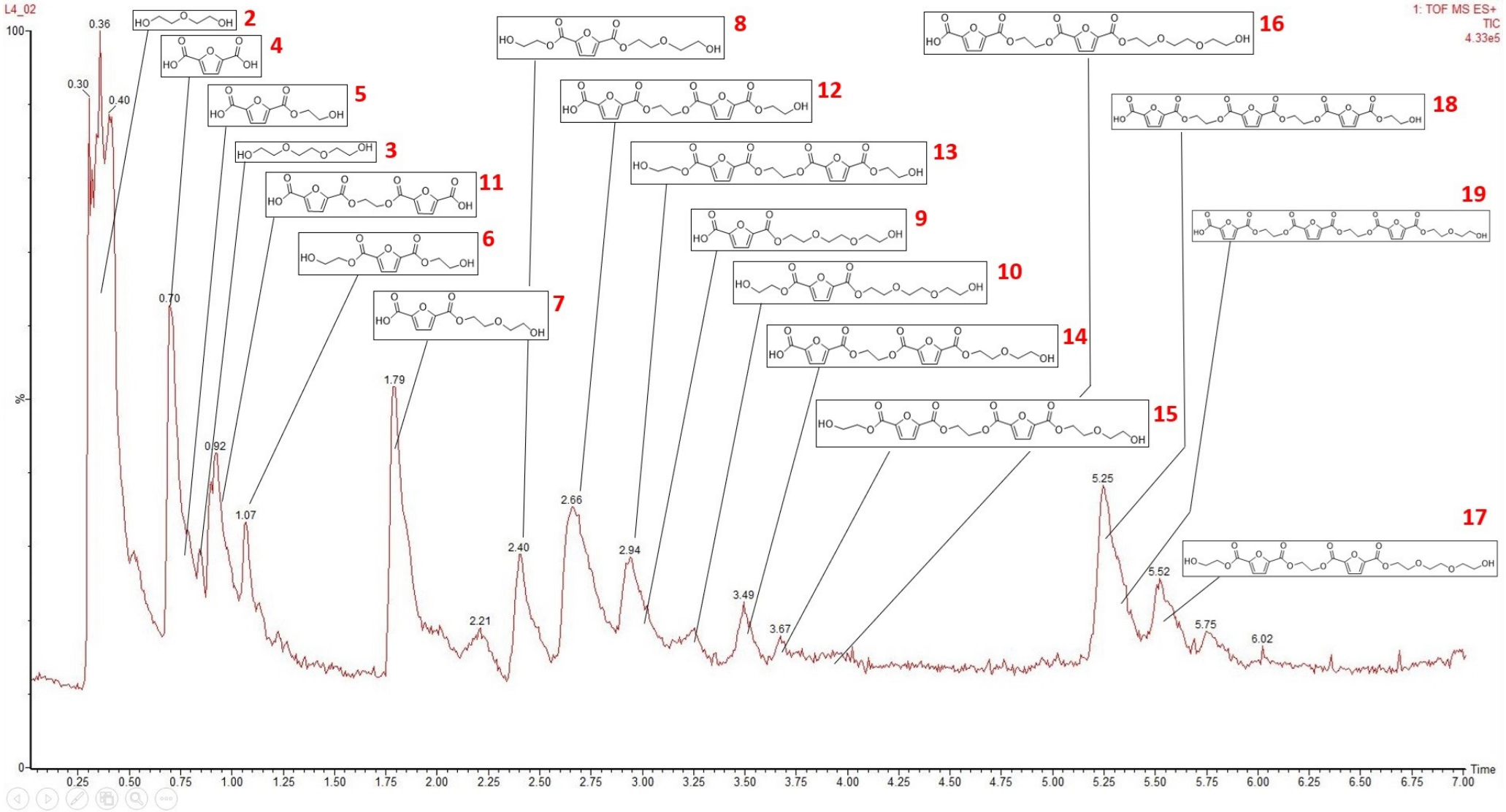

Figure S4. LC/MS chromatogram with assignment of the hydrolysis intermediates. According to the molecular masses identified via ESI-MS, all hydrolysis intermediates found in this reaction were assigned to the respective peaks for the reaction L4 in Fig. S3. Additional information can be found in Table 1. All hydrolysis intermediates eluted before 7 min. The numbers shown in red correspond to the entries in Table 1.

