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Abstract: Sequential electron beam-steam explosion (EB-SE) pretreatment was applied to hardwood (Birch) and softwood (Pine) substrates to enhance enzymatic saccharification. The effect of these two pretreatments on the structure and composition of the individual cell wall components was examined. The combination of these treatments showed a synergistic effect on the conversion of hemicelluloses into water soluble oligomers and enhanced the overall enzymatic saccharification of wood substrates. Even after the combined pretreatment Pine was more recalcitrant than Birch, which seemed to be due to different effects on the lignin. Model systems created from cellulose and isolated high molecular weight (HMW) lignin fractions were found to inhibit enzymatic conversion of cellulose by 20 % over a control. Conversely, low molecular weight lignin fragments were found to be slightly beneficial for enzymatic hydrolysis of cellulose substrates. This inhibition is likely related to the unproductive binding of the cellulose enzymes to the HMW lignin. Additionally, the presence of the HMW lignin reduces the swelling capacity of the wood substrate, and thus its accessibility to enzymes. These results provide insight to the complex interactions between lignin and cellulase enzymes, and highlight the need for pretreatment processes that can effectively cleave lignin into oligomeric fragments.

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Editor
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Dear Dr Overend,

Enclosed you will find our manuscript entitled ”E-beam irradiation & steam explosion as biomass pretreatment, and the complex role of lignin in substrate recalcitrance”, by myself, Dr. Stephen Kelley and Dr. Dimitris Argyropoulos. We would like to publish this manuscript in one of the future editions of Biomass & Bioenergy journal.

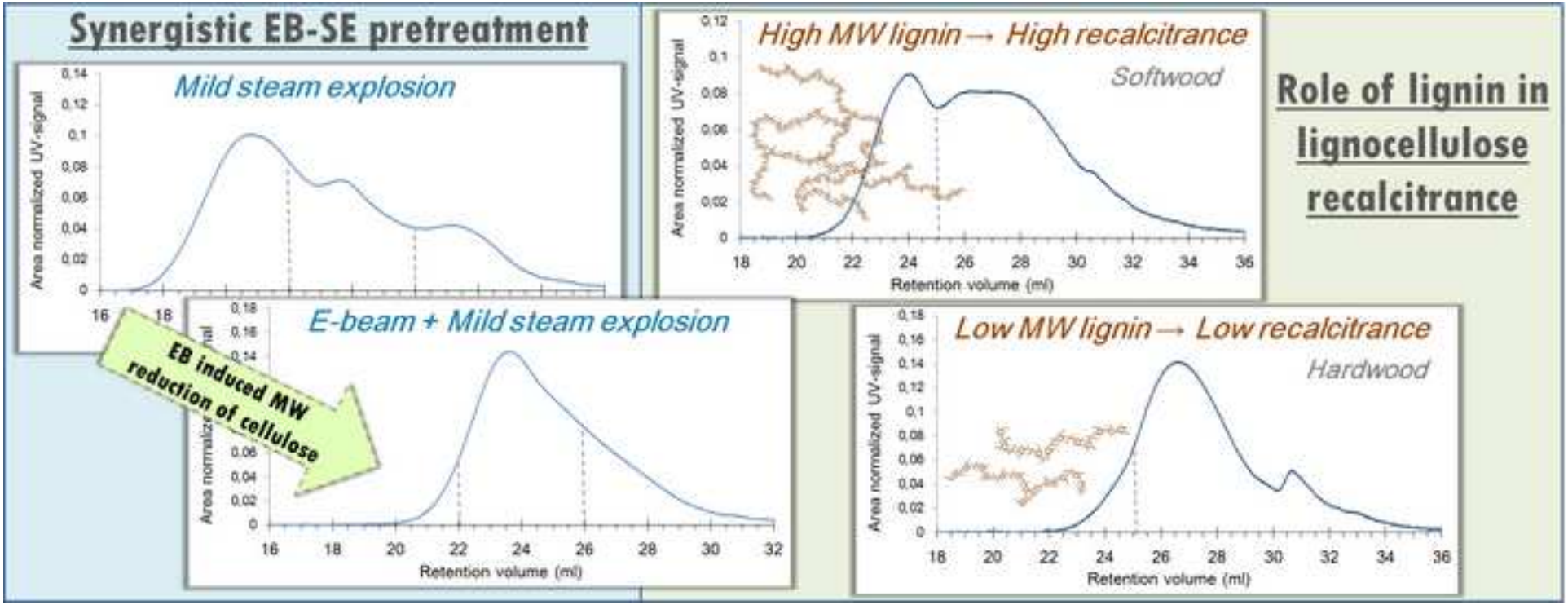
This manuscript describes our efforts in establishing mechanistic understanding of novel and efficient pretreatment technique combining electron beam irradiation and steam explosion. We have also shed light to the complex interplay of lignin structure in pretreated substrates and performance of cellulolytic enzymes therein. Our research could confirm some of the latest findings in the field of enzymatic hydrolysis of lignocellulose. Specifically that it is the polymeric structure of lignin that contributes to the substrate recalcitrance, and that conversion of lignin into oligomeric fragments can actually boost the enzymatic hydrolysis. These findings are important regarding the future progress of lignocellulose pretreatment and establishment of economically viable cellulosic ethanol production.

The manuscript has been prepared according to guidelines of Biomass & Bioenergy journal. We will be happy to address any shortcomings to improve its quality and provide most our message to the readers in efficient fashion. We look forward to yours and reviewers decision.

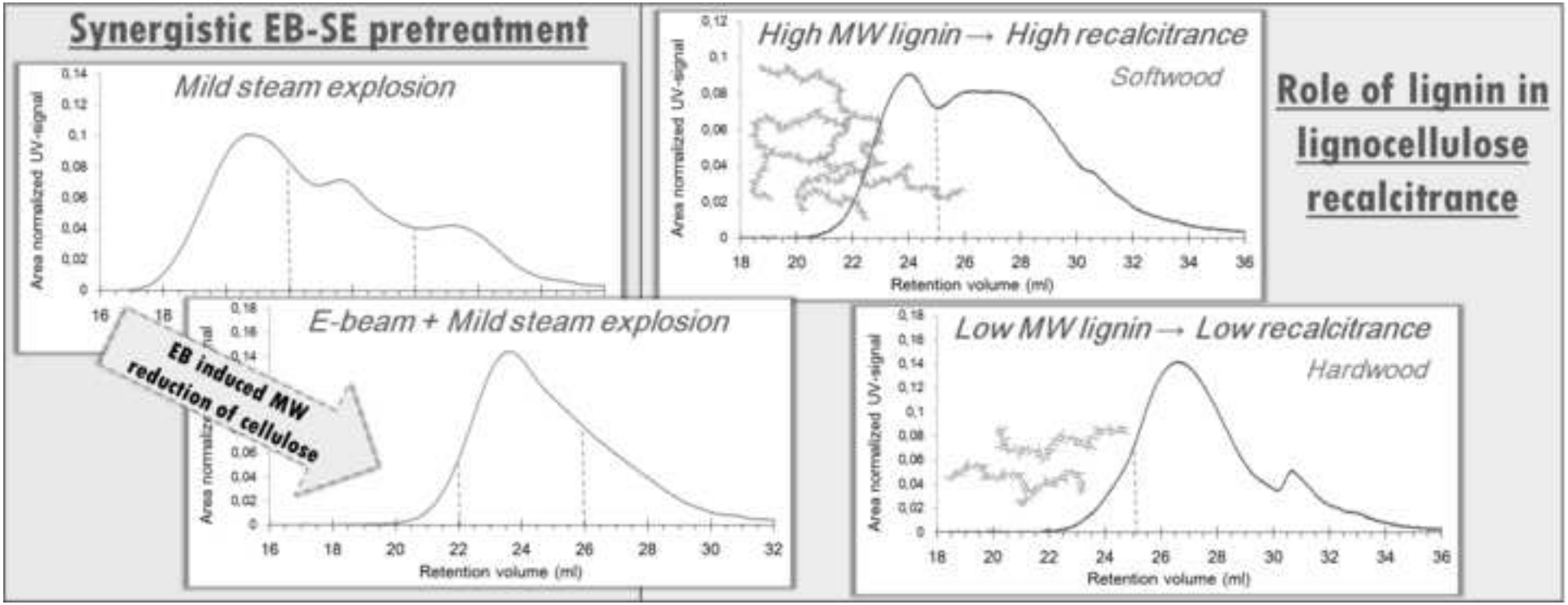
Sincerely yours,

Timo Leskinen, Ph.D.

Graphical Abstract



Graphical Abstract



Highlights

E-beam irradiation & steam explosion as biomass pretreatment, and the complex role of lignin in substrate recalcitrance

Timo Leskinen, Stephen S. Kelley, and Dimitris S. Argyropoulos

- Combined electron beam (EB) and steam explosion (SE) are an effective pretreatment
- EB mainly depolymerizes cellulose while SE hydrolyses hemicelluloses and lignin
- Degradation of lignin-hemicellulose matrix was crucial for enzymatic hydrolysis
- High molecular weight lignin contributes strongly to substrate recalcitrance
- Low molecular weight lignin deposited on substrate seems beneficial for enzymes.

E-beam irradiation & steam explosion as biomass pretreatment, and the complex role of lignin in substrate recalcitrance

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Abstract

Sequential electron beam-steam explosion (EB-SE) pretreatment was applied to hardwood (Birch) and softwood (Pine) substrates to enhance enzymatic saccharification. The effect of these two pretreatments on the structure and composition of the individual cell wall components was examined. The combination of these treatments showed a synergistic effect on the conversion of hemicelluloses into water soluble oligomers and enhanced the overall enzymatic saccharification of wood substrates. Even after the combined pretreatment Pine was more recalcitrant than Birch, which seemed to be due to different effects on the lignin. Model systems created from cellulose and isolated high molecular weight (HMW) lignin fractions were found to inhibit enzymatic conversion of cellulose by 20 % over a control. Conversely, low molecular weight lignin fragments were found to be slightly beneficial for enzymatic hydrolysis of cellulose substrates. This inhibition is likely related to the unproductive binding of the cellulose enzymes to the HMW lignin. Additionally, the presence of the HMW lignin reduces the swelling capacity of the wood substrate, and thus its accessibility to enzymes. These results provide insight to the complex

interactions between lignin and cellulase enzymes, and highlight the need for pretreatment processes that can effectively cleave lignin into oligomeric fragments.

Keywords: Pretreatment, Steam explosion, Electron beam, recalcitrance, lignin, molecular weight

1 Introduction

The global efforts to reduce carbon dioxide emissions, including the recently ratified Paris Agreement, and a desire to generate more sustainable domestic fuels continue to drive interest in lignocellulose based biofuels. [1] Woody plants are an abundant biomass resource, but they also possess formidable technical challenges for biochemical processing. [2] One major challenge for commercial utilization of lignocellulose is the difficulty of converting the structural polysaccharides of cell walls into fermentable sugars. This phenomenon is general termed as 'recalcitrance', although it originates from several different physical and chemical attributes of the biomass. [3-5]

Different pretreatment techniques have been developed in attempts to overcome the biomass recalcitrance, including chemical, mechanical, solvent based, and hydrothermal treatments. [3, 6] The effectiveness of different biomass pretreatments are based on their costs, e.g., capital and operating, and their effectiveness in terms of hydrolysis rate, and the amount of enzyme required to produce a given amount of soluble sugar.

Steam explosion (SE) is a well-established biomass pretreatment technique for bioethanol production, and also biomass composites. [7] Mechanistically SE can be classified as a hydrothermal pretreatment process that degrades both the lignin and carbohydrate fractions.

In many hardwoods and perennials the thermally induced hydrolysis reactions are enhanced by acetic acid generated from acetyl groups naturally present in the biomass. At the same time SE promotes the physical disintegration of the biomass increasing surface area. [3] SE induces extensive hydrolysis of hemicelluloses polymers, while there is only limited depolymerization of cellulose. [3, 8] In contrast, the effects of SE on lignin are complex, and both depolymerization and condensation reactions occur. [9] Alteration of the native lignin structure, and its re-deposition on the pretreated biomass has been suggested. [3] These complex interactions are dependent on the biomass source, and the detailed heat and mass transfer reactions that take place within the specific SE reactor; many of these details require further research.

Pretreatment by electron beam (EB) irradiation relies on completely different mechanisms than SE since it uses high energy electrons to create reactive radical species within the biomass. The secondary reactions of these radicals typically lead to scission of bonds within cell wall polymers. [10, 11] Unlike SE, EB-irradiation has been found to reduce the degree of polymerization (DP) of cellulose [10, 12] while also causing alteration of the lignin-hemicellulose matrix [13]. EB has been shown to have beneficial effects on subsequent treatments such as acid hydrolysis or mechanical crushing. [14, 15]. As such, EB has potential for complimenting the effects of SE, and together they may provide a synergistic effect.

Interactions between cellulose enzymes and pretreated biomass is still not completely understood. Although enzymes are commonly known to adsorb unproductively on lignin [16], it has also been shown recently that lignin can enhance enzymatic hydrolysis [17-19]. Recent molecular modeling work on lignin models in aqueous solvent that the lignin has

extended conformation with some amphipathic character, analogous to surface active compounds. Clearly the detailed interplay between the molecular structure of lignin and its impact on enzymes requires further in-depth investigation.

In the present study we have examined the application of EB and SE pretreatments separately, and in combination, with special attention to the interaction between cellulose enzymes and lignin structure. We applied a size-exclusion chromatographic (SEC) methodology [20] to probe the changes in the carbohydrates and lignin caused by pretreatment conditions. We also examined how the polymeric structure of lignin impacts enzymatic hydrolysis.

Materials and methods

1.1 Materials

The solvents were purchased from Sigma-Aldrich and Fisher scientific and used without further purification. 1-allyl-3-methylimidazolium chloride ([amim]Cl) was prepared as described elsewhere. [20] α-Cellulose (C8002 Sigma) was purchased from Sigma-Aldrich. The enzyme cocktail used was Ctec2 supplied by Novozymes (USA), with a determined activity of 107 FPU/mL.

The Norwegian Pine and Birch wood samples were supplied by BioOil AS (Norway). The EB irradiation of the wood was conducted by our commercial partner. A 200 kW 4 MeV electron accelerator (IBA, Belgium) was used for treatment. 1 kg of samples were set on aluminum trays and passed four times though the accelerator that was set to deposit 25kGy per pass. The SE was performed at the Norwegian University of Life Sciences (UMB),

Norway. Details of the SE facility are provided elsewhere. [1][21] The EB and SE conditions are summarized in Table 1.

Electron Beam irradiation	Steam explosion (6 min retention)
None	None
100 kGy	None
None	170 °C
100 kGy	170 °C
None	200 °C
100 kGy	200 °C

Table 1 Conditions of the electron beam and steam explosion pretreatments used in this study. All conditions were applied to Birch and Pine wood.

1.2 Extractions and determination of extractive compositions

All extractions were carried out in a Soxhlet extractor for 18 h and 24 h for dichloromethane (DCM) and water, respectively. The DCM extracts were recovered by evaporation of the solvent followed by drying in a desiccator to constant weight. Triplicate analyses of 200 °C SE sample of Birch were used to determine standard deviations 18.5 mg/g in extractive yields. The composition of the DCM extractives was examined by ^{1}H-NMR and further fractionation techniques as described elsewhere. [22] All aqueous extracts were frozen prior to further analyses. Carbohydrate and lignin content of the water extractives were determined using standard methods [23, 24] Standard deviations from triplicates were 20 mg/g for sugars and 4 mg/g for lignin.

1.3 Water retention value (WRV)

Prior to the WRV determination, all soluble hemicelluloses and lignin were removed by sequential extractions with DCM, water, acetone, and dioxane-water 85:15 v:v (yields shown in Table A.1 of Appendix). The WRV was determined in a manner similar to that described by Kumar et al., [25] with the exception that a medium grade sinter glass filter was used to drain a 1.0 g sample during centrifugation. The standard deviation of these determinations was 3 %.

1.4 Size-exclusion chromatography

The benzoylation and acetobromination procedures have been described elsewhere. [20] There was a minor alteration to the benzoylation procedure where the dissolution of a 10 mg sample was initiated using 1 g of 1-allyl-3-methylimidazolium chloride ([amim]Cl) ionic liquid, in the presence of 200 μL of pyridine. After 48 h of incubation at 80 °C, 300 μL of 1-methylimidazole was added and the incubation was continued at 60 °C for 72 h. Reaction and washing steps were then carried out as described in the original procedure.

Due to the multimodal lignin distributions observed in this study the typical molecular weight averages were not deemed useful. A qualitative discussion of the MWD profiles were sufficient for this study.