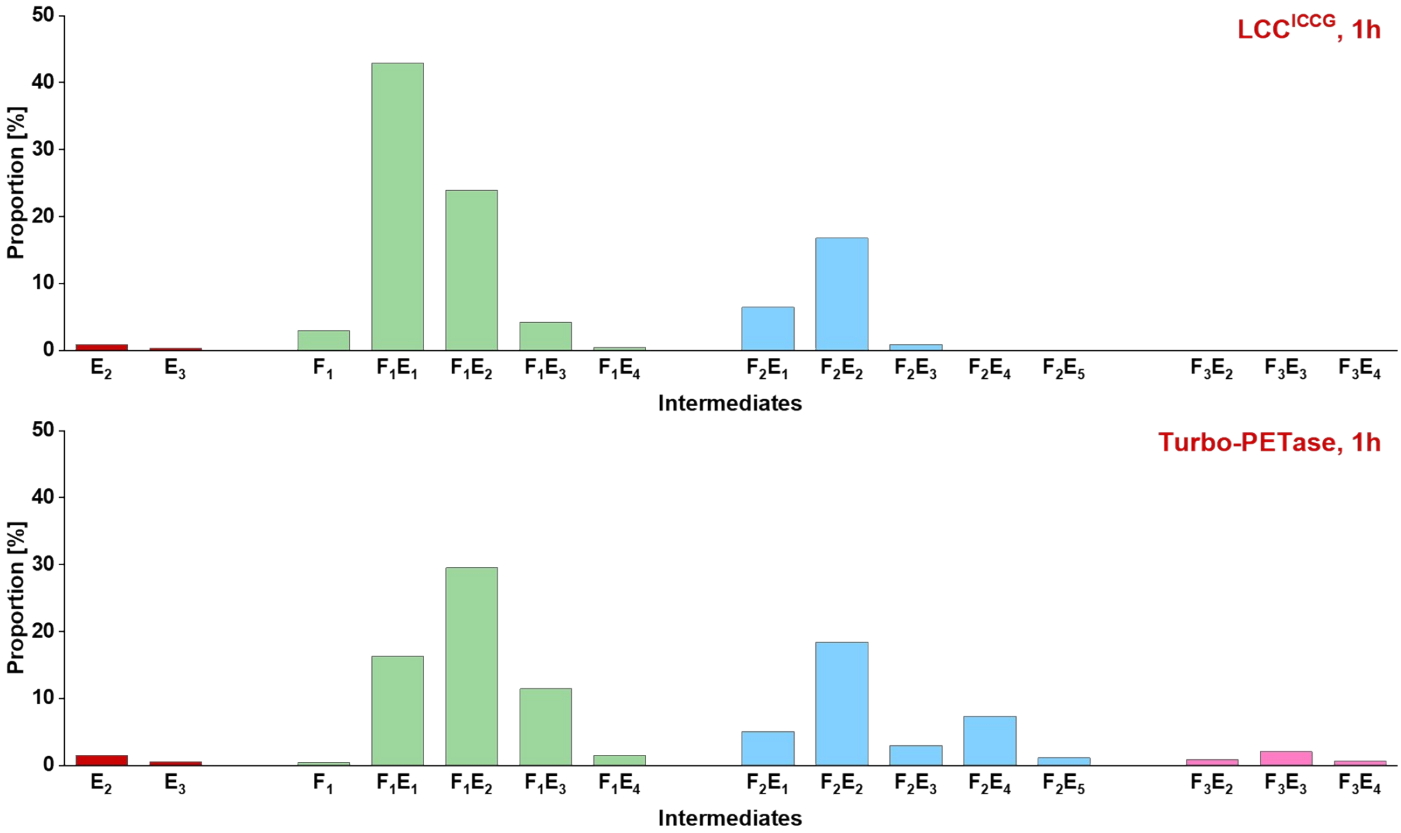

Figure S5. Proportion of enzymatic hydrolysis intermediates. Based on sum formulas determined via ESI-TOF mass spectrometry, the proportion of hydrolysis intermediates with equal F- and E-residues could be identified. Ethylene glycols are depicted in red (E2, E3; MEG was not identified in these samples), monomers in green (F1EX), dimers in blue (F2EX) and trimers in pink (F3EX). Shown is the sum of all molecules that have the same number of FDCA (F) and EG (E), while respective numbers of EGs shown represent the sum for each molecule and not the repetition in sequence; for example, the sum of all dimers that contain a total of 3 EGs is shown (e.g., E-F-E-F-E + F-E-F-E-E). Chromatograms used for this representation can be found in Fig. S3-S4.