1.5 Deposition of lignin on cellulose

The preparation and characterization of the ‘soluble’ and ‘insoluble’ lignin fractions have been reported elsewhere [23], denoted in the previous work as LSA and E lignins, and their insoluble residues, respectively. Soluble E-lignin and corresponding insoluble residue were obtained from extraction of lignin rich residue of enzymatically hydrolyzed EB-SE 200 °C

Birch using ethanol-water mixture at 80 °C. LSA-lignin and insoluble residue were obtained from the same material by extraction with aqueous 1.0 M NaOH at room temperature. Initially, 0.5 g of cellulose was weighed in a crimp seal bottle and 100 mg of a lignin preparation was added as a powder. The lignin, 100 mg, was dissolved/dispersed onto 0.5 g of cellulose with 5 mL of acetone-water 85:15 v:v. The lignin was dissolved (or simply finely dispersed in the case of residues) followed by an overnight refrigerated storage. After 24 h the solvent was allowed to evaporate at atmospheric pressure with constant orbital shaking, and any remaining water was removed by freeze drying. The dried samples were subjected to enzymatic hydrolysis (in triplicate, standard deviation typically less than 6 mg/g) as described below.

1.6 Enzymatic hydrolysis

All samples were dried and sieved to create a homogenized particle size ranging between 0.85 – 0.25 mm. Enzymatic hydrolyses were carried out at pH 4.9 in acetate buffer at 5 wt. % solids, by using 10 FPU/g of Ctec2 cellulase enzyme. The mixture was incubated in an orbital shaker at 50 °C for 72 h. The hydrolysis reactions were quenched by cooling the mixture to approximately 10 °C. An aliquot of the resulting liquor was withdrawn and filtered for HPLC analyses as described elsewhere. [22]

Standard deviations of triplicate analyses of pretreated materials were below 1 %, and error bars are not included in Figure 1. Figure 6 contains error bars due to larger determined variation.

2 Results and discussion

2.1 Enzymatic digestibility of pretreated Birch and Pine

The effects of the individual and combined pretreatment on sugar release was evaluated. The data of Figure 1 shows that the 100 kGy irradiation treatment alone or 6 min steam explosion at 170 °C did not impact enzymatic digestibility relative to the untreated control. However, the combination of EB and SE (170 °C) showed a three-fold increase in sugar release from Birch, and a doubling for Pine. As expected increasing the SE temperature to 200 °C increased sugar release to 660 mg/g (89 %) and 320 (47 %) mg/g for Birch and Pine, respectively. The addition of EB to the 200 °C SE only provided incremental added benefit. In all cases Pine was more difficult to hydrolyze. Figure 1 also shows the well-documented differences in enzymatic hydrolysis rates between hardwoods and softwoods. [26, 27] These differences remained even with the differential chemical pathways created by the combination of EB and SE.

Figure 1 Enzymatic sugar release from Pine and Birch substrates after various pretreatment conditions. Maximum theoretical yields from Birch and Pine substrates were 740 and 680 mg/g, respectively.

2.2 Depolymerization of cellulose

Reducing the degree polymerization (DP) of cellulose during pretreatment is known to increase sugar release although the mechanistic details are complex. [16] Under conditions used in this study, SE pretreatment is reported to cause very limited changes in the DP of cellulose, [8] while EB is effective at depolymerizing cellulose. [12] Thus, the EB-SE

sequence was expected to lower the cellulose DP, and also potentially 'open up' the cellulose structure. Changes in the molecular weight (MW) distributions of carbohydrates due to the different pretreatments are shown in Figure 2.

Figure 2 SEC chromatograms of pretreated substrates and starting materials after benzoylation. A) Birch no EB B) Birch with EB C) Pine no EB D) Pine with EB.

On the basis of our recent work, [20] the multimodal molecular weight distributions (MWD) displayed in Figures 2A and 2C can be assigned cellulose (21 mL retention volume), hemicelluloses (24 mL), and lignin (28 mL). Figure 2A and 2C confirm prior work that suggests SE at temperatures between 170 °C and 200 °C do not significantly depolymerize cellulose.

The middle peak (24 mL) that corresponds to the hemicelluloses clearly diminishes after 200 °C SE, in accordance with the literature. [28] Depolymerization of hemicelluloses at 200 °C was qualitatively similar between Birch and Pine (Figures 2A and 2C) and also in agreement with soluble sugars and oligomers recovered by water extractions (section 2.3).

The MWD in EB irradiated and EB-SE pretreated substrates (Figure 2B and 2D) showed significant differences compared to the non-irradiated ones. For samples subjected to EB alone the highest MW peak that corresponds to intact cellulose (21 mL) was essentially eliminated by EB treatment, i.e. shifted towards lower MW (approx. 24 ml retention). For

the EB-SE 200 °C pretreatment, a new, low MW peak emerged at 28 mL, which can be assigned to hemicellulose oligomers.

Surprisingly, the combination of EB-SE didn't further reduce the cellulose MW. This is in contrast to the enzymatic hydrolysis data shown in Figure 1, which shows clear differences between the EB-SE 170 °C and EB-SE 200 °C pretreatments. Taken together these results suggest that changes in the hemicellulose and lignin are controlling enzymatic hydrolysis of cellulose, and that in these systems the cellulose DP is not critical. This is reasonable given the view of the cell wall as a composite matrix, where cellulose fibrils are surrounded by matrix of hemicelluloses and lignin. [29] This cell wall matrix includes covalently bonded lignin-hemicellulose complexes (LCC). [30, 31] This work highlights the importance of disrupting this complex LCC-matrix to allow for effective enzymatic hydrolysis.

2.3 Hydrolysis and solubilization of hemicellulose-lignin matrix

The MWD profiles also provide insights into the depolymerization of the hemicelluloses by SE treatment. Conversion of hemicelluloses and lignin into water-soluble oligomeric fragments could be further quantified by hot water extractions. It has been demonstrated how LCC-fractions become soluble after depolymerizing treatments on wood substrate [32], and how conversion of hemicelluloses and lignin into oligomeric form enhances the enzymatic digestibility. [4, 5, 16] Thus the water solubility of LCC may offer a way to compare the effectiveness of pretreatments.

Figure 3 Composition of aqueous extractables. Approximate maximum theoretical yields of soluble hemicelluloses from contents in Birch and Pine are 270 and 230 mg/g, respectively, and 260 and 280 mg/g for lignins.

The data shown in Figure 3 supports the SEC analyses discussed in section 2.2, showing that extensive depolymerization of the hemicelluloses did not take place during the EB irradiation or even during the 170 °C SE pretreatment. The combination EB-SE, or SE at 200 °C alone shows significant disruption of the hemicellulose fraction, and to a lesser extent depolymerization of the lignin. Nevertheless, for both Birch and Pine SE temperatures approaching 200 °C were required to create a significant amount of the water soluble fraction

The effects of 200 °C SE pretreatment can be rationalized based on the kinetics of autohydrolysis reactions that occur during hydrothermal treatments. Garrote et al. [28] report nearly complete deacetylation of Eucalyptus at 200 °C during 6 minute retention, whereas at 170 °C the autohydrolysis reactions were limited. The EB pretreatment is reported to be capable of generating low concentrations of acetic acid, [13] which may account for the higher hemicellulose depolymerization by EB-SE 170 °C. Accordingly the EB-SE water extracts were in general slightly more acidic in comparison to SE extracts (data not shown). Reaching low pH conditions facilitates the hydrolysis of hemicellulose glycosidic bonds during the SE, and improves the effectiveness of pretreatment. [3, 28]

Lignin dimers and oligomers have very low solubility in water under the moderately acidic conditions used for these extractions, and so DCM was used to extract the lignin fraction from the pretreated samples. The DCM extracted materials from the EB or 170 °C SE samples were the common Birch and Pine extractives (Figure 4) (Figure A.1 of Appendix). The 200 °C SE pretreatment of Birch lead to significant fragmentation of lignin, producing up to 95 mg/g of DCM soluble material. The higher temperature pretreatment also showed

a significant increase in DCM extractives of Pine, albeit at a lower level of 40 mg/g. The naturally lower lignin content in Birch, relative to Pine, coupled with the more extensive degradation and extraction should contribute beneficially to the hydrolysis by enzymes.

Figure 4 Proportions of oligomeric lignin and extractive compounds that could be extracted by DCM after pretreatments.

Removal of hemicelluloses and lignin from pretreated substrate is known to increase the enzyme accessibility to the cellulosic surfaces of the substrate, since the removal of these components increases cellulose accessibility and overall surface area (i.e. nanoporosity). [5, 16] The mass of water soluble hemicelluloses removed from the pretreated samples accordingly showed a positive linear correlation with their enzymatic digestibility (see Figure A.2 in Appendix). This correlation was clearly stronger in the case of Birch, implying that the removal of hemicelluloses was an important pretreatment factor for hardwoods, while the effects for softwoods are more complex.

The water retention values (WRV) of SE pretreated Birch and Pine were measured to better understand the effects of nanoporosity and substrate composition on sugar release. The WRV has been shown to correlate with the substrate porosity and degree of delignification. [25, 33]

The WRV analyses confirmed the differences in the porosity, or swelling capability, between the two species. Under the same pretreatment conditions Birch had a WRV of 149 % while the WRV for Pine as only 96 %. These results imply to greater surface area and this greater enzyme accessibility for Birch relative to Pine.

Differences in the mass of hemicelluloses and lignin extracted from pretreated samples (Figure 3) are modest relative to the 53% difference in WRV between SE 200 °C samples of Birch and Pine. This raises questions about subtle differences in the macrofibrillar ultrastructure of the cell wall between the two species, or differences in molecular architecture of lignin. Crosslinking of lignin by a heat treatment is considered as mechanism that reduces swelling of wood [34], and similar lignin crosslinking effects may play a major role also in swelling behavior of pretreated biomass.

2.4 Molecular weight changes in lignin during the pretreatments

The MWD of lignin in native and EB/SE pretreated samples was examined to gain further insights into the behavior of lignin in the two woods. Acetobromination derivatization followed by SEC analysis [20] has been shown to allow for direct observation of the MWD of the lignin within the woody substrate with minimal side reactions. The MWD profiles of the lignins (Figure 5) correlated with the observed differences in sugar release between the softwood and hardwood, and can help explain the greater recalcitrance of Pine.

Figure 5 Molecular weight distributions of lignin in the pretreated substrates, analyzed by SEC after acetobromination. A) Birch samples without irradiation B) Irradiated Birch samples C) Pine samples without irradiation D) Irradiated Pine samples.

Softwoods are known to have more carbon-carbon bonds, e.g., C5-C5, than hardwoods, [35, 36] and these differences have been used to explain the difficulty in pretreating softwoods.. Hardwood lignins are also now understood to be more ‘linear’ than softwood

lignin, [36] which impact their hydrodynamic volume in a solvent, and also the solubility of a particular lignin.

Multimodal MWD profiles of the lignin fractions shown in Figure 5 reflect the extreme heterogeneity of the lignin after different pretreatments. Pine shows a bimodal pattern with a greater MWD, (with two peaks at 24 and 26 mL retention volume, Figure 5A), while Birch showed a lower, unimodal MWD (with a peak at 27 mL, Figure 5C). For the untreated controls the apparent weight average MW (M_w) in Pine is four times higher than that of Birch. This relative difference grew to 20-fold after severe EB-SE treatments (for determined average molecular weights see Table A.2 in Appendix). Lignin polymers are known to undergo various reaction pathways under the acidic conditions present during SE affecting its MWD. In agreement with the work of Li et al., [9] the observed MW profiles imply the presence of condensation and branching within the Pine lignin subjected to the SE treatment at 200^{o}C (Figure 5C).

The simultaneous formation of a lower MW peak (elution volume of 28-29 mL) also shows the presence of a very low MW fraction, which is consistent with the extraction data of Figures 3 and 4. The condensation reactions are less apparent in Birch (Figure 5A), while depolymerization reactions were more significant.

Occurrence of depolymerization and condensation reactions within lignins subjected to irradiation dosages below 200 kGy are known to be low [11]. In this work, minor alteration in lignin structure by 100 kGy irradiation dosage was observed, although compared to SE, the EB irradiation had less impact on the MWD of lignin (Figures 5B and 5D). Subtle

changes in the MW profiles suggest some condensation reactions for the EB treated samples from both wood species

2.5 Relationship between lignin structure and cellulose enzyme inhibition

Lignin is known to limit accessibility of enzymes to biomass substrates, reduce the swelling of the substrate and contribute to unproductive binding with the enzymes. [16] Also lignin's structural features, such as condensed phenolic structures, in addition to its overall hydrophobicity, have been shown to limit sugar release. [16, 27]

To better understand the relationship between the macromolecular structure of lignin and unproductive binding of cellulose enzymes, two lignin fractions were isolated from the residue of enzymatically hydrolyzed EB-SE Birch. These fractions were then re-deposited on clean α-cellulose fibers. Two soluble lignin fractions were created, one using ethanol-water (7:3 w:w) and the second using aqueous 1.0 M sodium hydroxide (NaOH). The lignin-rich insoluble residues from these extractions were also tested. The insoluble nature of residues appear to be due to their higher MW, and they contained minor proportions of non-hydrolyzed cellulose and hemicelluloses (determined by FT-IR). Further details about the isolation and characterization of these fractions have been provided elsewhere. [23]

The lignins were added to the clean cellulose (see Methods 1.5). The soluble lignins were to effectively "coat" the surfaces of cellulose fibers, whereas the insoluble fraction should mainly be dispersed among the fibers as larger particulates. These anticipated differences were visible in macroscopic scale as the low MW soluble lignin fractions produces a very uniform brownish cellulose fibers, and the high MW lignin fractions produced a visibly heterogeneous suspension of submillimeter lignin particulates and incompletely coated

cellulose fibers. Thus only the soluble lignin could theoretically act as a barrier film on the fibers, and the insoluble lignin should influence mainly via unproductive binding of the enzymes. The sugar release data shown in Figure 6 suggest that deposition of the low MW lignin did not limit hydrolysis unlike suggested previously [25], while unproductive adsorption of enzymes on complex insoluble lignin structures clearly reduces enzyme action. This may link to previously suggested inhibition arising especially from condensed phenolic units. [27]

Figure 6 Influence of deposited lignin fractions on enzymatic hydrolysis of α-cellulose. Fractions were isolated from solid lignin rich residue of EB-SE 200 °C pretreated Birch after enzymatic hydrolysis. The residue was divided into two fractions based on solubility in either ethanol-water (7:3 w:w) or 1.0 M NaOH solution, resulting two fractions of different macromolecular structures for both extraction systems.

Presence of the soluble, lower MW lignin fraction provided a small but reproducible increase in sugar release, similarly to recent findings of Lai et al. [17]. This is also consistent with our recent work that showed that wood extractives and selected hydrophobic model compounds deposited on fresh cellulose fibers could increase sugar release. [22] It may be that the amphipathic nature of low MW lignin fractions makes this material to act in similar fashion than surfactants that are known to be beneficial for enzymatic hydrolysis.

3 Conclusions

Birch and Pine was pretreated with EB, SE or a combination of EB/SE pretreatments to enhance enzymatic hydrolysis. The EB treatment was found to lower the MW of cellulose, while SE had a minimal impact on cellulose MW, and there was not significant interaction between these pretreatments. The enzyme digestibility of SE pretreated samples was attributed to the removal of hemicelluloses. EB induced depolymerization of cellulose alone showed no changes in sugar release. These results suggest the majority of recalcitrance effect to arise from the hemicellulose-lignin matrix of the cell wall.

Based on changes in the MW distributions of benzoylated carbohydrates EB alone, or SE at 170 °C alone did not significantly alter the LCC structures. Significant improvements in the rate of cellulose hydrolysis were only seen after extensive autohydrolysis of the hemicelluloses and lignin from the SE 200 °C pretreatments. Introduction of the EB prior SE 200 °C enhanced the depolymerization process of the LCC matrix.

Lignin condensation reactions at 200 °C SE created a high MW lignin fraction, which was most obvious for Pine. EB only showed minor synergistic influence on lignin depolymerization. The presence of residual high MW lignin reduced both swelling capacity of the treated biomass and resulted enzyme inhibition likely via unproductive binding of the enzymes on complex lignin structures.

Hydrolysis of model cellulose substrates highlighted detrimental effects of high MW residual lignin on sugar release. Conversely, a low MW lignin fractions slightly enhanced sugar release. This observation was attributed to a reduction in non-productive binding between the cellulose and enzyme. In pretreated substrates the negative impacts of residual

seem to be a combination of lignin being a physical barrier to the cellulose surfaces, limiting the swelling of the biomass substrate, and also creating a surface for non-productive binding with the enzyme.

Acknowledgements

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Supporting material

^{1}H -NMR of Birch extractives; Graph of hemicellulose solubility against enzymatic sugar release; Table of yields of extracted material from Birch and Pine; Table of average molecular weights of lignin in pretreated samples; Graph about influence of alkali deposited lignin on cellulose hydrolysis.