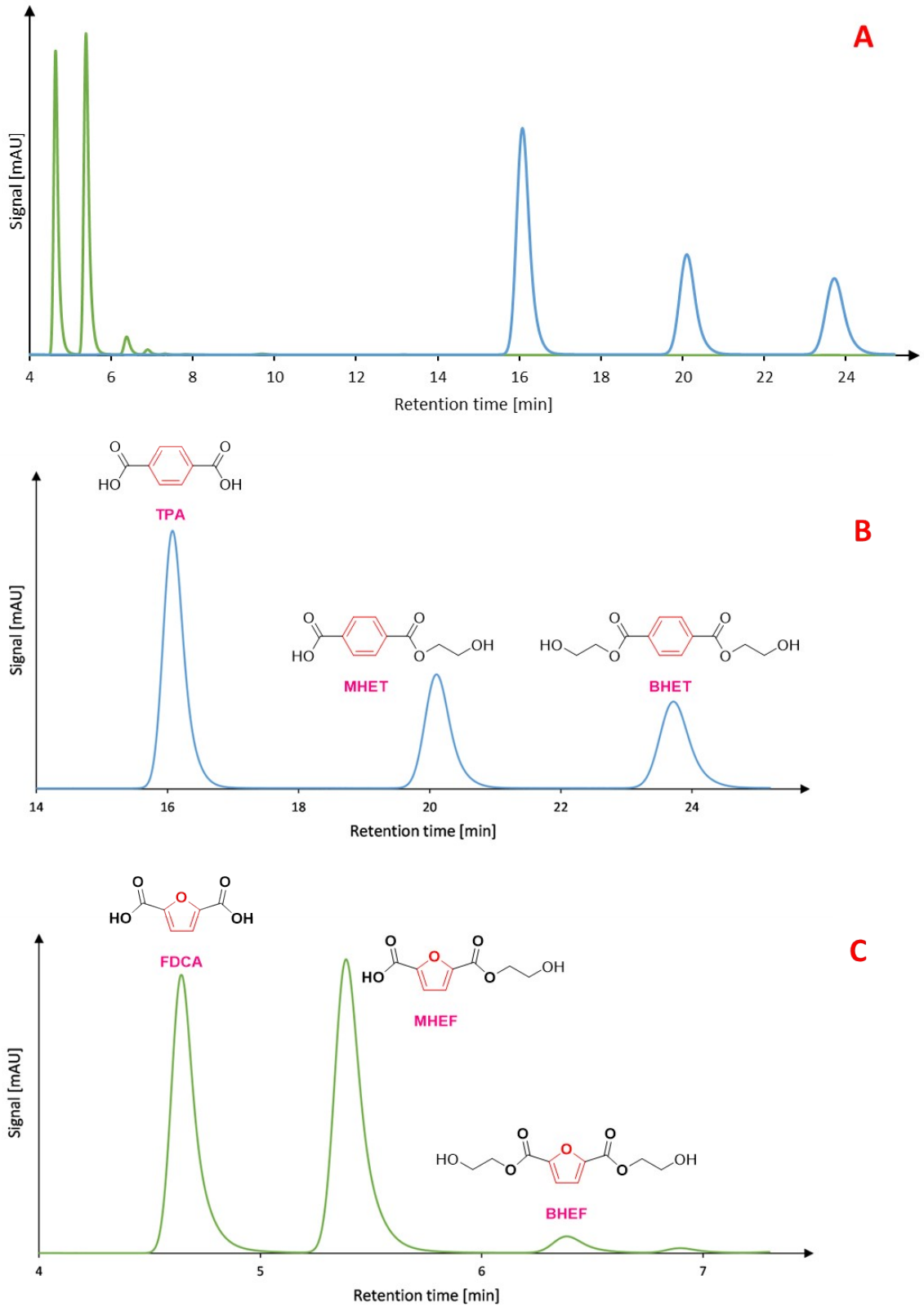

Figure S6. Comparison of elution profiles of hydrolyzed Nano-PET and Nano-PEF. Nano-PET (blue curve) and Nano-PEF (green curve) samples were analyzed using analytical HPLC under isocratic conditions using 15% (v/v) ethanol and 85% (v/v) of 0.1% (v/v) formic acid in water. **A**: Overview of both samples in B and C; **B**: Hydrolyzed Nano-PET with TPA, MHET, and BHET identified by standards; **C**: Hydrolyzed Nano-PEF with FDCA identified by a FDCA standard, and MHEF and BHEF tentatively identified based on PET analysis. As the structures of FDCA, MHEF and BHEF are very similar to TPA, MHET and BHET, respectively, similar running behaviors were assumed.