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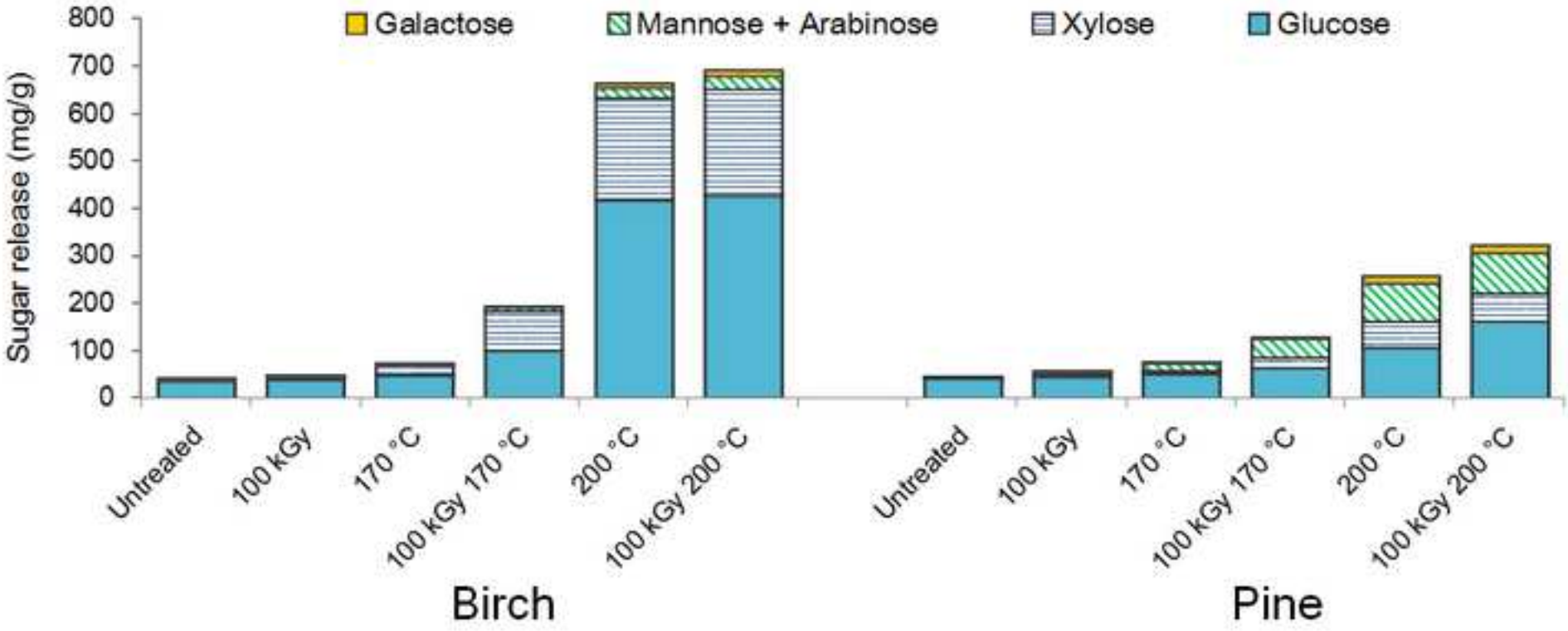

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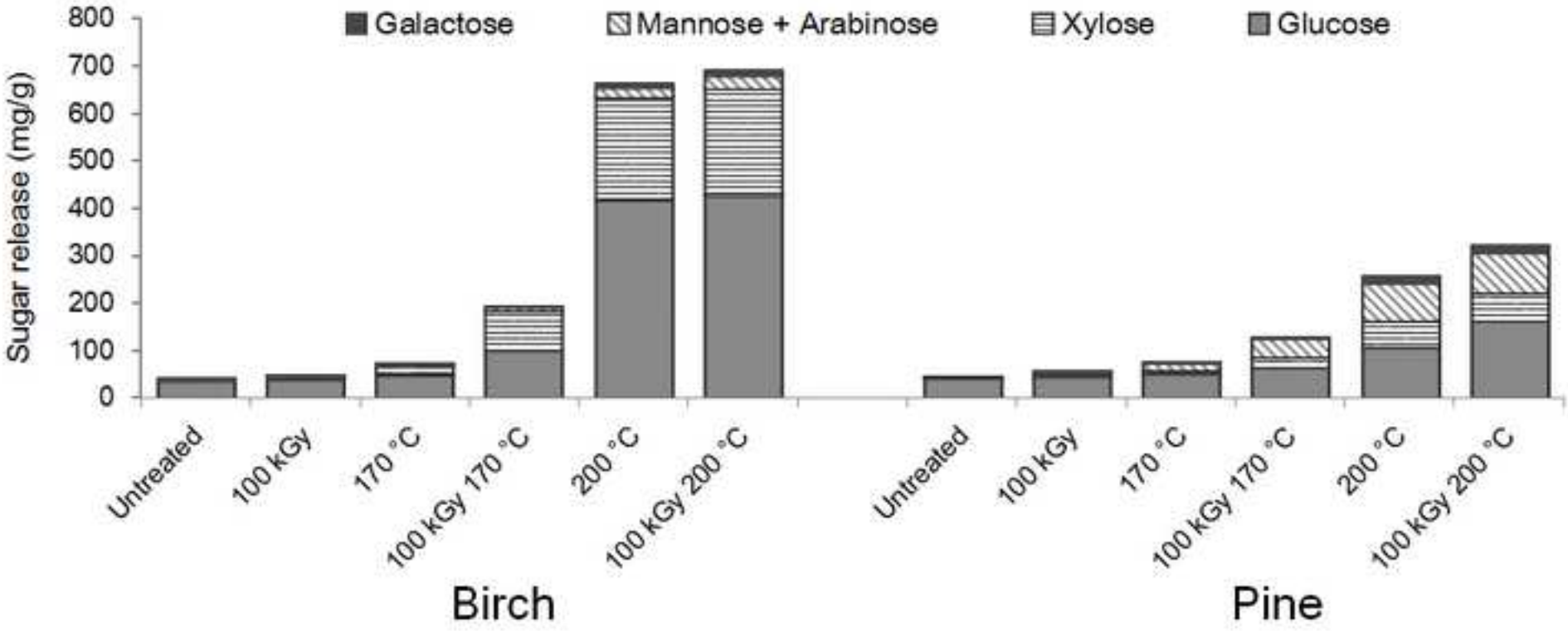

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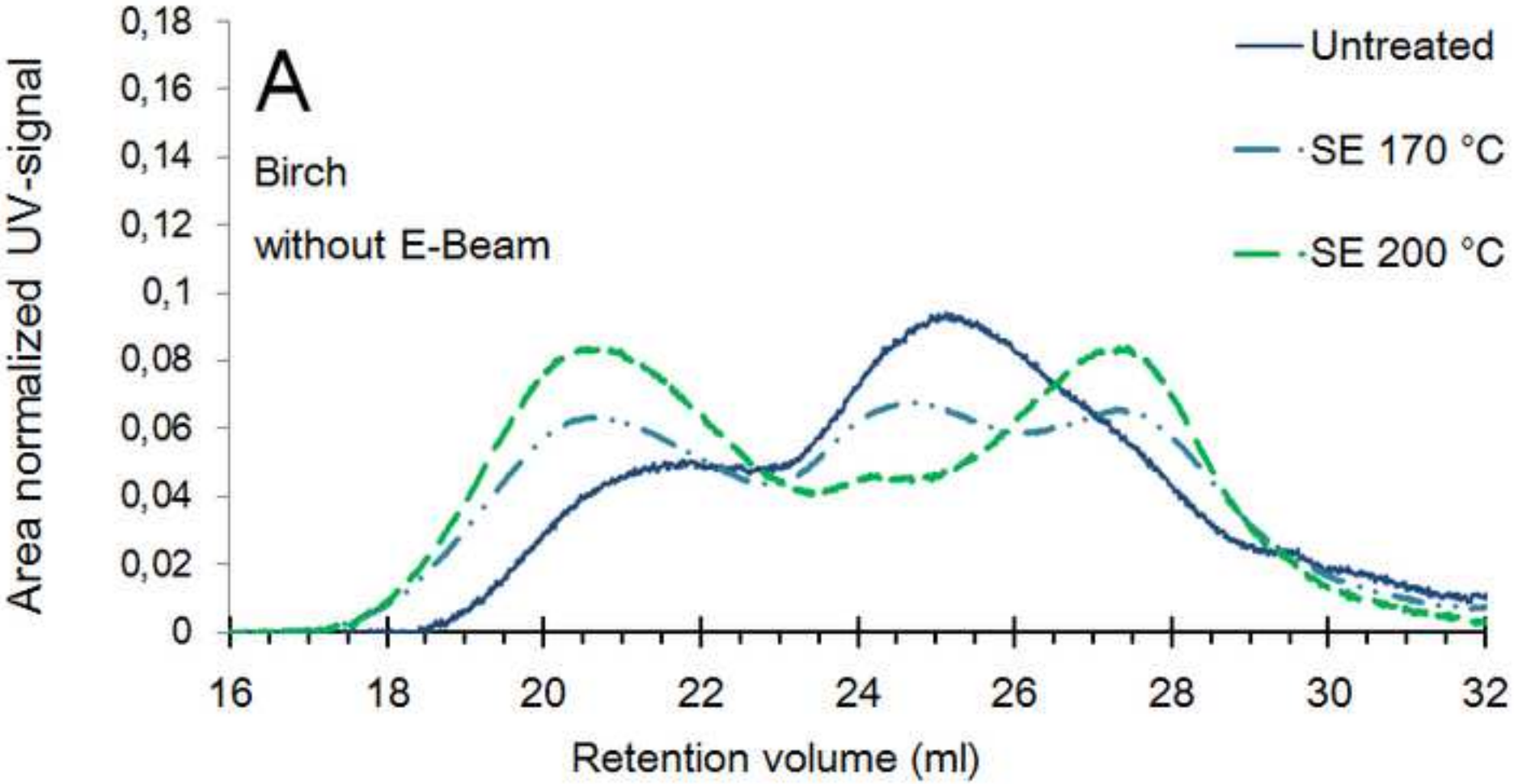

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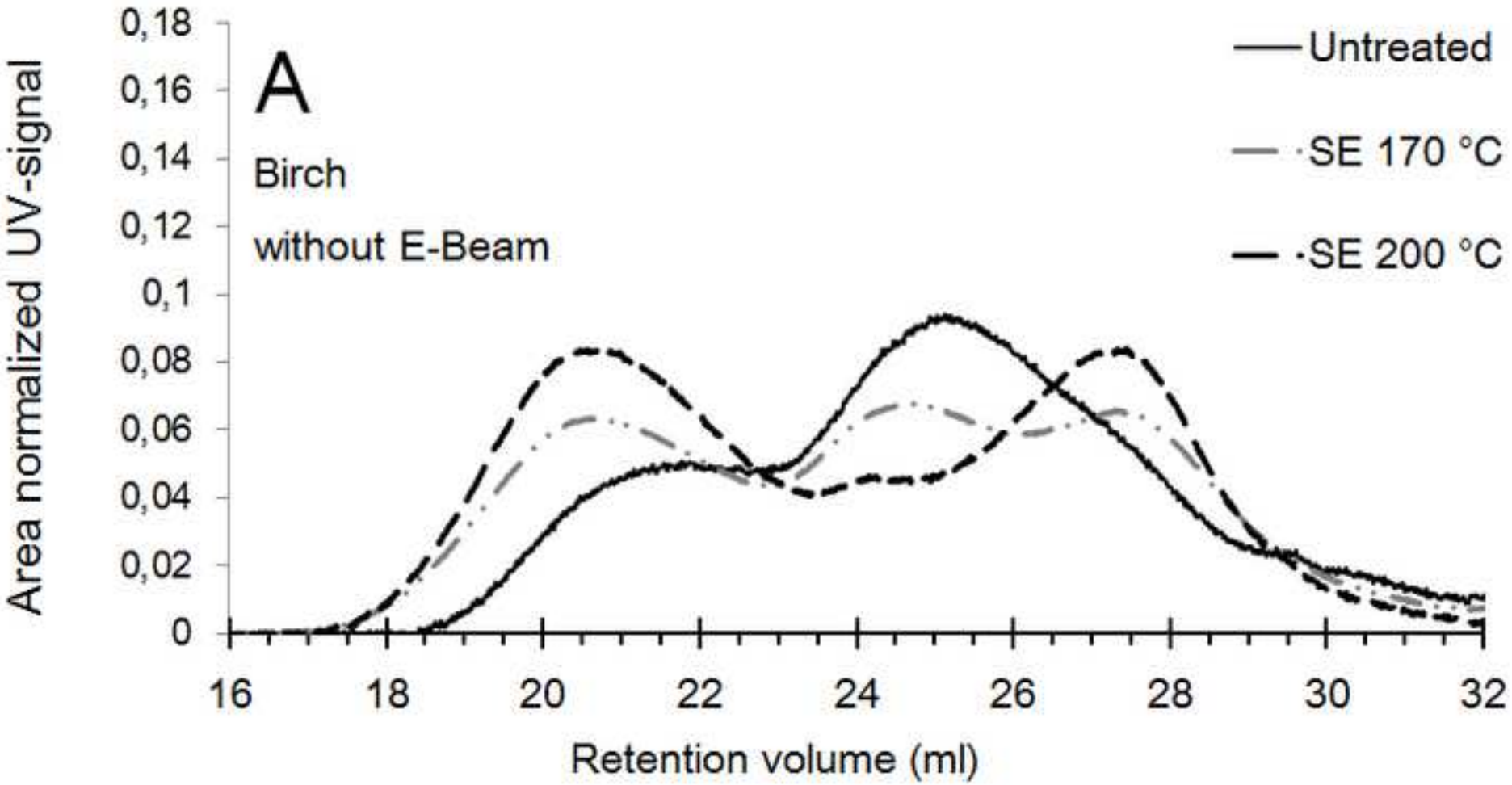

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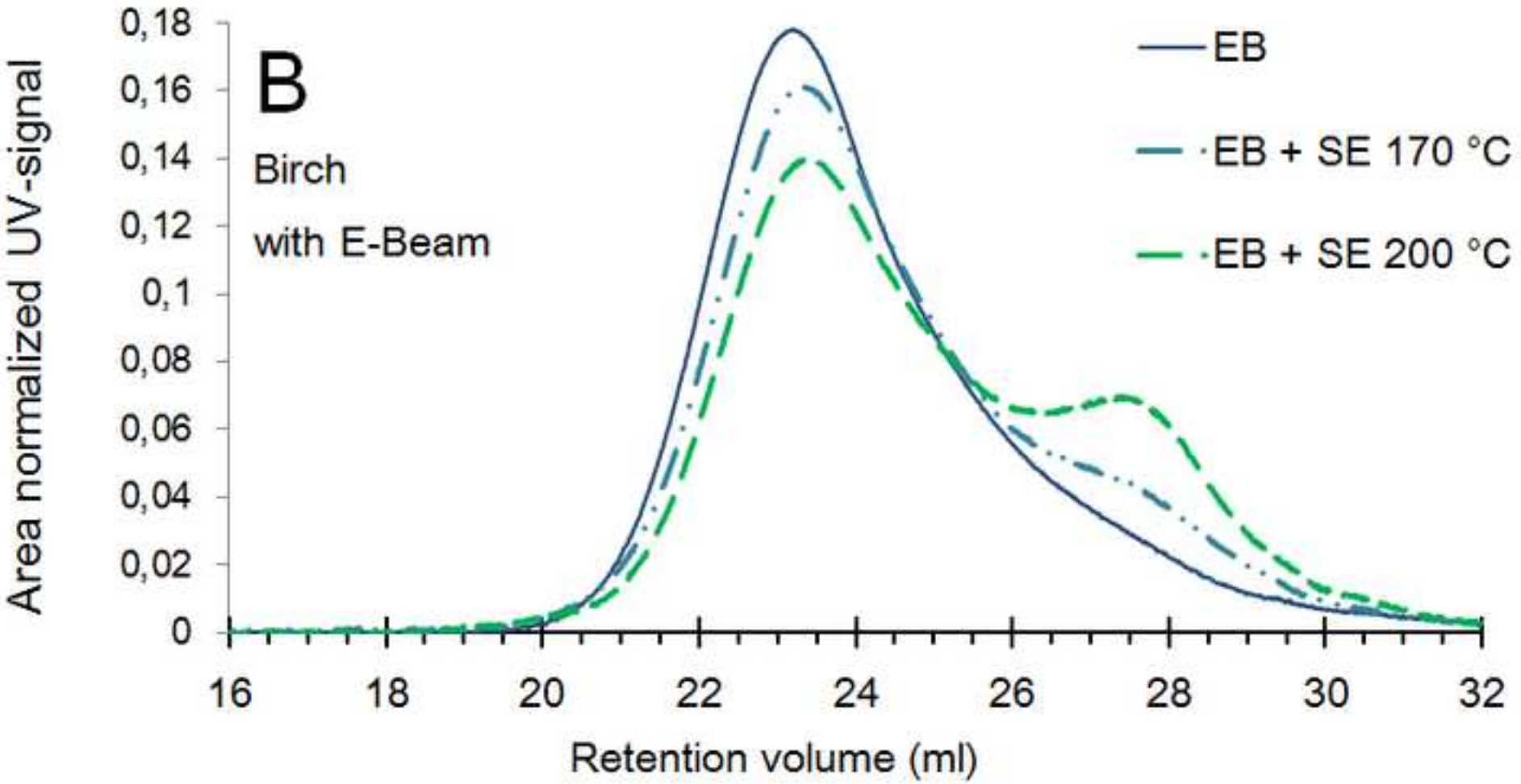

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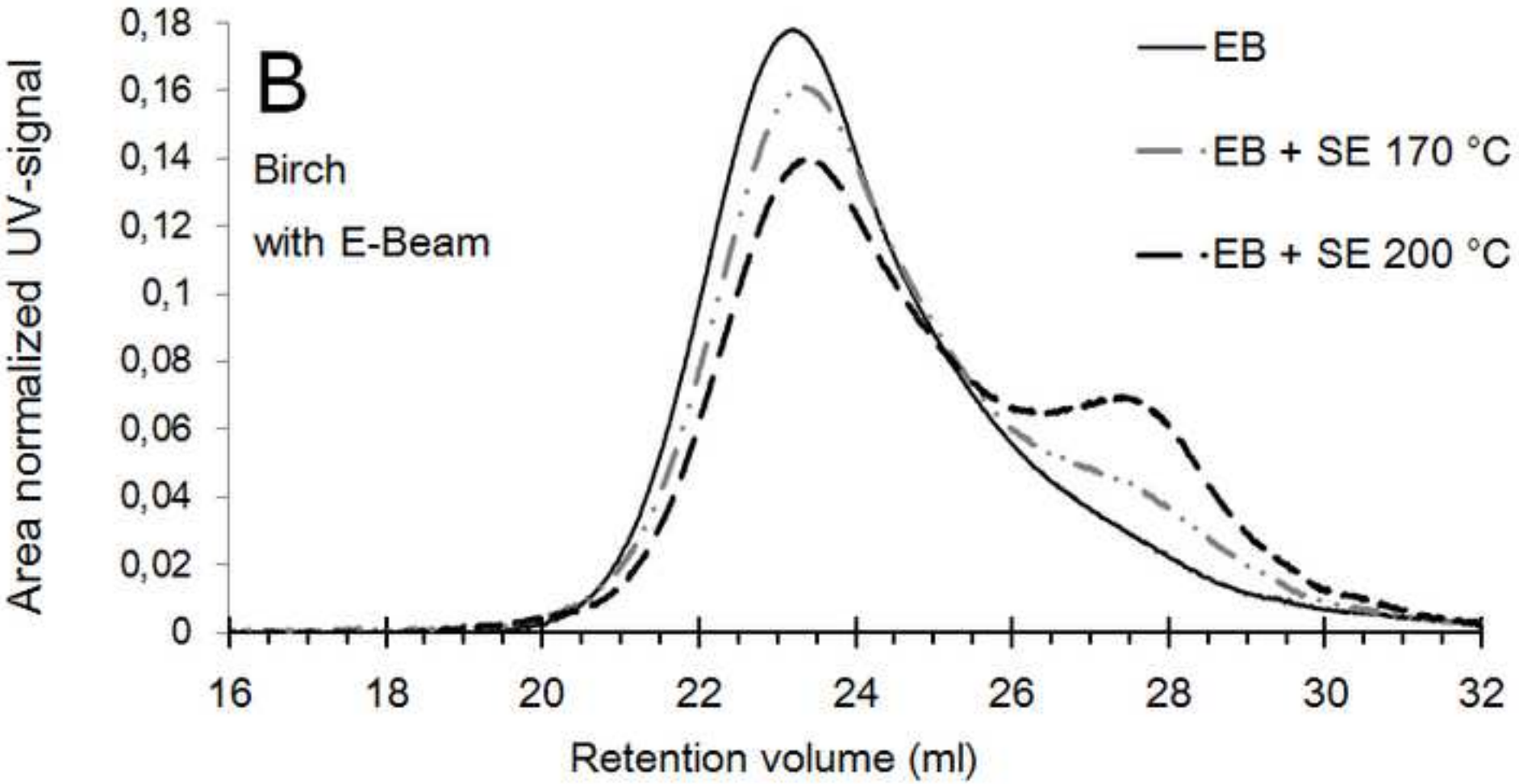

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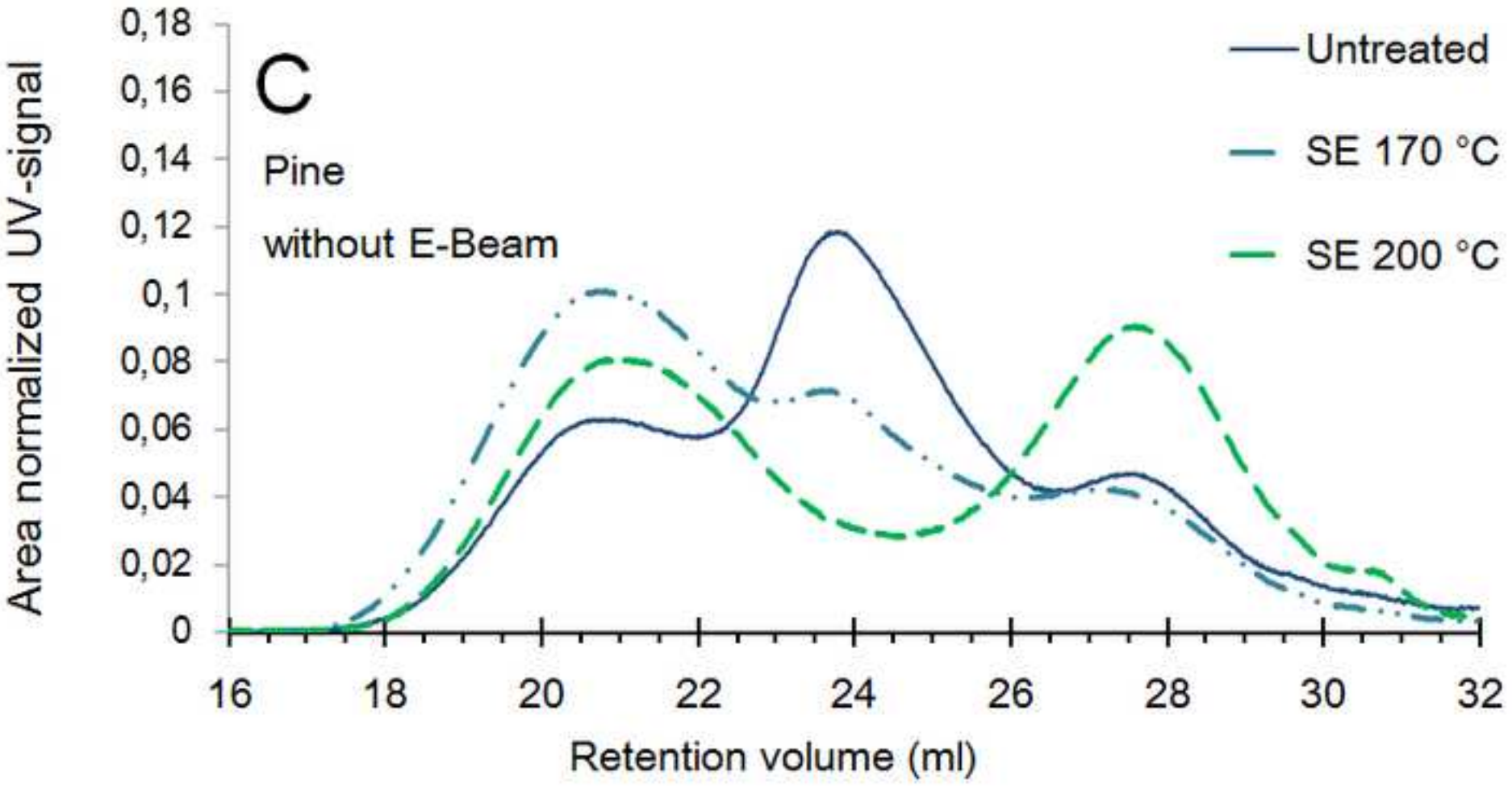

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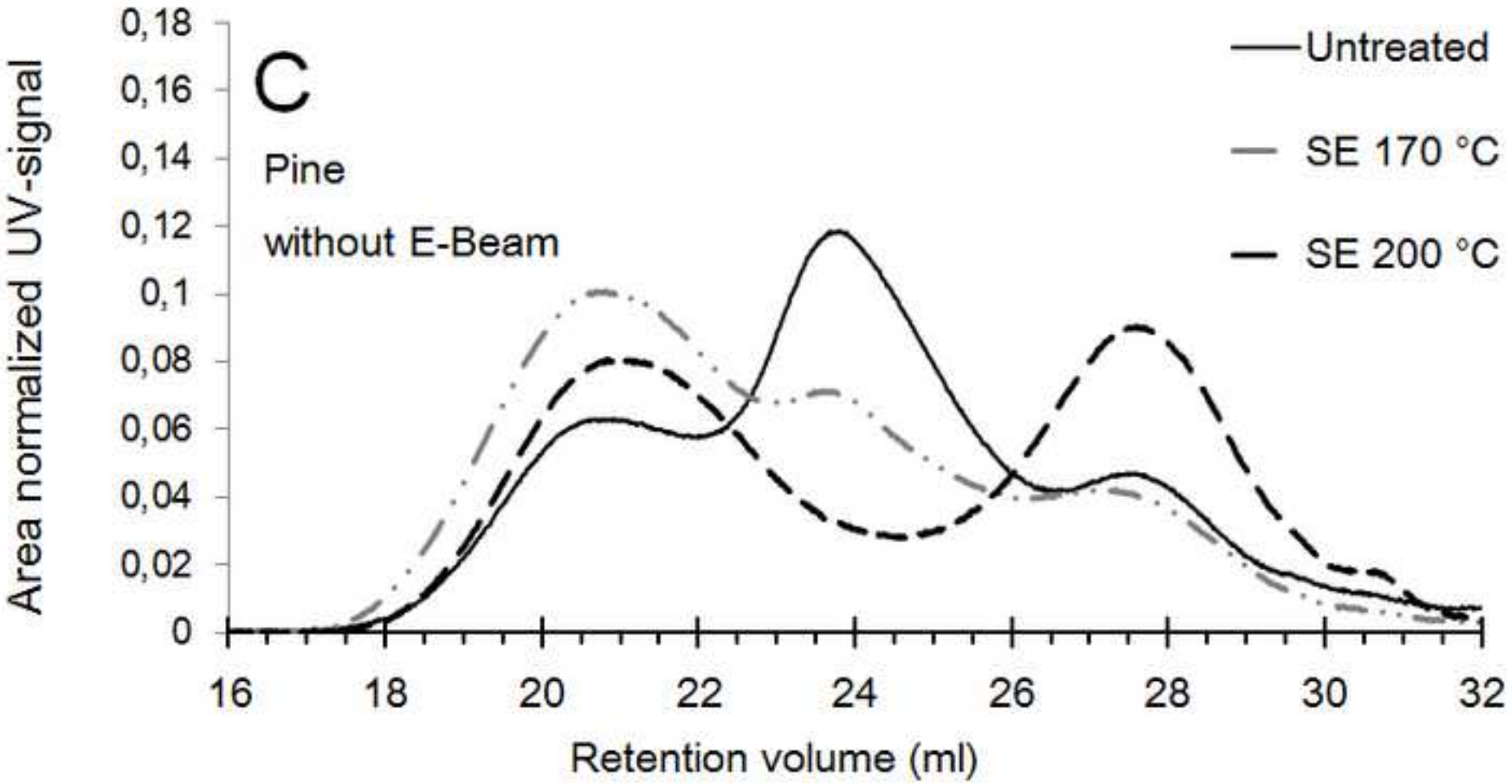

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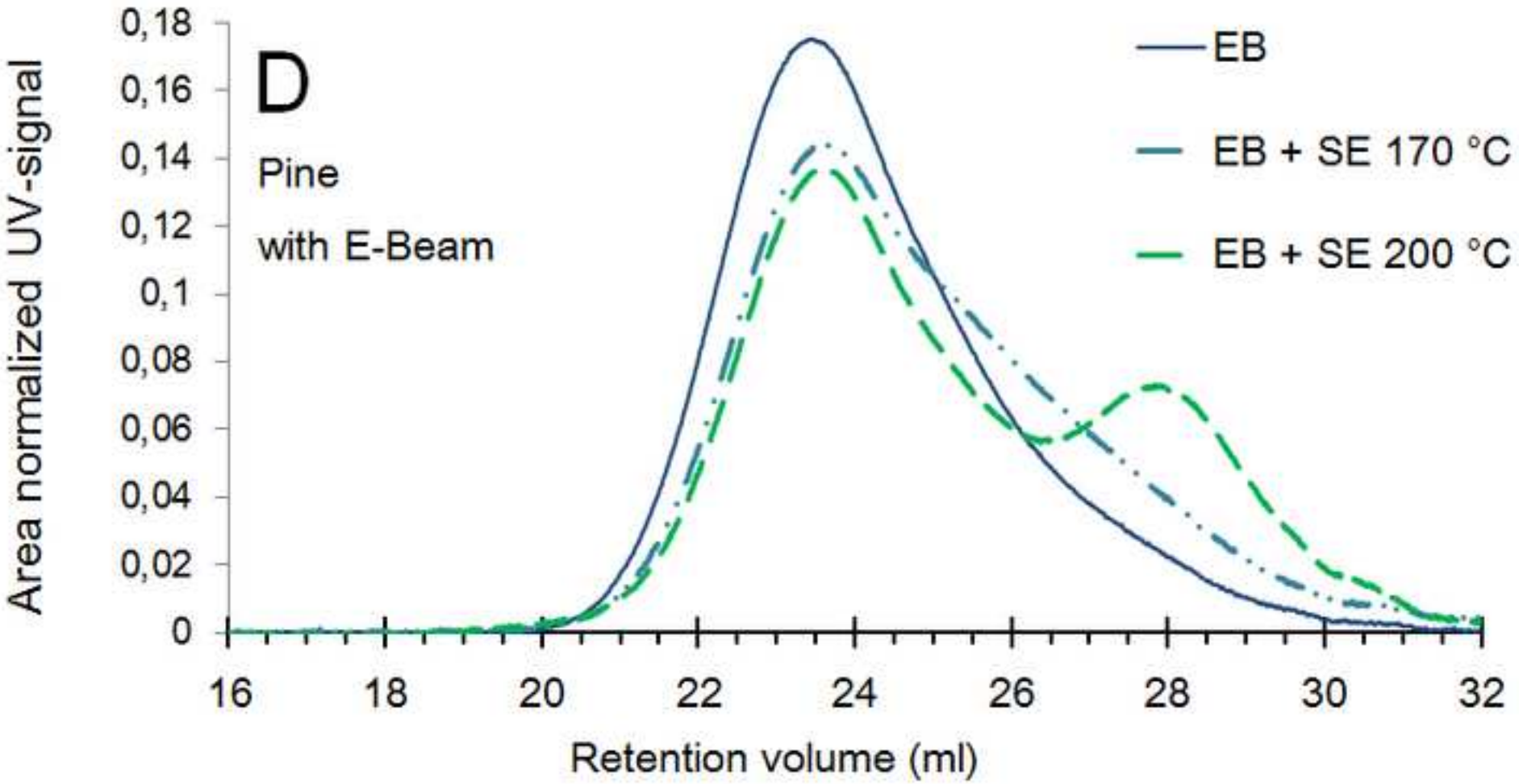