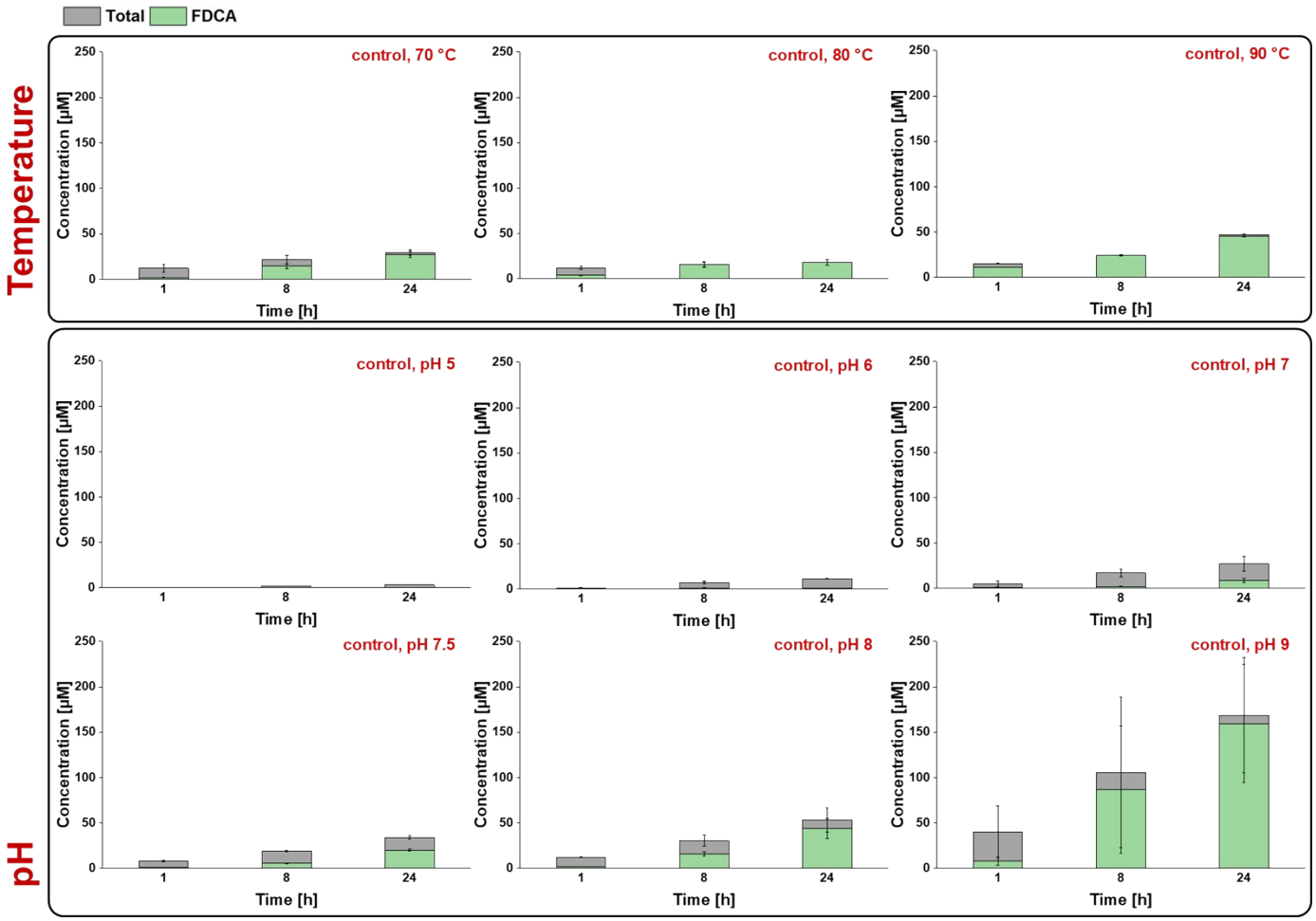

Figure S7. Self-hydrolysis of Nano-PEF under different conditions. In parallel to the biocatalytic reactions, controls were always carried out with the samples that only contain Nano-PEF and no enzymes to analyze the self-hydrolysis of the polymer under certain conditions. The temperature and pH control experiments were performed at different temperatures and with different pH values (as stated). The product profile was analyzed after 1, 8 and 24 h reaction time via HPLC. The controls were done in triplicates, and the error bars represent the standard deviation.