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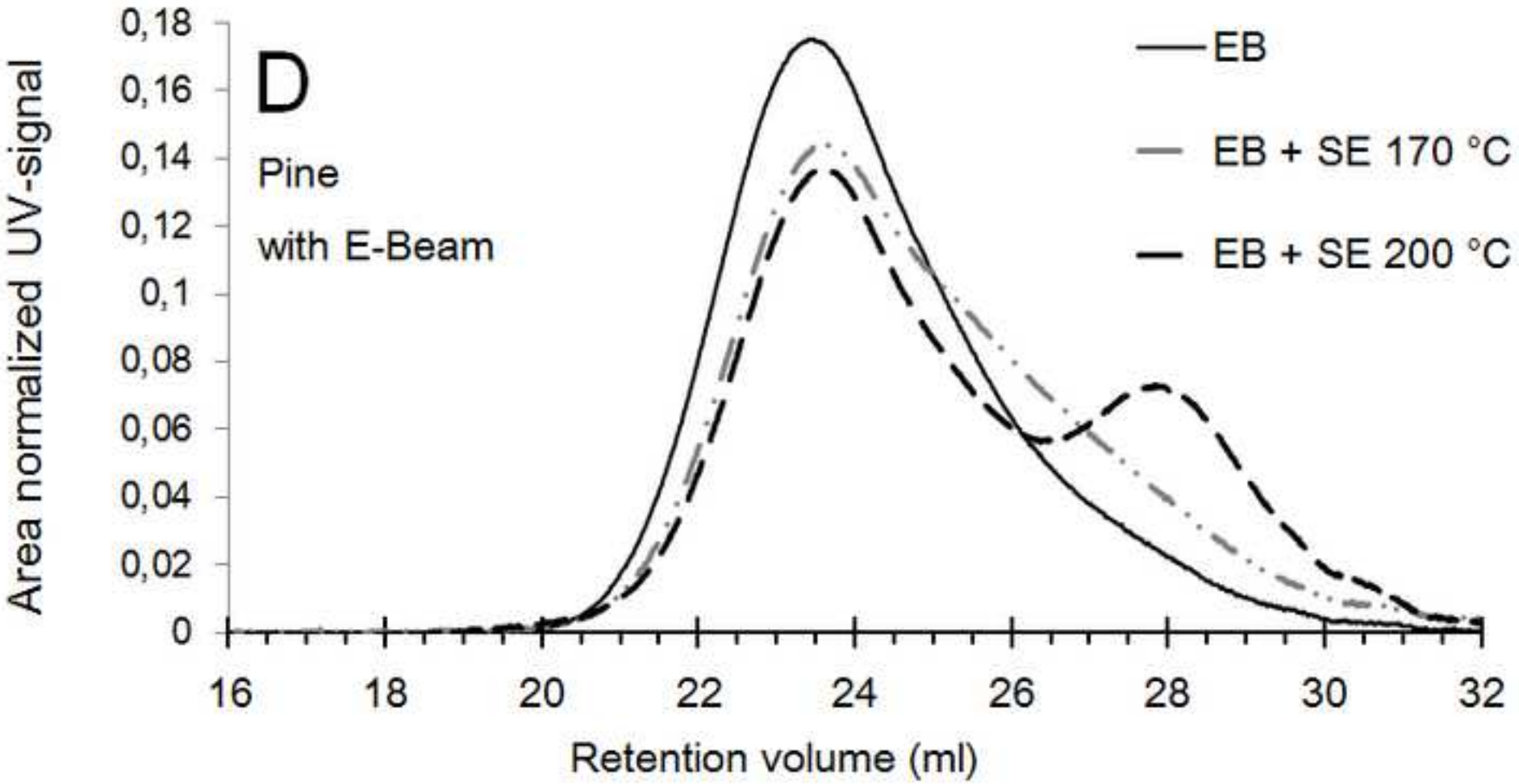

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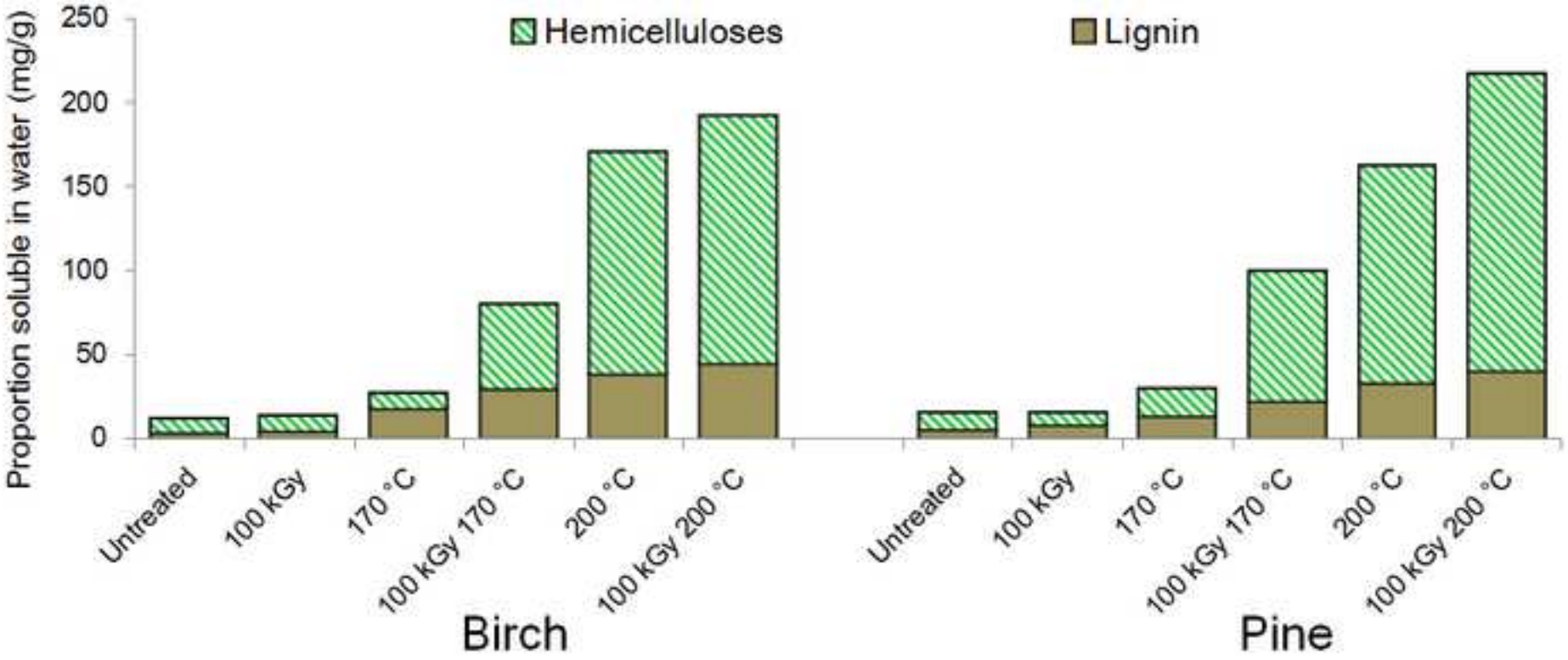

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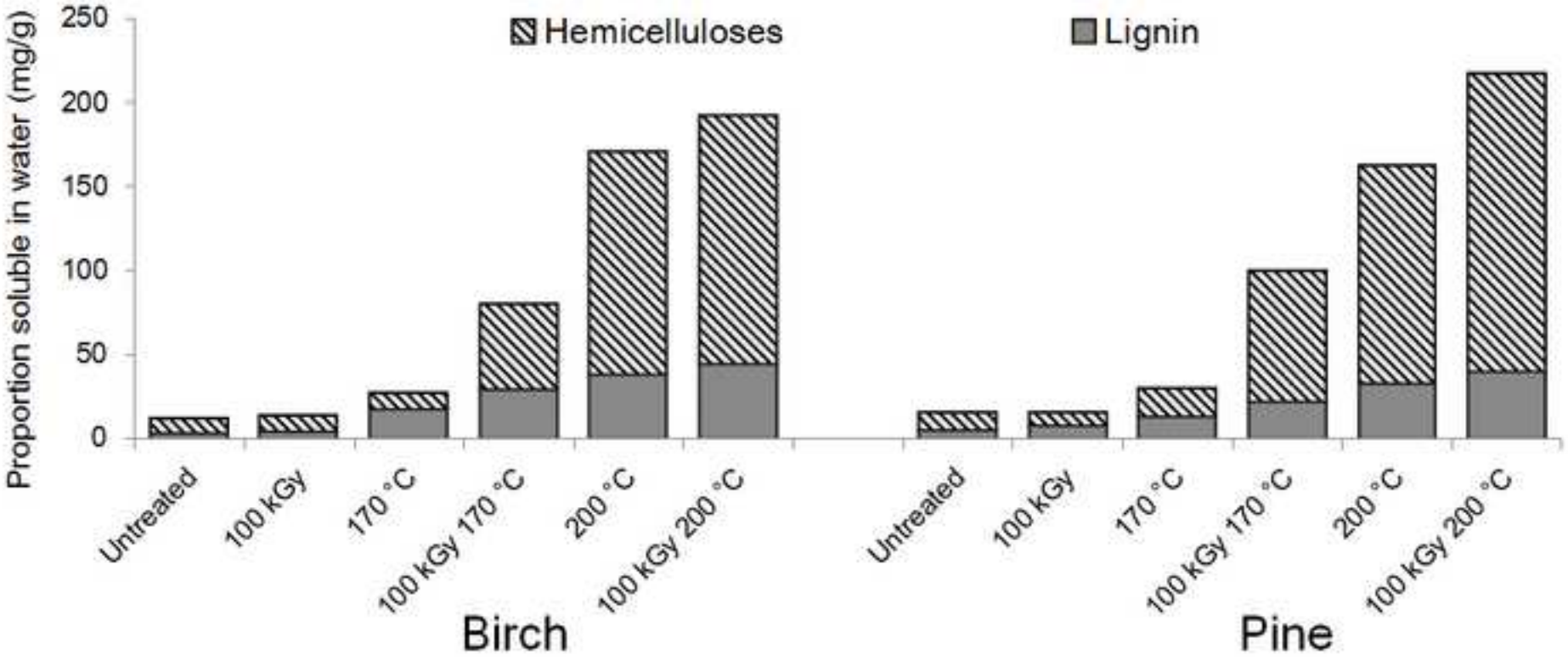

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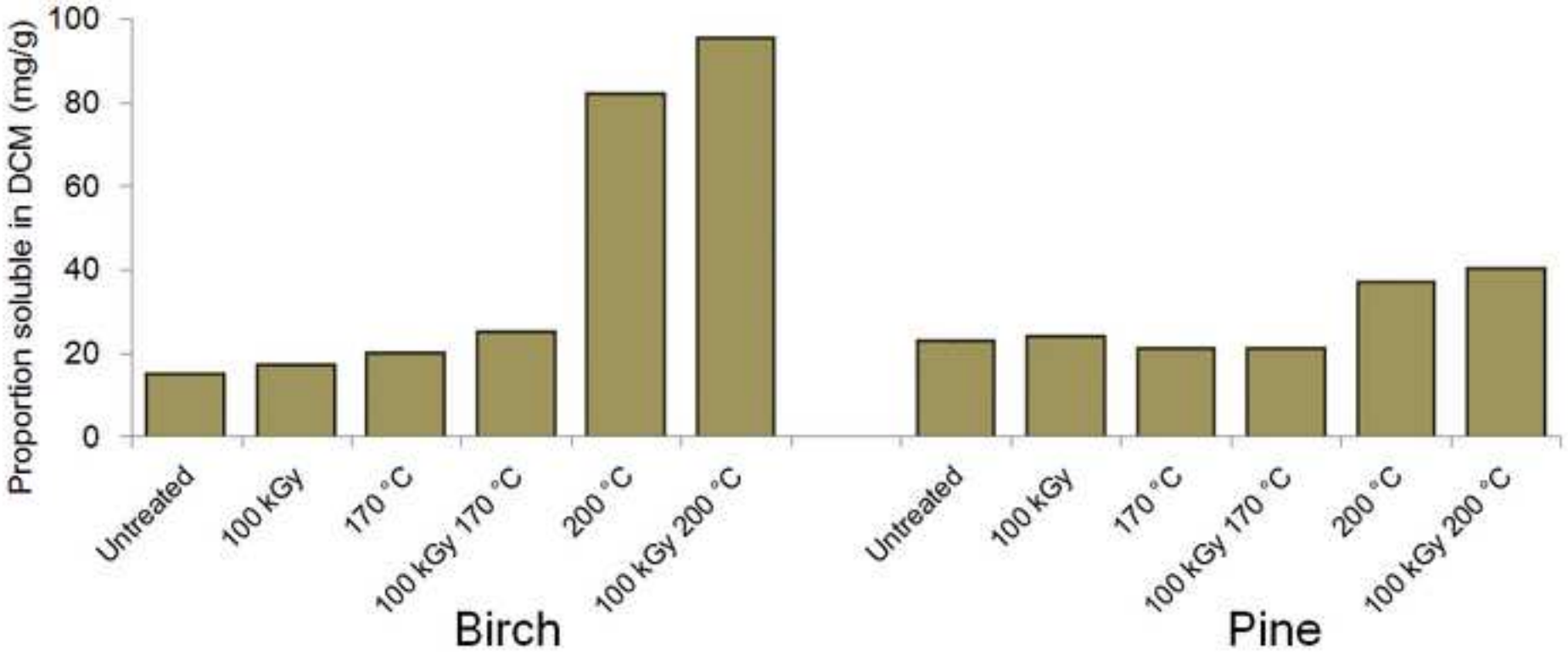

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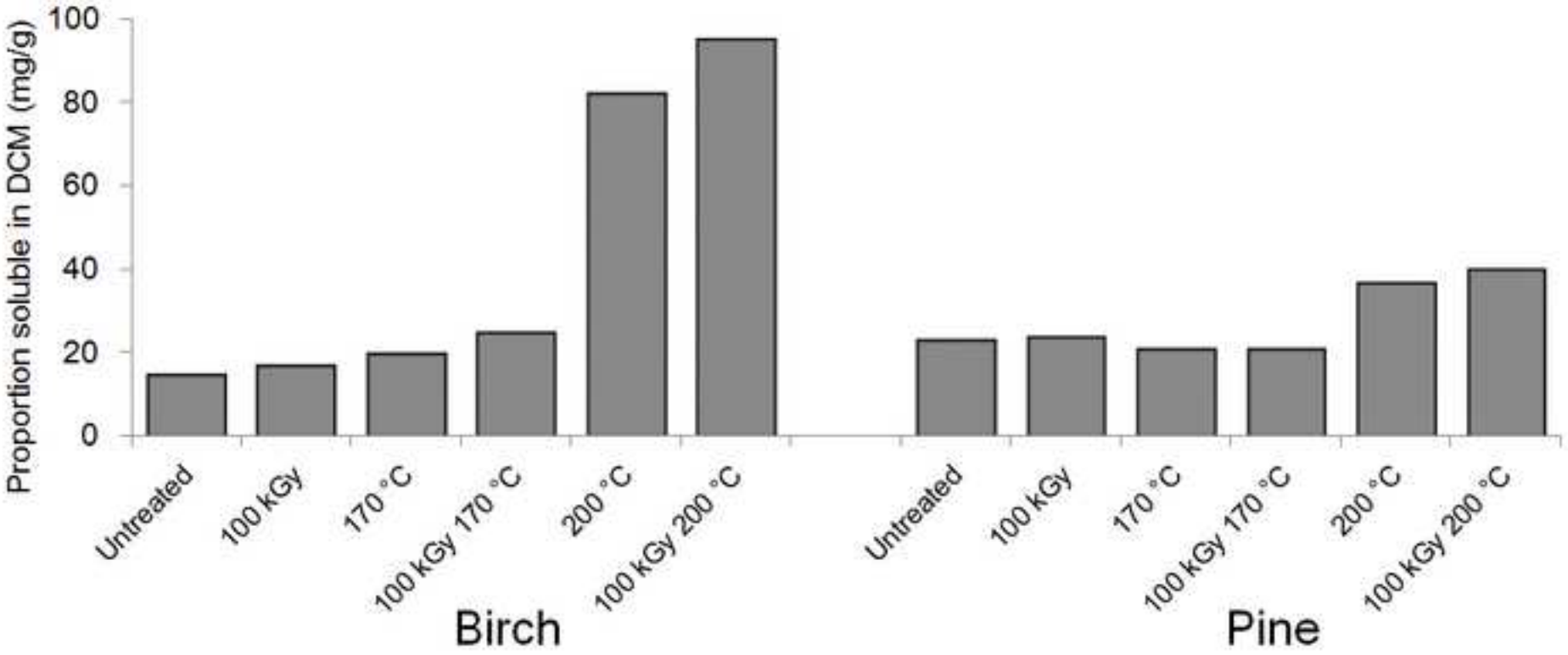

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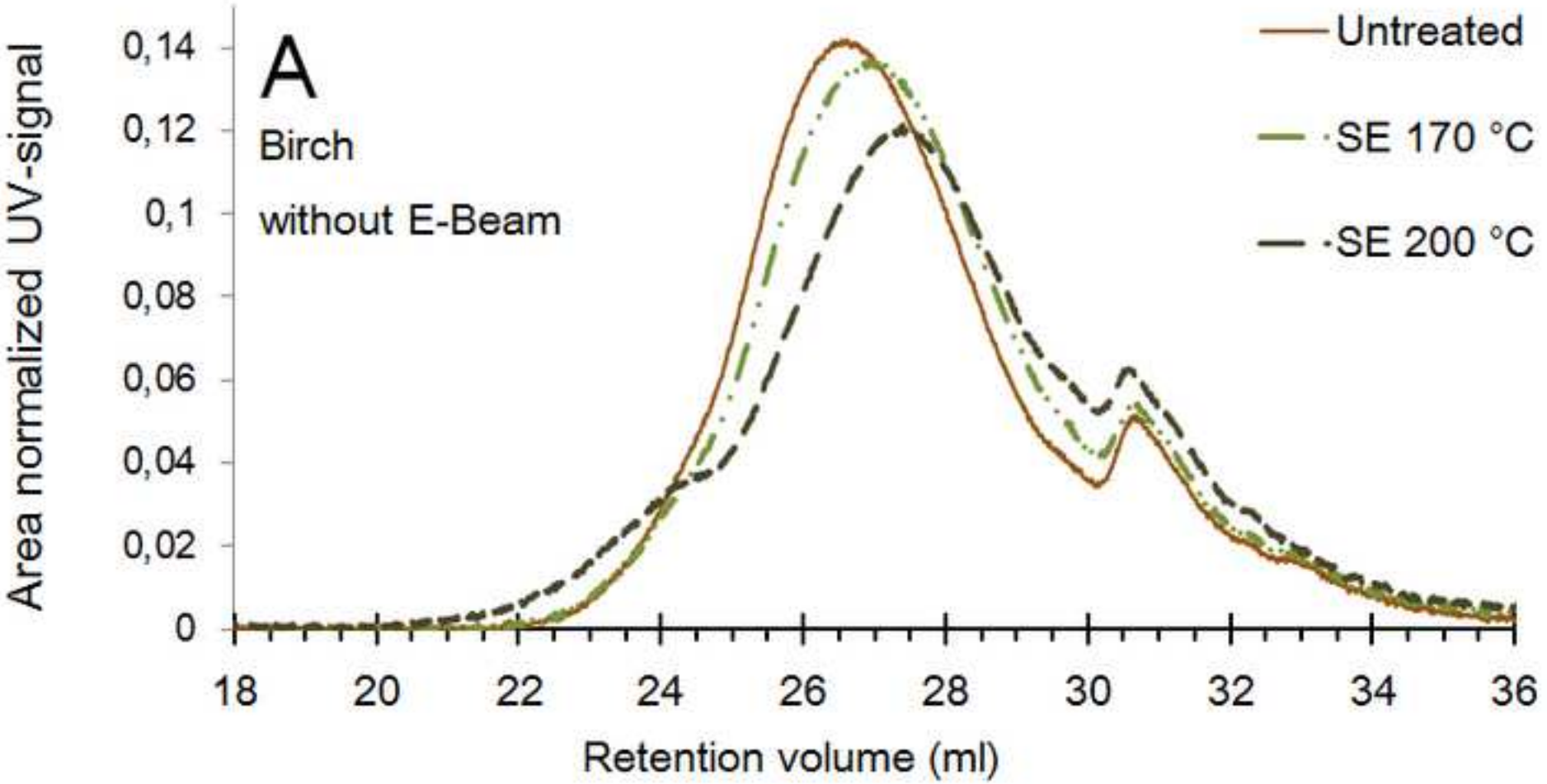

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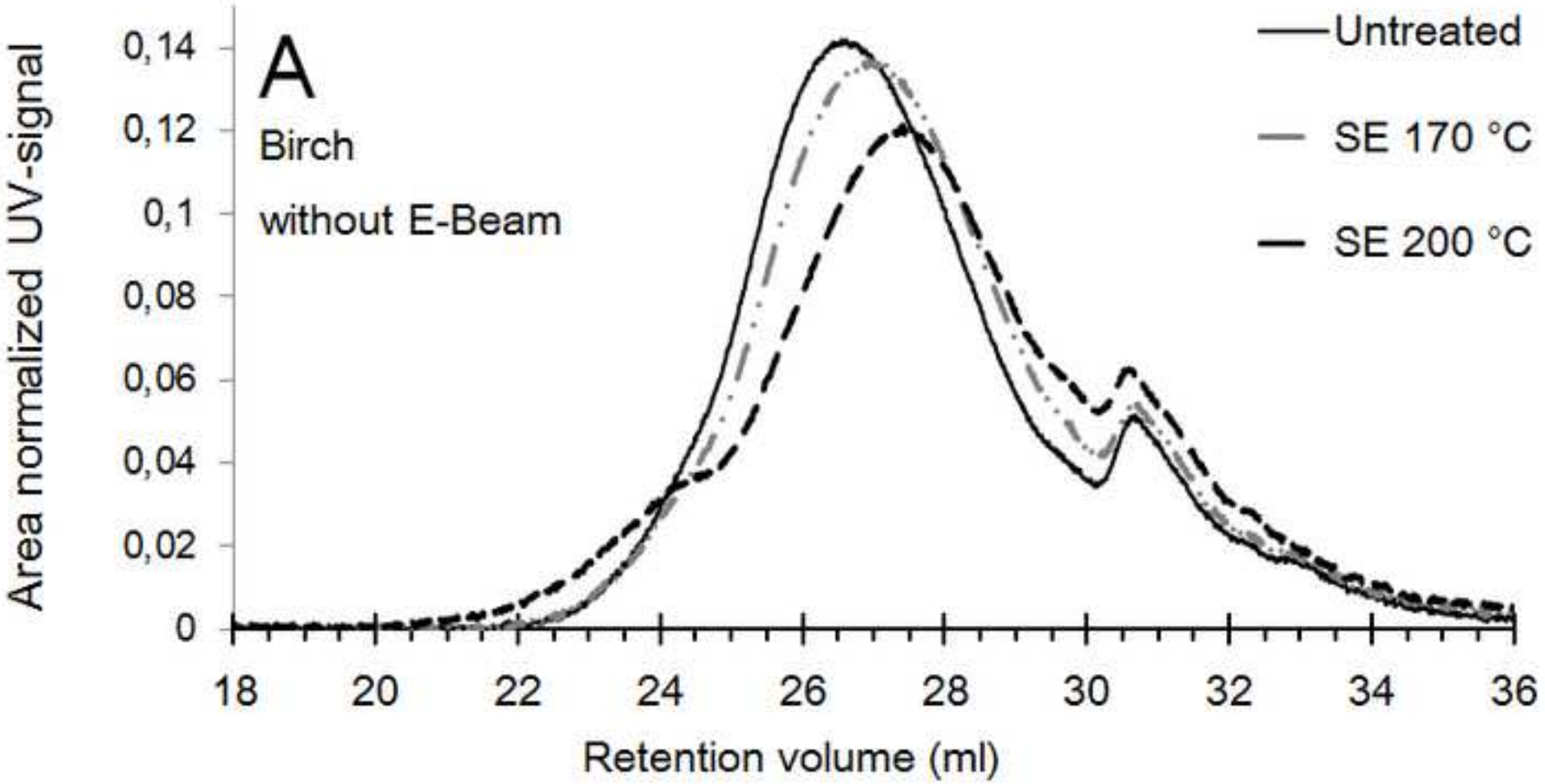

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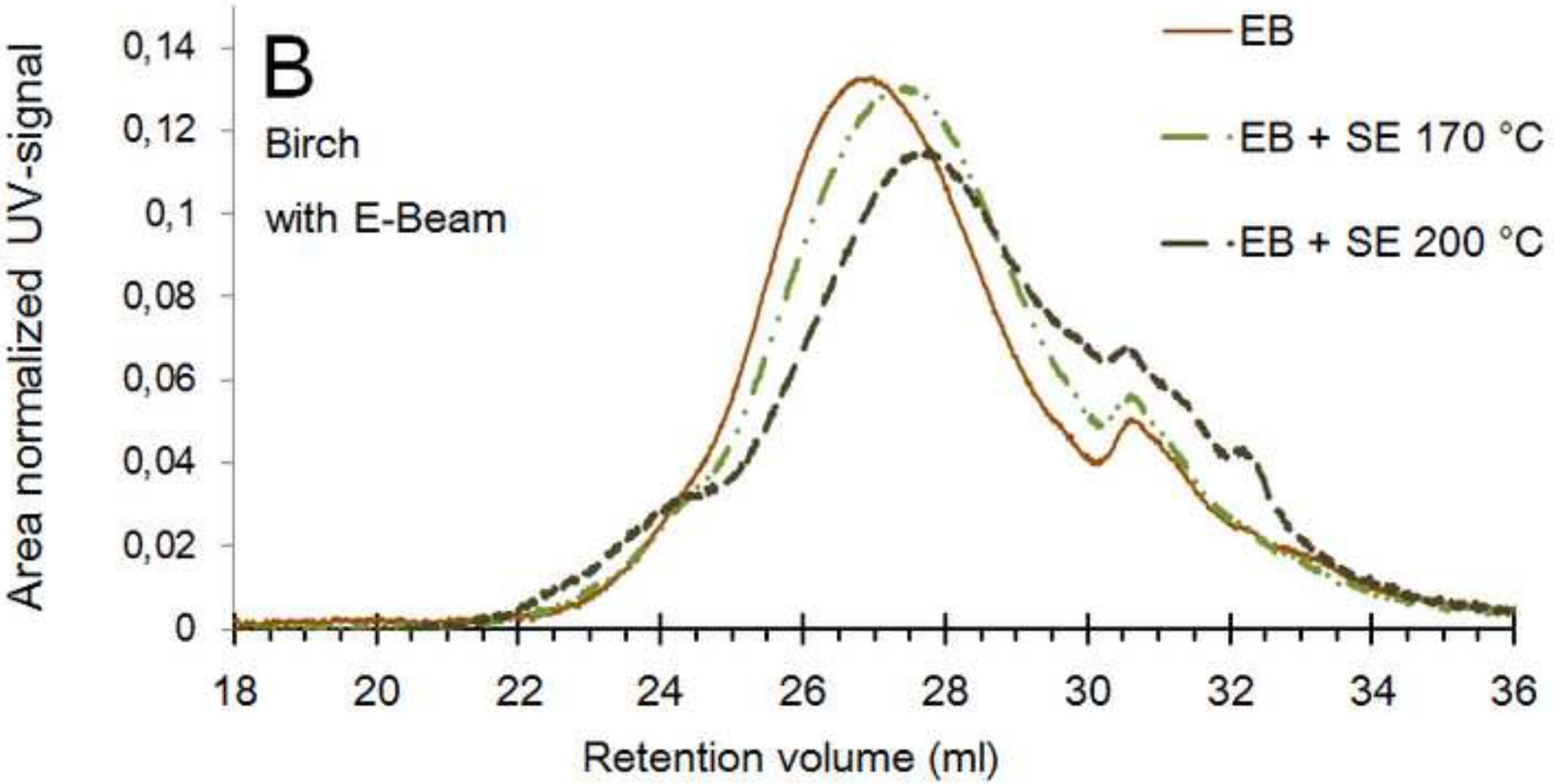

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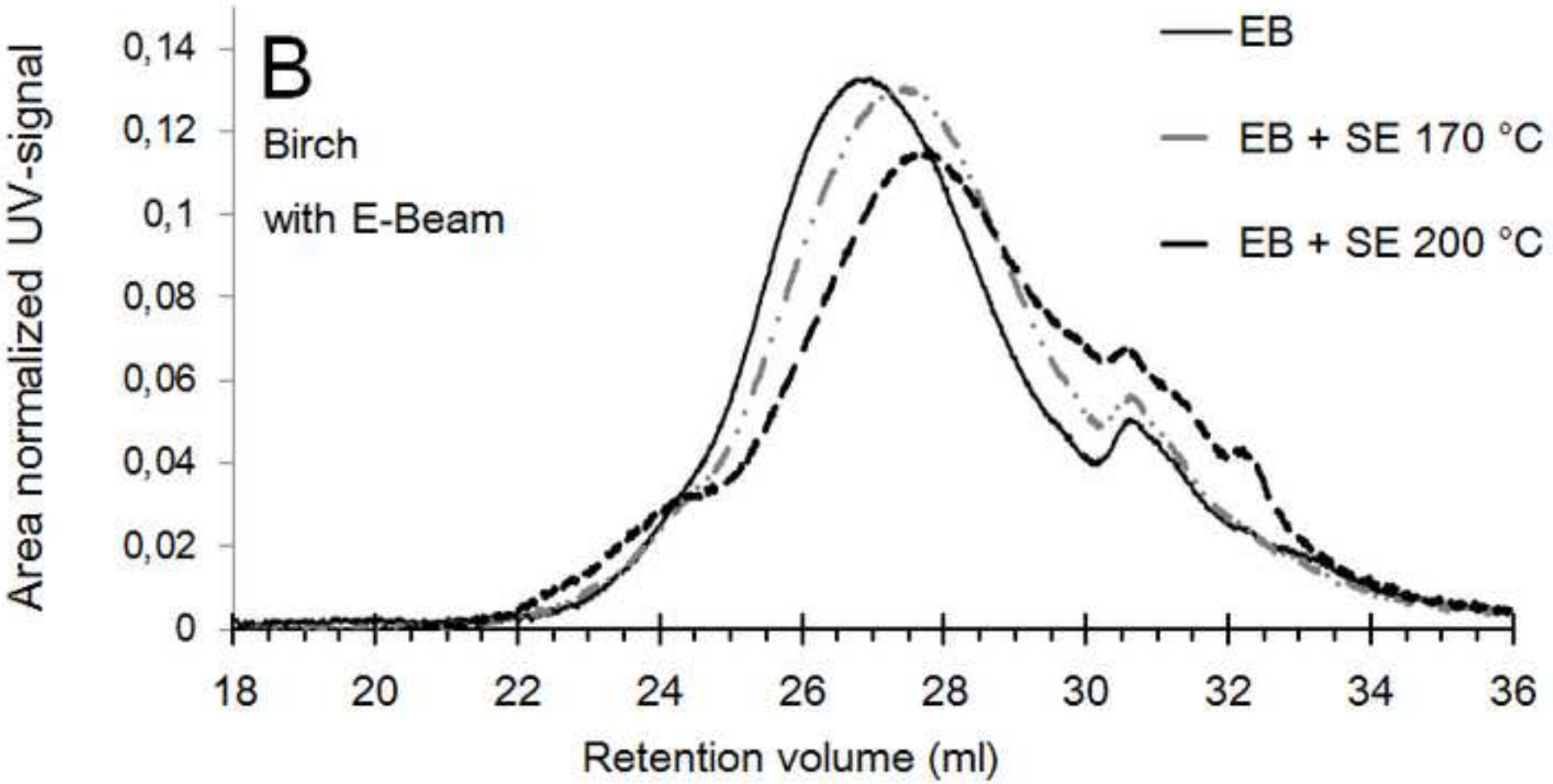

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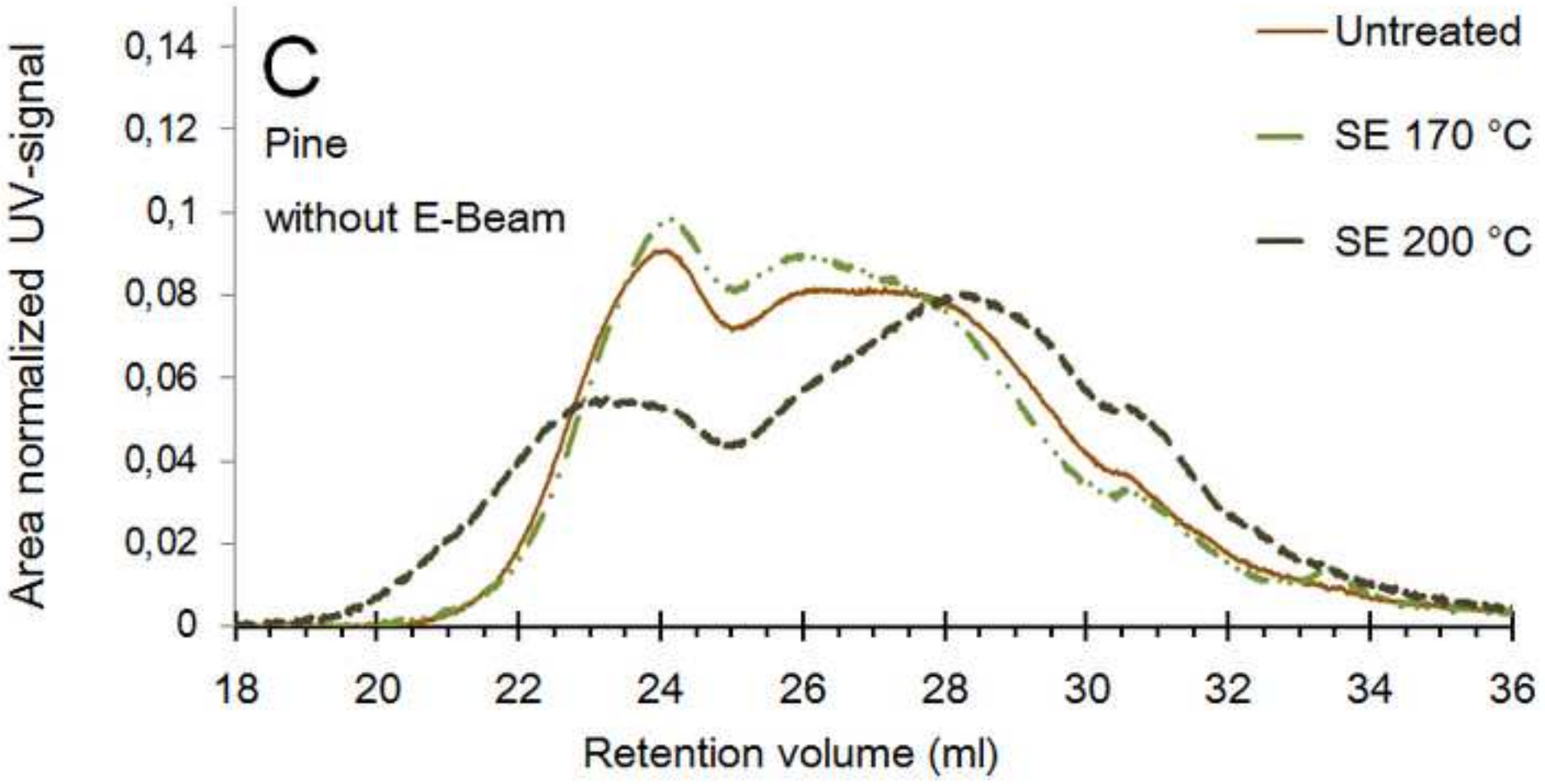