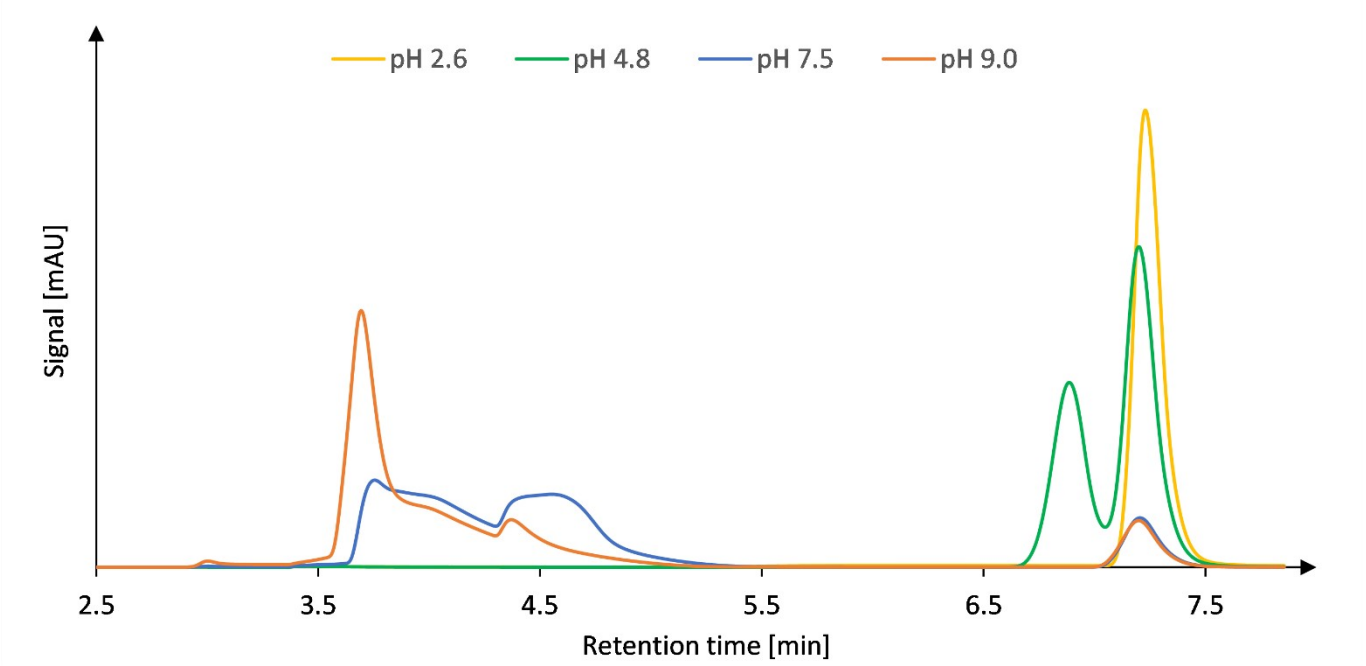

Figure S8. pH dependence of FDCA during HPLC analysis. The pH of samples significantly influences the interactions between the column and FDCA; thus, the HPLC samples should be maintained below 2.6 to ensure the presence of a single peak for accurate detection and quantification.

Table S1. HPLC gradient used for the quantitative analysis of the PEF hydrolysis products.