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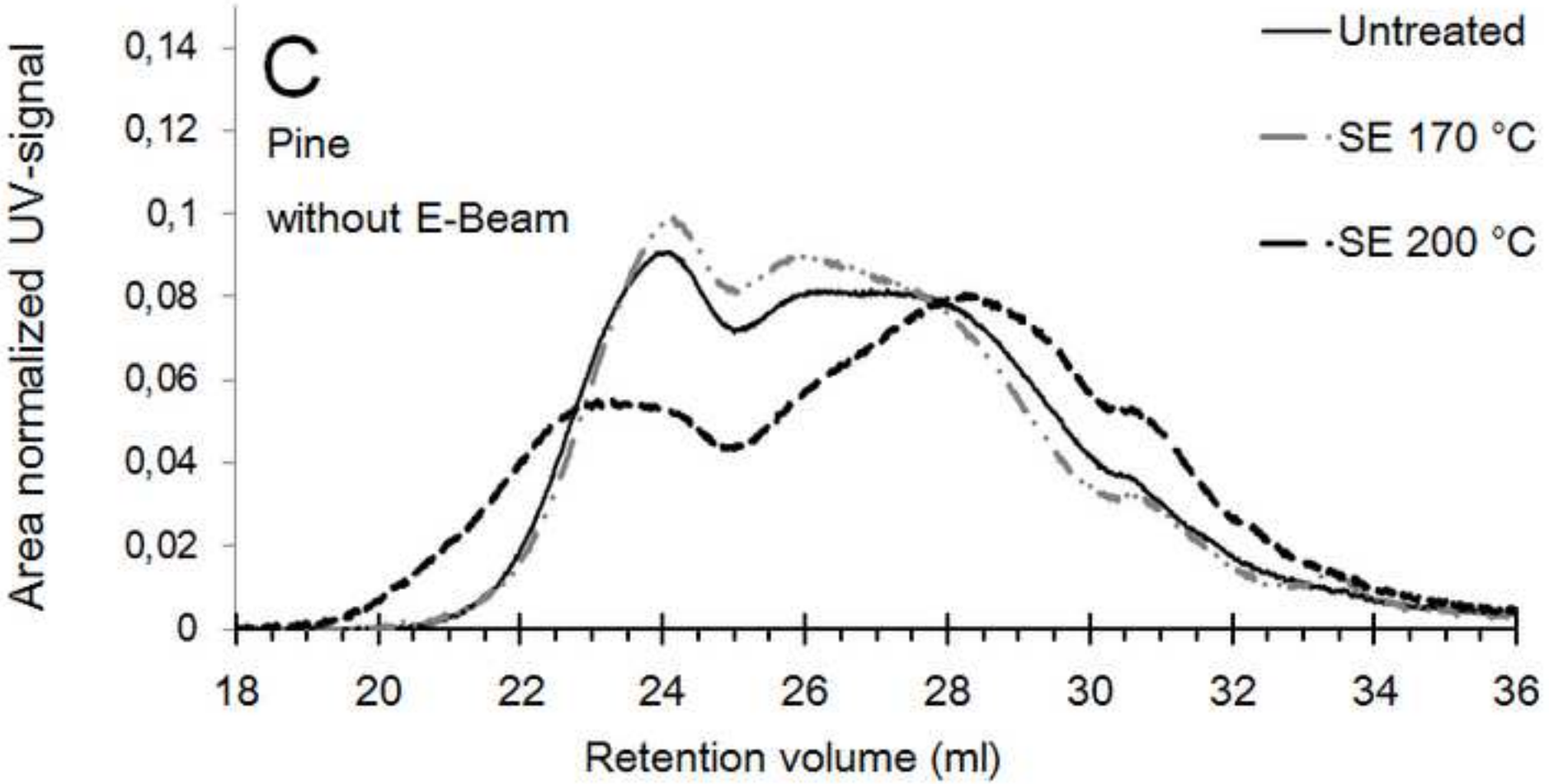

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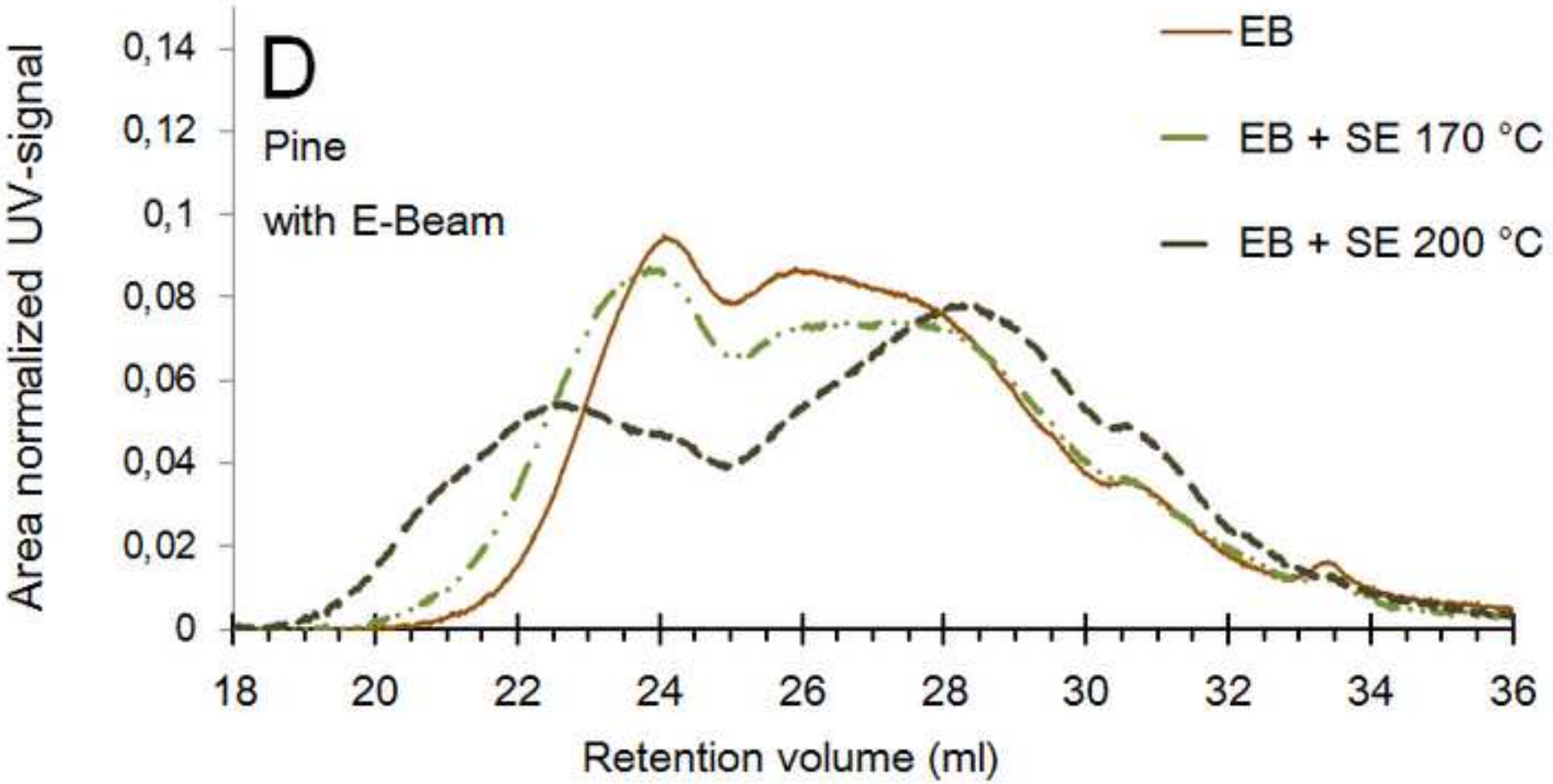

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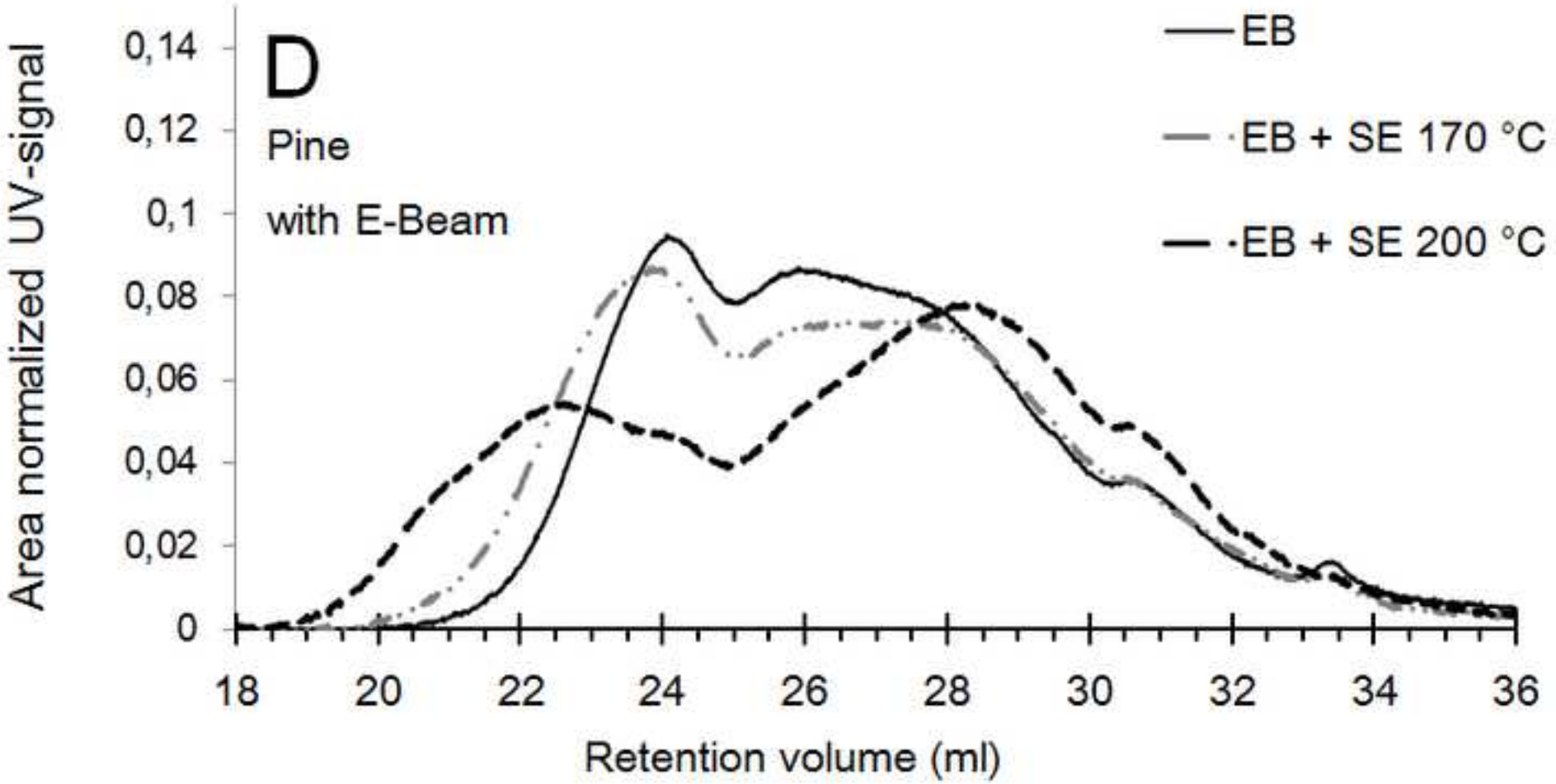

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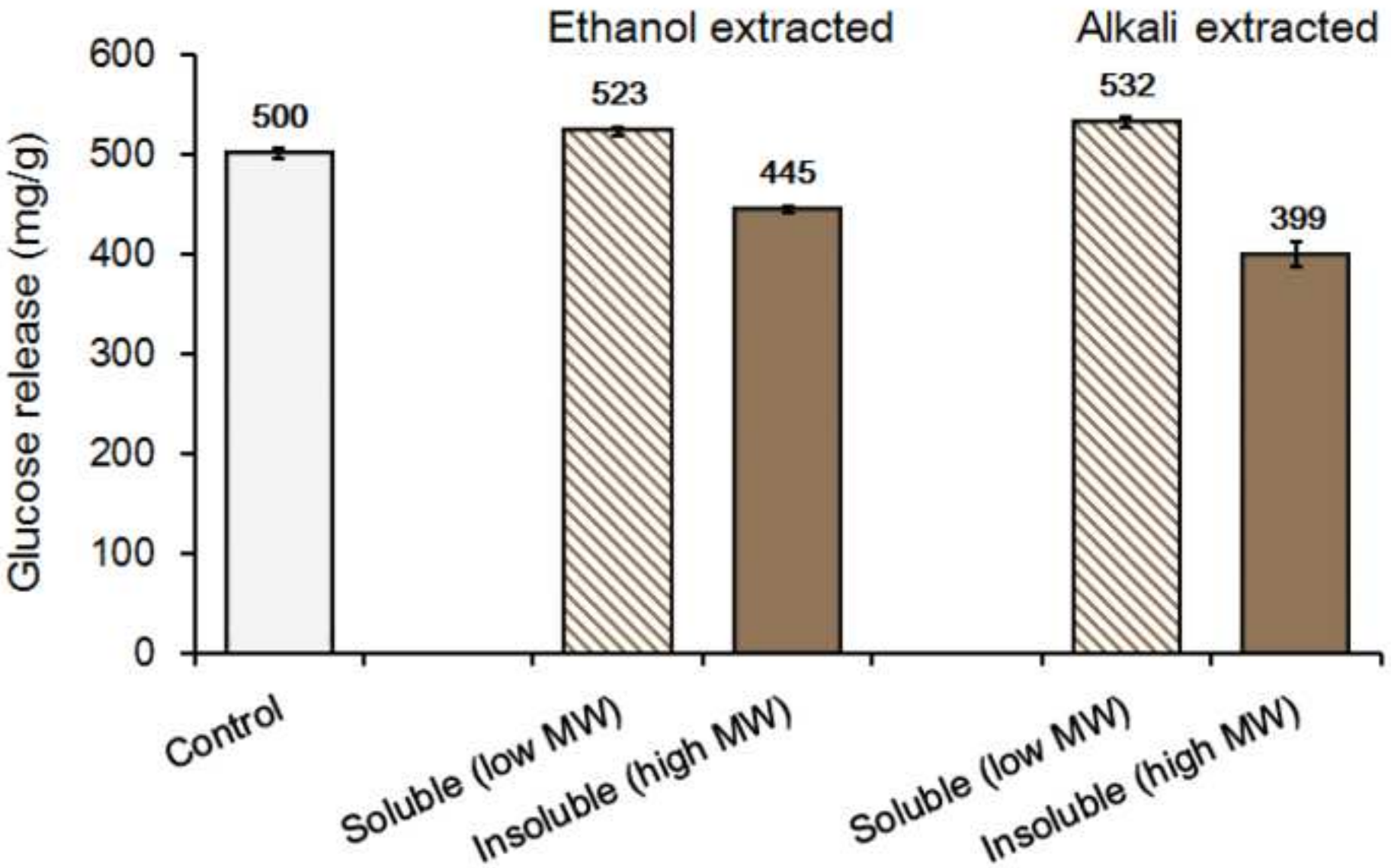

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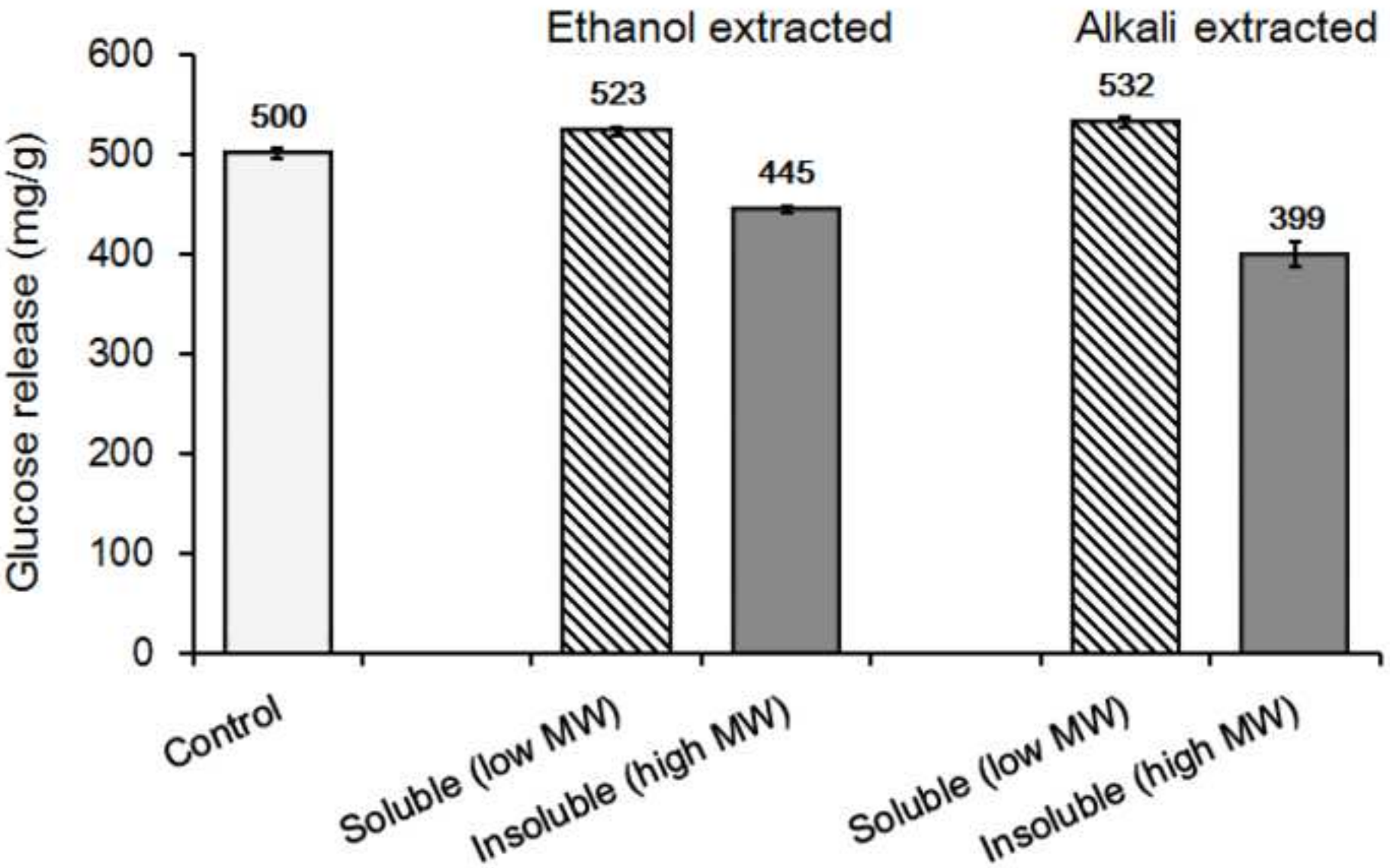
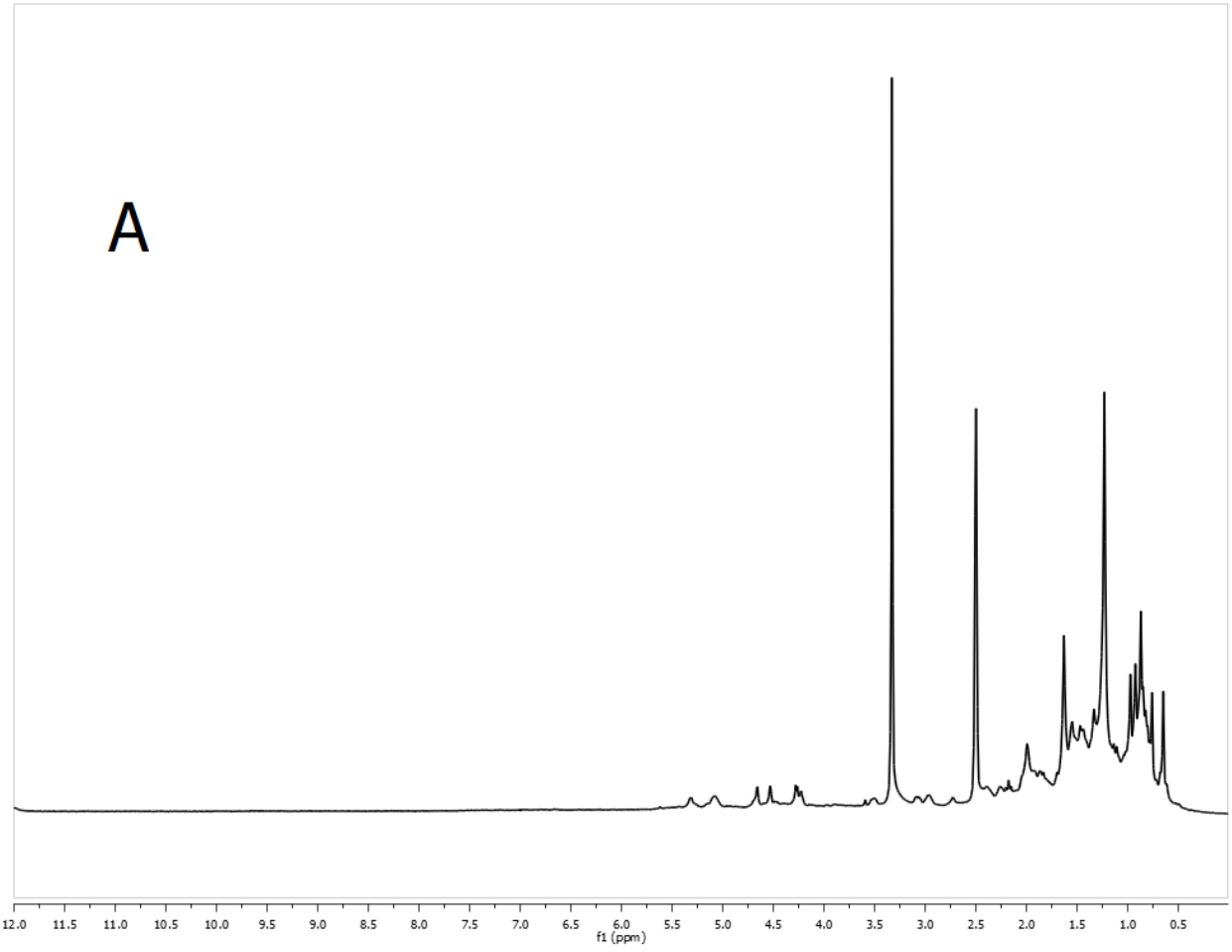


Appendix

E-beam irradiation & steam explosion as biomass pretreatment, and the complex role of lignin in substrate recalcitrance

Timo Leskinen, Stephen S. Kelley, and Dimitris S. Argyropoulos

Appendix A.1 ^{1}H-NMR analysis of DCM extractives



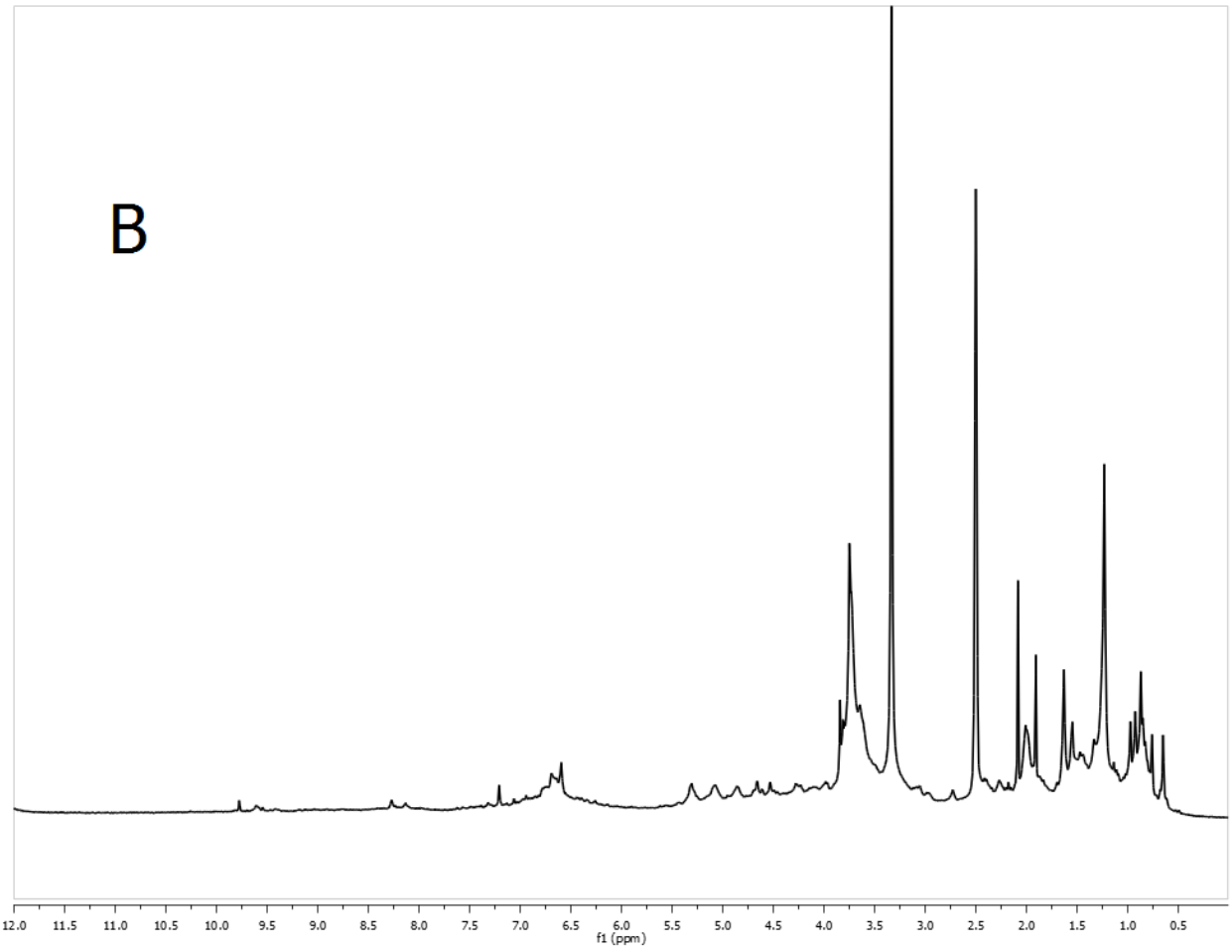


Figure A.1 Analysis of DCM extractives from Birch by ^{1}H-NMR spectroscopy, showing the presence of phenolic moieties of lignin fragments after severe pretreatments. A) Extractives from untreated starting material B) Extractives from 200 °C steam exploded Birch.

Appendix A.2 Correlation between hemicellulose solubility and enzymatic saccharification

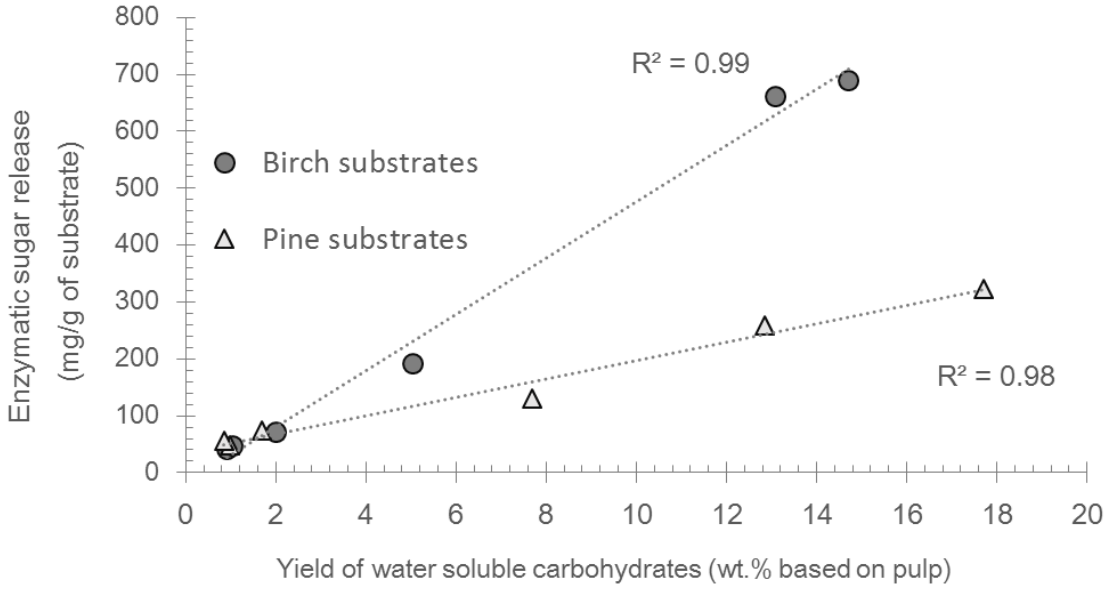


Figure A.2 Correlation of hemicellulose solubility from pretreated substrates with determined enzymatic sugar release.

Appendix A.3 Yields of soluble material from sequential solvent extractions

Extraction solvent	**Birch** Steam exploded at 200 °C (wt. % based on pulp)	**Pine** Steam exploded at 200 °C (wt. % based on pulp)
Dichloromethane	6.5	3.5
Water	Lignin: 2.3 Carbohydrates: 14.6	Lignin: 1.8 Carbohydrates: 11.5
Acetone	2.8	2.4
Dioxane-water 9:1	2.4	1.2
Total extracted	28.6	20.4

Table A.1 Yields of extracted materials from sequential solvent extractions done prior to WRV determination. Carbohydrates and lignin were determined separately in case of water extraction. In other fractions, lignin was the main component. Values are from single experiments.

Appendix A.4 Calculated molecular weight averages of lignin in pretreated substrates

	Mn	Mw	MP	Mz	PD
Pine					
Untreated	2,100	15,000	19,000	98,000	7.1
EB	2,100	14,000	19,000	100,000	6.5
SE 170 °C	2,200	15,000	17,000	115,000	6.7
EB + SE 170 °C	2,200	25,000	22,000	262,000	11.5
SE 200 °C	1,800	43,000	1,000	783,000	24.1
EB + SE 200 °C	1,900	102,000	1,000	3,593,000	54.4
Birch					
Untreated	1,700	4,000	3,000	13,000	2.4
EB	1,600	5,000	3,000	53,000	2.8
SE 170 °C	1,600	4,000	3,000	25,000	2.6
EB + SE 170 °C	1,500	4,000	2,000	42,000	2.8
SE 200 °C	1,500	7,000	2,000	140,000	4.5
EB + SE 200 °C	1,400	5,000	2,000	70,000	3.9

Table A.2 Calculated average molecular weight for lignin in untreated and pretreated materials, based on SEC analysis of acetobrominated samples (Figure 5 of main text).

Appendix A.4 Influence of deposited lignin fractions on enzymatic hydrolysis of model cellulose substrate

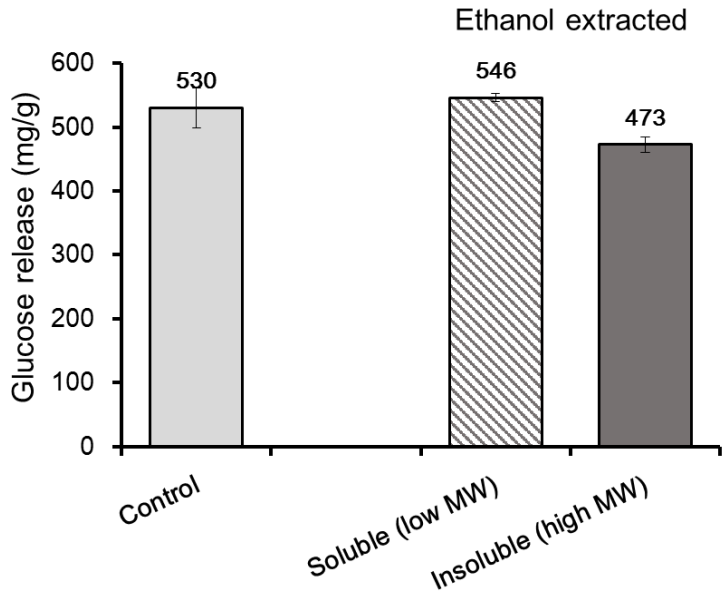


Figure A.3 Influence of deposited lignin fractions on enzymatic hydrolysis of α-cellulose. Fractions were isolated from solid lignin rich residue of EB-SE 200 °C pretreated Birch after enzymatic hydrolysis. The residue was divided into two fractions based on solubility in ethanol-water (7:3 m:m). Deposition onto the cellulose substrate was done using 1.0 M NaOH solution instead of acetone-water 85:15 v:v that was used in other experiments reported in the main text. The NaOH deposition procedure resulted significant errors even in control sample due to material losses during a required washing step to remove residual NaOH.