Time [min]	0.1% (v/v) formic acid in water [% v/v]	Acetonitrile [% v/v]
0	88	12
2	88	12
7	60	40
9	30	70
10	30	70
12.5	88	12
16	88	12

Table S2. HPLC gradient used for separating PEF hydrolysis products for MS analysis.

Time [min]	2 mM Ammonium acetate buffer [% v/v]	Acetonitrile [% v/v]
0	88	12
2	88	12
7	60	40
9	30	70
10	30	70
12.5	88	12
16	88	12

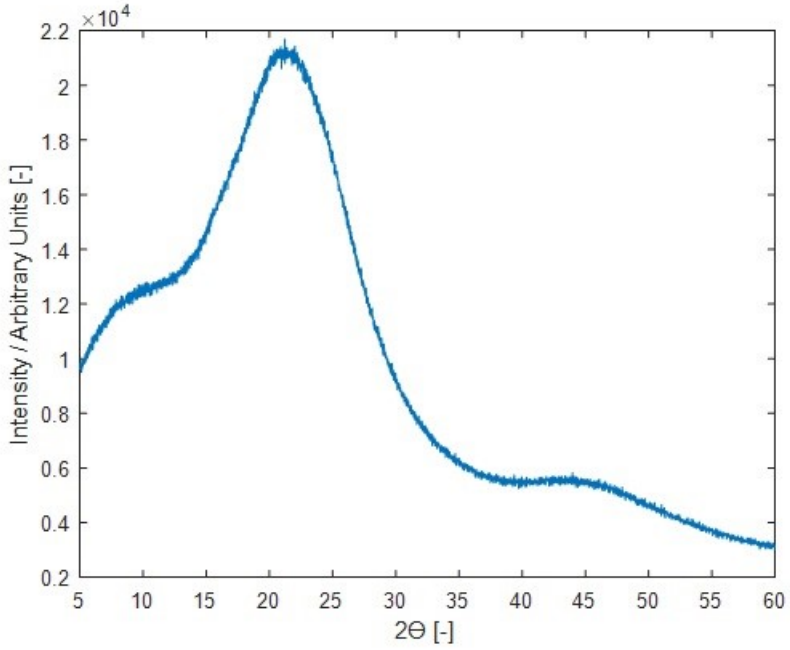

Figure S9. Measurement of a PEF sample using PXRD.

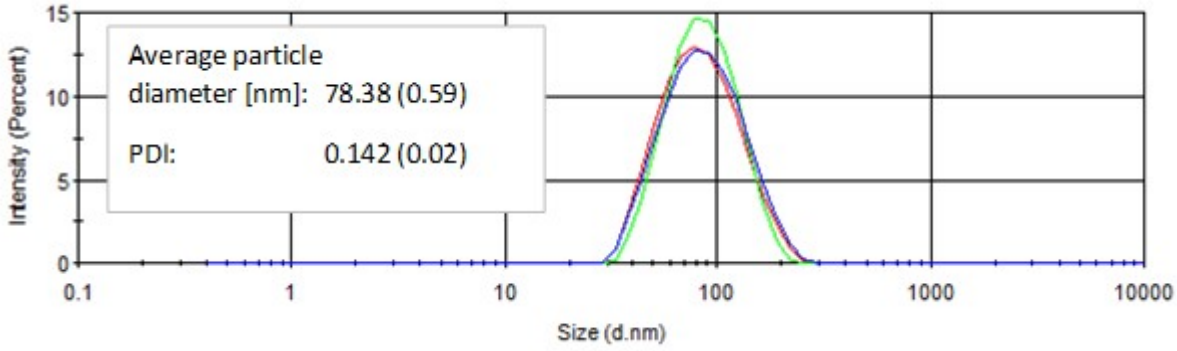

Figure S10. Particle size distribution of the Nano-PEF suspension. The reported values were measured in triplicates, the average particle size and PDI are given as mean with the standard deviation in brackets.

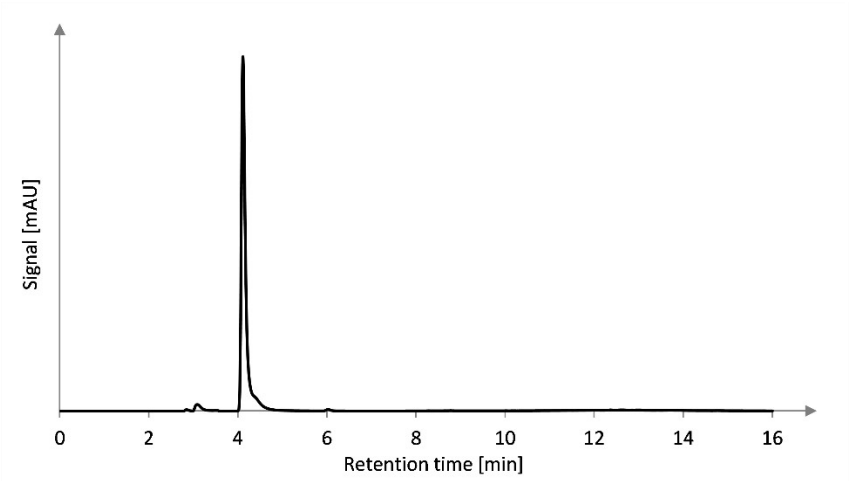

Figure S11. Total hydrolysis of Nano-PEF. 1 mL Nano-PEF was totally hydrolyzed to FDCA as mentioned in the methods. Per mg of applied Nano-PEF, 4.74 µmol FDCA were obtained (or 5048 (µM*mL)/mg) without byproducts and FDCA as the single product. The experiments were done in triplicates.

References

[1] T. Heinks, K. Hofmann, S. Last, I. Gamm, L. Blach, R. Wei, U. T. Bornscheuer, C. Hamel, J. von Langermann, *Selective Modification of the Product Profile of Biocatalytic Hydrolyzed PET via Product-specific Medium Engineering*, ChemRxiv, **2024**.